Activation of a dormant replication origin is essential for *Halofex mediterranei* lacking the primary origins

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The use of multiple origins for chromosome replication has been demonstrated in archaea. Similar to the dormant origins in eukaryotes, some potential origins in archaea appear to be inactive during genome replication. We have comprehensively explored the origin utilization in *Halofex mediterranei*. Here we report three active chromosomal origins by genome-wide replication profiling, and demonstrate that when these three origins are deleted, a dormant origin becomes activated. Notably, this dormant origin cannot be further deleted when the other origins are already absent and vice versa. Interestingly, a potential origin that appears to stay dormant in its native host *H. volcanii* lacking the main active origins becomes activated and competent for replication of the entire chromosome when integrated into the chromosome of origin-deleted *H. mediterranei*. These results indicate that origin-dependent replication is strictly required for *H. mediterranei* and that dormant replication origins in archaea can be activated if needed.
Chromosome replication starts at specific sites known as origins, where initiator proteins bind and recruit replication machinery components. Bacteria use a single origin, which is usually adjacent to the initiator gene dnaA, to replicate their chromosome, whereas eukaryotes perform chromosome replication with multiple replication origins that are recognized by origin recognition complexes. Dormant origins are replication origins that are not used in a normal cell cycle. If DNA encounters replication stress or if adjacent origins fail to fire, dormant origins can be activated to help complete chromosome replication, providing an important mechanism for cells to deal with replication stress and to maintain genomic stability. For organisms with multiple replication origins on a chromosome, the coordination of origin utilization is vital to ensure complete and accurate genome duplication.

Chromosome replication using multiple replication origins has been widely recognized in archaea. The combination of an autonomously replicating sequence (ARS) assay or two-dimensional gel electrophoresis with marker frequency analysis (MFA) has led to the identification of multiple replication origins in Sulfolobus species, haloarchaea, and Pyrococcus furiosus, thus greatly expanding our knowledge of archaeal replication origins. Archaeal replication origins generally contain an AT-rich region flanked by conserved repetitive DNA sequences designated as origin recognition boxes (ORBs). The initiator Cdc6 shows homology to both Orc1 and Cdc6 in eukaryotes and is responsible for ORB recognition and minichromosome maintenance (MCM) helicase recruitment. As in bacteria, an archaeal replication origin usually lies upstream of the initiator gene orc1/cdc6 (ref. 20). Studies in Sulfolobus islandicus and Haloarcula hispanica have demonstrated that the firing of specific origins is dependent on their adjacent initiators, which facilitate the direct regulation of each origin. Based on the origin features described above, multiple cdc6-associated replication origins in 15 fully sequenced holoarchaeal genomes have been predicted. However, not all of these origins were shown to be active in the genome-wide analysis of replication profiles, similar to the presence of dormant origins in eukaryotes. However, no dormant origin has been experimentally identified in archaea.

The origin usage in one cell cycle was investigated in Sulfolobus acidocaldarius, which possesses three origins on its chromosome. The three replication origins fire once per cell cycle, with oriC1 and oriC3 firing slightly earlier than oriC2 (ref. 23). For the three origins in S. islandicus and the two origins on the chromosome of H. hispanica, gene knockout experiments demonstrated that no single origin was essential, but at least one orc1/cdc6 gene (for S. islandicus) or one origin (for H. hispanica) was required for chromosome replication. Intriguingly, all active replication origins on the chromosome in H. volcanii H26 can be knocked out, and the resultant strain grows even faster than the parent strain. Owing to the requirement for RadA recombinase, which is essential for homologous recombination, homologous recombination was proposed to account for replication initiation in this origin deletion strain of H. volcanii.

In this study, the utilization of multiple replication origins and the consequences of origin deletion were investigated in Haloferax mediterranei, which belongs to the same genus as H. volcanii. We identified the replication origins on the chromosome and examined origin utilization throughout the growth phase. Surprisingly, knocking out all active origins on the chromosome led to the activation of a dormant replication origin, oriC4-cdc6H. The oriC4 is essential for chromosome replication in the absence of all other active origins, and the three active origins cannot be deleted simultaneously if oriC4 has been knocked out. Interestingly, when oriC4-cdc6H was replaced with oriP2-oriC3, a putative dormant origin from H. volcanii, oriP2-oriC3 could also efficiently initiate the replication of the entire chromosome of H. mediterranei in the absence of all other active origins. These results demonstrate that dormant origins can be activated in archaea if needed, and that chromosome replication in H. mediterranei, unlike that in H. volcanii, strictly requires origin-dependent initiation.

**Results**

**Coordinated utilization of three chromosomal origins.** Genome sequencing has revealed that multiple initiator genes (cdc6) are encoded in the H. mediterranei genome, with eight in the main chromosome and five in the three megaplasmids. Because replication origins are dependent on adjacent initiator proteins (Cdc6) for firing, the replication origins of the H. mediterranei chromosome were predicted using Cdc6 and potential ORBs associated with these initiator genes. In addition, as the genomes of H. mediterranei and H. volcanii share high homology over the entire chromosome, except for a 1-Mb fragment inversion between the two rRNA operons, the predicted origins and cdc6 genes were also compared with those of H. volcanii. Of the eight cdc6 genes in the H. mediterranei chromosome, cdc6A, cdc6C and cdc6G share high identity (>90%) with the three main replication initiation genes orcl, orc2 and orc5, respectively, in H. volcanii. These three cdc6 genes were also adjacent to inverted ORB repeats, representing a conserved characteristic of archaeal replication origins (Fig. 1a and Supplementary Fig. 1). Therefore, the three predicted cdc6 genes with adjacent ORB-containing regions were designated as orci1-cdc6A, orci2-cdc6C and orci3-cdc6G (Fig. 1a).

Genetic assays were performed to investigate the ARS activities of the three predicted origins. Three fragments, orci1-cdc6A, orci2-cdc6C and orci3-cdc6G, were cloned into the non-replicating plasmid pB1501 (Supplementary Table 1), which cannot be replicated in any holoarchaeal genome. The resulting plasmids were transferred into H. mediterranei strains (Table 1) with the corresponding chromosome fragments deleted to avoid integration of the plasmids into the chromosome. As expected, the transformants were obtained. The presence of autonomously replicating plasmids in the corresponding transformants was determined by Southern blot analysis (Fig. 1b), which was further confirmed by retransferring the plasmids from H. mediterranei back into Escherichia coli (Supplementary Fig. 2). These results clearly showed that the three predicted origins were all capable of autonomous replication.

Whole-genome DNA microarray-based MFA was then used to examine the replication profile of H. mediterranei and the utilization of the three predicted origins throughout the entire growth phase. According to the growth curve, samples for DNA extraction were collected in the early exponential phase (12 h), middle exponential phase (18, 24 h), late exponential phase (36, 42 h) and stationary phase (66 h) (Fig. 1c). The MFA results for the 12-h sample revealed three peaks, suggesting that three origins were used to initiate chromosome replication. The three peaks, located proximal to cdc6A, cdc6C and cdc6G, represented orci1, orci2 and orci3, respectively (Fig. 1d). The variable heights of the three peaks could reflect different initiation efficiencies of these origins, indicating that orci1 is likely more efficient at all of the time points (Fig. 1d). The MFA peak heights of the origins decreased gradually from 12 to 24 h, but the pattern of the differential initiation efficiencies of the origins was maintained (Fig. 1d). At 36 h, only the peak corresponding to orci1 was slightly visible, probably because only a small percentage of cells were cycling at this time point. The profile of the 42-h sample
showed no prominent peaks (Fig. 1d), which was consistent with strain growth near the stationary phase. Therefore, despite the different initiating efficiencies, the three replication origins were used in coordination with cell growth.

**Activation of a dormant origin.** The above results demonstrated that three origins were used to replicate the chromosome, but with different initiating efficiencies. To investigate origin utilization when specific origins were deleted and to test the viability of the strain lacking all of these active origins on the chromosome, the three origins were targeted for knockout. Interestingly, knockout of each origin, double knockout of the oriC3 and oriC1 and simultaneous knockout of all three origins could be successfully obtained (Fig. 2a). These origin-deleted strains grew more slowly than the wild-type strain (Fig. 2b), with growth defect of ~3.7% (for ΔoriC1) to 12.4% (for ΔoriC1ΔoriC2ΔoriC3) (Fig. 2c). However, the observed survival and growth of the DF50ΔoriC1ΔoriC2ΔoriC3 strain indicated that efficient chromosome replication could still occur in the absence of the three main origins.

Microarray-based MFA was then used to examine the replication profile of strains with deletions of one, two or all three of the origins. In the single origin deletion strains, the peaks corresponding to the deleted origins disappeared (Fig. 2d), supporting the observation that the three origins on the chromosome were active. Notably, a new prominent peak of replication initiation emerged in the strain that had all three origins deleted, whereas the three peaks corresponding to the original origins disappeared. This new peak, located near the cdc6H gene, represented a new replication origin and was designated oriC4 (Fig. 2d). Remarkably, the new peaks corresponding to oriC4 were also observed in DF50ΔoriC1ΔoriC3 and perhaps in DF50ΔoriC1, but not in DF50ΔoriC2 or DF50ΔoriC3 (Fig. 2d). Therefore, when the active origins on the chromosome were knocked out, a dormant replication origin was activated to replicate the entire chromosome either alone or in combination with other active origins. Interestingly, we noticed that the degree of activation or the initiation efficiency of oriC4 was variable, as the peaks corresponding to oriC4 in the replication profiles of the origin deletion strains had different heights (Fig. 2d). In the
replication profile of DF50\(\text{oriC1}\), the oriC4 peak was only slightly visible, whereas in that of DF50\(\text{oriC1}\text{-\text{oriC3}}\) the oriC4 peak was nearly the same height as the oriC2 peak. Finally, in DF50\(\text{oriC1}\text{-\text{oriC2}}\text{-\text{oriC3}}\), the oriC4 peak was the only peak and had a prominent height. Therefore, the activation of oriC4 was positively correlated with the number of replication origins knocked out. To the best of our knowledge, this is the first report of the activation of a dormant origin in the domain of Archaea.

The dormant oriC4 depends on adjacent cd6H for firing. The new peak, corresponding to oriC4, observed in the replication profile of DF50\(\text{oriC1}\text{-\text{oriC2}}\text{-\text{oriC3}}\) was proximal to cd6H; however, its precise location and the crucial elements responsible for its replication initiation remained undetermined. Interestingly, cd6H was adjacent to inverted ORB repeats (within the predicted oriC4), as was observed for cd6A, cd6C and cd6G, among the eight cd6 genes in the \textit{H. Mediterranei} chromosome (Fig. 3a and Supplementary Fig. 1). Therefore, oriC4 was proposed to be dependent on cd6H for firing, and an ARS assay was used to test this proposal. The fragments oriC4, cd6H and oriC4-cd6H were individually cloned into pBluescript, and their autonomous replication abilities were examined via transformation of the DF50\(\text{oriC1}\text{-\text{oriC2}}\text{-\text{oriC3}}\) strain. Although no transformants were observed for the single oriC4 or cd6H fragment, transformants corresponding to the oriC4-cd6H fragment were obtained, and Southern blot analysis confirmed the presence of autonomous replicating plasmids in the transformants (Fig. 3b). Therefore, oriC4-cd6H is a novel replication origin. Although oriC4-cd6H is dormant in the chromosome of wild-type \textit{H. Mediterranei}, it is capable of autonomous plasmid replication and can be activated for chromosome replication in \textit{H. Mediterranei} in the absence of other active origins.

Recent acquisition of oriC4 may account for its dormancy. To determine the evolutionary difference between the dormant origin and the active origins, we analysed the distribution of \textit{H. Mediterranei}-type origins in the Haloferax genus by comparing Cd6 from \textit{H. Mediterranei} against the other 14 species via BLAST. The three active origins oriC1-cd6A, oriC2-cd6C and oriC3-cd6G were found to be conserved in these Haloferax species (Table 2). However, the dormant origin oriC4-cd6H was much less conserved and was present only in \textit{H. Mediterranei} and two other species, namely \textit{H. Larsenii} and \textit{H. Elongans} (Table 2).

Interestingly, sequence context analysis revealed that oriC1-cd6A, oriC2-cd6C and oriC3-cd6G were located in the conserved chromosomal context, whereas oriC4-cd6H was located in the variable chromosomal context (Fig. 4). The conservation of these three active replication origins indicated that these origins were present in the initial Haloferax in the evolutionary process. However, in contrast to these original active replication origins, the dormant origin oriC4-cd6H was likely acquired more recently. We propose that the recently acquired oriC4-cd6H may be less competitive in the recruitment of inherent replication initiation factors (such as MCM), which may contribute to its dormant state in the wild-type strain.

### Table 1 | Strains used in this study.

| \textit{H. Mediterranei} strains | Relevant characteristics | Source or reference |
|----------------------------------|--------------------------|---------------------|
| DF50                             | \(\Delta\text{pyrF} \) strain of \textit{H. Mediterranei} ATCC 33500 | Liu et al.²⁶ |
| DFA50                            | trpA deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-cd6A}) | oriC1-cd6A deletion mutant of DF50 | This study |
| DF50\(\text{oriC2}\text{-cd6C}) | oriC2-cd6C deletion mutant of DF50 | This study |
| DF50\(\text{oriC3}\text{-cd6G}) | oriC3-cd6G deletion mutant of DF50 | This study |
| DF50\(\text{oriC4}\text{-cd6H}) | oriC4-cd6H deletion mutant of DF50 | This study |
| DF50\(\text{oriP1}\text{-cd6L}) | oriP1-cd6L deletion mutant of DF50 | This study |
| DF50\(\text{oriP2}\text{-cd6M}) | oriP2-cd6M deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}) | oriC1 deletion mutant of DF50 | This study |
| DF50\(\text{oriC2}) | oriC2 deletion mutant of DF50 | This study |
| DF50\(\text{oriC3}) | oriC3 deletion mutant of DF50 | This study |
| DF50\(\text{oriC4}) | oriC4 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC2}) | oriC1 and oriC2 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC3}) | oriC1 and oriC3 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) | oriC1, oriC2 and oriC3 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC2}\text{-oriC4}) | oriC1, oriC2 and oriC4 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC3}\text{-oriC4}) | oriC1, oriC3 and oriC4 deletion mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}\text{-oriC4}) | oriC1, oriC2, oriC3 and oriC4 deletion mutant of DF50 | This study |
| DF50\(\text{oriC4}\text{-trpA}) | \(\Delta\text{oriC4}\text{-trpA} \) mutant of DF50 | This study |
| DF50R                            | p\text{tna-radA} mutant of DF50 | This study |
| DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}\)R | p\text{tna-radA} mutant of DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) | This study |
| H13                             | \(\Delta\text{oriC4}\text{-cd6H}\text{-trpA}\text{-oriP2}\text{-oriC3}) mutant of DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) | This study |

The oriC4 is essential in absence of other active origins. The dormant origin oriC4 was activated for chromosome replication in the absence of the other three active origins. To verify the necessity and importance of oriC4 in strains lacking the primary active origins, we attempted to knock out oriC4 in the DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) strain and in the DF50 and DF50\(\text{oriC1}\text{-oriC3}) strains. Although knocking out oriC4 in DF50 and DF50\(\text{oriC1}\text{-oriC3}) was easy, we failed to knock out oriC4 in DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) after screening more than 600 FOA\(^{+}\) (5-fluoroorotic acid resistant) colonies from two independent experiments (Table 3). This result indicated that oriC4 is essential for chromosome replication in the absence of the other three origins. The necessity of oriC4 was also tested using gene disruption experiments. Because the archaeal replication origin depends on an adjacent initiator protein for firing, oriC4 inactivation was performed by disrupting the cd6H gene. Notably, the disruption of cd6H in DF50 and DF50\(\text{oriC1}\text{-oriC3}) resulted in a large number of large colonies, whereas the disruption of cd6H in DF50\(\text{oriC1}\text{-oriC2}\text{-oriC3}) only resulted in sporadic tiny transformants (Supplementary Fig. 3a). PCR analysis of the tiny transformants revealed both disrupted and intact cd6H genes (Supplementary Fig. 3b). As \textit{H. Mediterranei} has multiple copies of its chromosome²⁵, the heterozygosity of cd6H genes in this disruption approach.
Further supports the requirement for oriC4 in the DF50ΔoriC1ΔoriC2ΔoriC3 strain.

To confirm the requirement for oriC4 in DF50ΔoriC1ΔoriC2ΔoriC3, we also developed a trpA-marked positive selection system in H. mediterranei based on the pyrF-based gene knockout system\(^2\). First, trpA was knocked out to construct the tryptophan auxotrophic strains DFA50 and DFA50ΔoriC1ΔoriC2ΔoriC3 (Fig. 5a). Then, oriC4 was replaced with the trpA marker. Although we successfully established the trpA-marked knockout of oriC4 in DFA50, we did not obtain any isolates for the oriC4 knockout in DFA50ΔoriC1ΔoriC2ΔoriC3 (Fig. 5b), reconfirming the requirement for oriC4 in the absence of the other three origins. Remarkably, knocking out oriC3 in DF50ΔoriC1ΔoriC2ΔoriC4 and knocking out oriC2 in DF50ΔoriC1ΔoriC3ΔoriC4 were also unsuccessful. All of these results clearly indicate that origin-dependent initiation is strictly required for chromosome replication in H. mediterranei and that oriC4 is essential for chromosome replication in the H. mediterranei DF50ΔoriC1ΔoriC2ΔoriC3 strain.

Interestingly, radA expression was significantly higher in DF50ΔoriC1ΔoriC2ΔoriC3 than in DF50 (Supplementary Fig. 4a). To test whether radA is essential in DF50ΔoriC1ΔoriC2ΔoriC3, as was observed in H. volcanii lacking all active origins\(^2\), radA was placed under the control of the inducible promoter p.tna, as described by Hawkins et al.\(^2\). Although the growth of DF50ΔoriC1ΔoriC2ΔoriC3 was slower in the absence of RadA (when radA expression was not induced), unlike H. volcanii, RadA was not strictly required for its viability (Supplementary Fig. 4b). This finding further confirms that the chromosome replication of this origin-less H. mediterranei is origin-dependent, which differs from the recombination-dependent replication recently proposed for H. volcanii\(^2\).

Identification of the dormant origin oriP1-cdc6L on pHM500.

We also assessed extrachromosomal replicons in H. mediterranei to determine whether other dormant origins exist. Two cdc6 genes encoded on pHM500, cdc6L and cdc6M, are adjacent to inverted ORB repeats, indicating that both genes are associated with replication origins (Supplementary Fig. 1). However, the pHM500 replication profile in the wild-type strain displayed only one peak located adjacent to cdc6M (Fig. 6a), indicating that...
oriP2-cdc6M is the active origin for pHM500 replication in the wild-type strain. We hypothesized that oriP1-cdc6L, acting as a dormant origin in the wild-type strain, would be activated for replication in the absence of oriP2-cdc6M. To test this hypothesis, we generated mutants lacking oriP1-cdc6L or oriP2-cdc6M; the autonomous replication abilities of the two origins were confirmed using an ARS assay (Fig. 6b and Supplementary Fig. 5a), indicating that either origin could be used to replicate the chromosome in the absence of the other origin. In our attempt to knock out oriP1-cdc6L in ΔoriP2-cdc6M, this was unsuccessful, and the disruption of cdc6L in ΔoriP2-cdc6M resulted in only a few tiny colonies (Supplementary Fig. 5b, 5c), indicating that oriP1-cdc6L is essential in the absence of oriP2-cdc6M. Therefore, we proposed that the dormant origin oriP1-cdc6L would be activated for pHM500 replication in the absence of oriP2-cdc6M.

Heterogeneous oriP2-orc13 initiates chromosome replication. The replication origins of *H. volcanii* have been extensively characterized, but no dormant origins have been reported in previous investigations. In the case of *H. mediterranei*, the two identified dormant origins oriC4-cdc6H and oriP1-cdc6L displayed the common features that both initiator genes were adjacent to the inverted ORB motifs and that no peaks of replication initiation emerged at the corresponding locations in the replication profiles of the wild-type strain. When these features were used to evaluate the potential origins in the *H. volcanii* main chromosome and in pHV4, which is integrated into the main chromosome of *H. volcanii* H26 (ref. 22), three orc1/cdc6 genes associated with adjacent intergenic regions were found to fulfill these criteria: orc4 on the main chromosome, and orc13 and orc7 on pHV4 (refs. 12, 22). Although no initiating activity was detected for these three fragments (putative origins) in the replication profiles of the origin-deleted *H. volcanii*22, we wondered whether they could be activated to replicate the *H. mediterranei* chromosome, given that dormant origins can be activated in *H. mediterranei* if needed. First, we tested the autonomous replicating abilities of the three fragments with an ARS assay in *H. mediterranei* and used the active origin oriP1-orc3 as a positive control. In this assay, the DF50ΔoriP2-cdc6M strain was selected as the transformation host because orc3 and cdc6 share high identities (98%), whereas no cdc6 gene in *H. mediterranei* shares high identity with orc4, orc13 or orc7 (<55%). Consistent with the results obtained for *H. volcanii*11, no transformants were observed for the corresponding orc4 fragment transformation. However, the other three fragments, oriP1-orc3, oriP2-orc13 and orc3-orc7, had autonomous replicating ability in *H. mediterranei* (Fig. 7a,b).

To test the initiating activity of the putative dormant origins from *H. volcanii* for chromosome replication in *H. mediterranei*, we attempted to replace the only origin (oriC4-cdc6H) on the chromosome of *H. mediterranei* DFA50ΔoriP1ΔoriC2ΔoriC3 with the *H. volcanii* oriP2-orc13 origin. Considering the autonomous replicating ability of oriP2-orc13, we expected that
The cell density of the corresponding culture of AS-168SY plates supplemented with uracil and 5-FOA and without propagated culture of one pop-in colony in the AS-168 broth was plated on without tryptophan. (oriC4 of genomes12. However, not all of these predicted origins have been predicted in 15 fully sequenced haloarchaeal very common in archaea. Based on the conserved characteristics of archaeal origins, multiple cdc6-associated replication origins have been predicted in 15 fully sequenced haloarchaeal genomes12. However, not all of these predicted origins are active, as revealed by genome-wide MFA14,22. Therefore, it is likely that dormant replication origins are widely distributed in haloarchaea. Because dormant origins can be activated for replication in the absence of other active origins, they can act as backup sites for replication initiation when surrounding origins are inactivated or when replication forks stall, as has been demonstrated for dormant origins in eukaryotes27,28. In contrast to active origins, dormant origins in haloarchaea were proposed to have been recently acquired (Fig. 4), possibly from environmental plasmids, viruses or other haloarchaea. Interestingly, similar to our results that the recently acquired dormant origin is competent to replicate the entire chromosome of the origin-deleted H. mediterranei, suppression of the initiation defect of chromosome replication (for example, in the dnaA or oriC-deleted mutants) by plasmid or prophage integration has also been reported in bacteria29,30. As horizontal gene transfer among haloarchaea is common and the acquisition of replication origins is favourable for the transfer of new genetic content31,32, we propose that the acquired origins that accompanied foreign genetic contents are important for haloarchaea to shape the chromosomal structure and to adapt to harsh and variable environments. In addition, these recently acquired origins can be active or dormant as a result of different intracellular and extracellular conditions; this may act as an additional adaptive feature for these haloarchaea.

Interestingly, oriC4 is dormant in wild-type H. mediterranei but is significantly activated in DF50ΔoriC1ΔoriC2ΔoriC3. In eukaryotes, competition among origins for limiting replication factors such as CDC45, Sld2 and Sld3 determines their initiation time and efficiency33,34. Because the H. mediterranei chromosome is polyploid35, as observed in other haloarchaea35, and all origins share the common helicase MCM, MCM may be one of the limiting factors, as previously proposed for H. volcanii22. Therefore, we can speculate that the ability of oriC4-cdc6H to recruit MCM may be weaker than that of other active origins, resulting in the dormant state of oriC4 in the wild-type strain. The deletion of active origins would liberate more MCM molecules, allowing Cdc6H to recruit MCM and initiate replication at oriC4. The gradually increased activation of
oriC4-cdc6H in the origin deletion strains supports this hypothesis (Fig. 2d). In particular, the firing ability of oriC4 appears to be suppressed by oriC1, the most efficient origin on the chromosome, as oriC4 could be slightly activated in the absence of oriC1. Interestingly, according to several phylogenetic analyses of Cdc6, Cdc6A is the most conserved Cdc6 and is distributed among all haloarchaeal genomes as well as other archaeal genomes

Figure 7 | The dormant origin oriP2-orc13 from H. volcanii can initiate the replication of the entire H. mediterranei chromosome. (a) ARS assays for H. volcanii oriP1-orc3, orc4+ and the potential dormant origins oriP2-orc13 and oriP3-orc7. Plasmids pBI501-oriP1-orc3, pBI501-oriP2-orc13, pBI501-oriP3-orc7 and pBI501-orc4+ (Supplementary Table 1) were transferred into DF50ΔoriP2-cdc6M. Fragment orc4+ refers to the orc4 gene plus the adjacent upstream intergenic region. Colonies were observed after 3 days at 37 °C against the chromosome position. The position of the replication origin (Fig. 4). (b) Southern blot analysis using the bla gene as the probe. Lane P contains the plasmid pBI501-oriP3-orc7 isolated from E. coli, whereas the other lanes contain crude DNA extracted from the corresponding H. mediterranei transformants. (c) Schematic diagram of the replication origin of the H. mediterranei H13 chromosome. H13 was constructed by replacing the oriC4-cdc6H region in DF50ΔoriC1ΔoriC2ΔoriC3 (DF50Δ123) with the oriP2-orc13 region from H. volcanii. (d) The origin-replaced mutant H13 was confirmed by PCR analyses, with DF50 and DF50ΔoriC1ΔoriC2ΔoriC3 (Δ123) as controls. The primers were specific for oriC1, oriC2, oriC3, oriC4, oriP2-orc13 and 7S; 7S was used as an internal control. (e) The growth phenotype of H13 compared with DF50ΔoriC1ΔoriC2ΔoriC3 (Δ123). Serial dilutions of stationary phase cells (equal amounts) were spotted onto AS-168 plates supplemented with uracil. (f) Marker frequency distribution for the H13 chromosome. High-throughput sequencing was performed for the total DNA from the exponential phase of H13, and the number of reads was calculated for every 1-kb window and plotted against the chromosome position. The position of the replication origin oriP2-orc13 is marked with an arrow.

oriC4-cdc6H on the chromosome was decreased in the stationary phase compared with the exponential phase; this finding is consistent with the active utilization of origins during the exponential phase. Apart from the usage of distinct initiator proteins, archaeal replication origins (including the dormant origins) in one cell share common MCM and GINS, and other unknown factors. These factors may interact with the distinct oriCs and Cdc6s with different efficiencies, thereby coordinating the firing or inactivation of distinct origins, as discussed above. More importantly, multiple origins acting as a group may also be regulated by these factors via the same mechanisms to ensure the coordination of DNA replication with cell growth.

Notably, although both H. mediterranei and H. volcanii belong to the Haloferax genus, share high homology over the entire chromosome and grow well after the deletion of all known active origins, we demonstrated that the growth of H. mediterranei lacking known active origins is dependent on the activation of a dormant origin. It has been proposed that the H. volcanii H26 lacking known active origins may initiate replication using a recombination-dependent mechanism. Importantly, our results demonstrated that activation of the dormant origin is essential for chromosome replication in the absence of the other active origins. This is consistent with results observed for other archaea such as S. islandicus and H. hispanica, in which at least one origin is required for chromosome replication. For H. volcanii H26 (ref. 22), Michel and Bernander have proposed an alternative explanation, suggesting that dormant or newly integrated origins...
may be responsible for the viability of *H. volcanii* strains lacking the main replication origins; however, no experimental data are available yet to support this speculation. In this study, we have provided the first evidence that a dormant replication origin can be activated and become essential for chromosome replication on the deletion of all other primary origins. Interestingly, although oriP2-oriC3 and oriP3-oriC7 in *H. volcanii* were previously shown to stay dormant in the absence of other active origins, these origins exhibited ARS activities in *H. mediterranei* (Fig. 7a,b), and at least oriP2-oriC3 (oriP3-oriC7 has not yet been tested) was able to initiate replication of the entire *H. mediterranei* chromosome without other active origins (Fig. 7d). It is possible that *H. mediterranei* but not *H. volcanii* may encode factors that are important for the activities of oriP2-oriC3 and oriP3-oriC7 or that *H. volcanii* may encode suppressive factors for these putative origins. Difference also exists for the essentiality of RadA for the origin-deleted *H. mediterranei* and *H. volcanii*. RadA is strictly required for *H. volcanii* in the absence of all active origins. In contrast, although the growth of DF50ΔoriC1ΔoriC2ΔoriC3 was slowed in the absence of RadA and the gene expression in DF50ΔoriC1ΔoriC2ΔoriC3 was significantly increased, RadA was not strictly required for the viability of *H. mediterranei* in which the main origins had been deleted (Supplementary Fig. 4). This further supports the notion that activation of the dormant origin but not recombination-dependent initiation is responsible for genome replication in *H. mediterranei* DF50ΔoriC1ΔoriC2ΔoriC3. Surprisingly, *H. volcanii* H26 lacking the known active origins exhibits accelerated growth, whereas *H. mediterranei* lacking the main origins exhibits slightly slower growth (Fig. 2b,c and Fig. 7e). The reason for this growth discrepancy is unknown, but all of these results indicate that there might be substantial differences between *H. volcanii* and *H. mediterranei*, even though they belong to the same genus. In summary, our characterization of origin utilization in *H. mediterranei* reveals for the first time that a dormant replication origin can be activated and becomes essential for chromosome replication in the absence of the other active origins in archaea. This further supports the notion that chromosome replication may generally require at least one replication origin. In addition, our results also suggest that replication initiation from multiple origins (including dormant ones) could be extensively regulated, thus providing a model system for investigating the regulatory mechanisms of Cdc6-dependent replication initiation. The observed difference between originless *H. mediterranei* and *H. volcanii* in the dependence on dormant origin activation may permit the identification of factors that are important for origin activity in halarchaea.

**Methods**

**Strains and plasmids.** The *H. mediterranei* strains used in this study are listed in Table 1, and the constructed plasmids and corresponding oligonucleotides are listed in Supplementary Table 1. The plasmids used for gene knockout and gene overexpression in Table 1, and the constructed plasmids and corresponding oligonucleotides are provided in Supplementary Table 1. The plasmids used for gene knockout and gene overexpression were transferred into DFA50 and a pop-in event was verified by PCR analysis.

**DNA preparation.** The genomic DNA used for microarray analysis or high-throughput sequencing was prepared using the phenol–chloroform method. The DNA was resuspended in Tris-EDTA buffer and stored at −20 °C. The crude genomic DNA used for Southern blot analysis was extracted by directly lysing *H. mediterranei* transformants with 200 µl double-distilled H2O and 100 µl phenol–chloroform, and the liquid supernatant was collected for the following analysis.

**Whole-genome DNA microarray-based MFA.** Whole-genome DNA microarrays (12 × 135 K) were designed and manufactured by CapitalBio (Beijing, China) and NimbleGen (Madison, USA), respectively, according to the whole-genome sequence of *H. mediterranei*. Approximately 34 50-mer probes per kb, encompassing the entire genomic sequence, were designed.

Genomic DNA was extracted from the *H. mediterranei* cultures at specific time points, as indicated in Fig. 1c, or during the exponential phase (OD600nm < 0.5) and stationary phase (OD600nm > 0.5) for the origins deletion strains. The ratios were calculated as exponential phase values versus stationary phase values for each probe, and outliers (ratio > 2 or < 0.5) were excluded from the analyses. For each analysis, the excluded outliers accounted for <0.4% of the total probe; thus, ~34 valid probes per kb remained. Owing to the high density of the probes, the ratios of the 30 probes were averaged as 1 point located at the first probe. The MFA graph was plotted with a 5-point window and a 1-point slide.

**High-throughput DNA sequencing and data analysis.** Owing to the lack of probes specific for oriP2-oriC3, the replication profile of *H. mediterranei* H13 was measured by high-throughput DNA sequencing. The genomic DNA was extracted from *H. mediterranei* H13 culture during the exponential phase (OD600nm ≈ 0.35). Library preparation and sequencing with Illumina HiSeqTM 2500 were performed by BGI (Biogenius-sequencing Technologies (Beijing) Co., Ltd., Shenzhen, China), following the BGI Marker technologies protocol (San Diego, CA). Clean reads recovered after filtering low quality reads and artificially redundant reads were mapped to the *H. mediterranei* genome with the Burrows–Wheelier Alignment tool. The number of reads was calculated for every 1-kb window and plotted against the chromosome position.

**pyrf marker-based genetic manipulation.** The gene knockout experiments were conducted according to previously described procedures. Briefly, two fragments (500–800 bp) located upstream and downstream of the target gene or fragment were amplified from *H. mediterranei* genomic DNA and ligated onto the pHEF plasmid. The constructed plasmids were transferred into *H. mediterranei* strains. The polyethylene glycol-mediated transformation method was used, and the knockouts were achieved via double-crossover homologous recombination.

**trpA marker-based genetic manipulation.** The trpA-marked positive selection system was constructed based on the pyrf-based gene knockout system. First, the tryptophan auxotrophic strain DFA50 was constructed by knocking out the trpA gene with the plasmid pHEF-ΔtrpA (Supplementary Table 1). Then, the trpA open reading frame under the trp promoter was inserted into DF50 to obtain pHFA101.

For the knockout of oriC4, the upstream and downstream fragments of oriC4 were placed flanking *pyrf*-tag-trpA. The resulting plasmid pHFA101-ΔoriC4 was transferred into DFA50, and a pop-in event was verified by PCR analysis. For positive selection of the pop-out event, the cultured population of one pop-in colony in a 36-broth plate was plated on AS-1685Y plates supplemented with uracil and 5-FOA but without tryptophan. The oriC4 knockouts could be selected on these plates because of the replacement of oriC4 with *pyrf*-tag-trpA.

For the replacement of oriC4-ΔtrpA on the DFA50ΔoriC1ΔoriC2ΔoriC3 chromosome with oriP2-oriC3, the upstream and downstream fragments of oriC4-ΔtrpA were placed flanking trpA on the oriP2-oriC3 fragment (Supplementary Table 1). The oriC4-ΔtrpA cassette was then cut from the resultant plasmid pHFA101-ΔoriC4-ΔtrpA and transferred into DFA50ΔoriC1ΔoriC2ΔoriC3. Transformsants were selected on AS-1685Y plates supplemented with uracil but without tryptophan to ensure the replacement of oriC4-ΔtrpA with trpA-oriP2-oriC3.

**ARS assay.** The ARS assay experiments were performed as previously described, with some modifications. The target fragment was amplified and cloned into the non-replicating plasmid pBI501 (Supplementary Table 1), which could not be replicated in *H. mediterranei* unless an ARS was inserted. To avoid integration of
the plasmids into the chromosome, the resulting plasmids were transferred into *H. mediterranei* strains (pyrF) with the corresponding fragments deleted. If the cloned fragments could confer autonomous replication to the plasmid pEM501 with a pyrF selection marker gene, the transformants could be obtained on an AS-1685Y plate without uracil. Plasmid recovery in the transformants, indicating the ARS ability of the cloned fragment, was verified using Southern blot analysis, as previously described [3]. This was further confirmed by retransferring the plasmids from *E. coli* to *H. mediterranei*. As the plasmid for the ARS assay contained a replication origin of an *E. coli* plasmid, the corresponding *E. coli* transformants will and can only be obtained in this retransformation if the plasmids can autonomously replicate in *H. mediterranei* and can thus be isolated from the *H. mediterranei* transformants.

Full-length, uncropped Southern blot images are provided in Supplementary Fig. 7.

**Cell growth comparison and pairwise growth competition.** To compare growth of the *H. mediterranei* DF50 (wild type) and the origin-mutant strains on solid plates, a serial dilution spotting assay was performed. Briefly, the wild-type and mutant strains were grown in AS-168 broth supplemented with uracil to the stationary phase (*OD*$_{600}$ ~ 4.0). The cell densities of the strains were adjusted to be equal before 5–10 fold serial dilutions were made with AS-168 broth (10$^{-2}$ to 10$^{-5}$). Then, 5 µl of each dilution was spotted on an AS-168 plate supplemented with uracil. Photographs were taken 2 to 3 days after spotting.

To compare growth in liquid culture, pairwise growth competition assays were performed and described [12] with minor modifications. Briefly, the *H. mediterranei* DF50 and the origin-mutant strains (1:1 ratio, and DF50 and the origin-mutant strains (1:1 ratio, and 10$^6$ cells were then inoculated in 10 ml of the same fresh medium and cultured for an additional day; this process was repeated once. To count the cells, the culture from each time point was diluted, and 150 µl aliquots were plated on AS-168 plates supplemented with uracil. The wild-type and origin-mutant cells were identified by PCR, and the ratio was calculated based on > 120 random colonies.

**Prediction and distribution of replication origins.** The intergenic region immediately upstream of *cdk6* was assessed for the presence of consensus repeats using MEME software [28] (motif size: 18-35; OMOPS model). The consensus repeats were manually evaluated, and those with a ‘G-string’ [11] were selected as the ORB elements. Only the intergenic regions with inverted ORB elements were considered as replication origins. To analyse the distribution of *H. mediterranei*-type replication origins in the Halofexus species, the BlastP programme (BLOSUM62 matrix) was used, comparing origin-associated Cdc6 proteins from *H. mediterranei* with those from certain *Haloferax* species, the BlastP programme (BLOSUM62 matrix) was used, comparing origin-associated Cdc6 proteins from *H. mediterranei* with those from certain *Haloferax* species [28]. Only those Cdc6 proteins with identities > 80% (ref. 12) were recognized.

**Genome resources and comparative genomics.** Two complete genomes, *H. mediterranei* ATCC 33500 and *H. volcanii* DS2 (ref. 30), and the contigs of 13 draft genomes, *H. sulfuriintis* ATCC BAA 897 (ref. 51), *H. maccosum* ATCC BAA 1512 (ref. 51), *H. definititica* ATCC 35960 (ref. 51), *H. pruhoense* DSM 18310, *H. larsenii* JCM 13917, *H. gibsonii* ATCC 33959, *H. elongans* ATCC BAA-1513, *H. alexandrinus* JCM 10717, *H. lucentense* DSM 14919, *H. halophila* sp. ATCC BAA-644, *H. halophila* sp. BAA-2207, were available through NCBI (http://www.ncbi.nlm.nih.gov/Genome). All comparative genomic analyses were performed with the Cgview Server [22] using the default parameters (http://stothard.afns.ualberta.ca/cgview_server/).

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Acknowledgements
This work was partially supported by grants from the National Natural Science Foundation of China (31100893, 31271334 and 31330001) and the Hundred Talents Program of the Chinese Academy of Sciences (to H. X.).

Author contributions
H.X. conceived the study; H.Y., Z.W. and H.X. designed the experiments; H.Y., J.L., X.L. and S.C. performed the experiments; H.Y., Z.W., J.L., L.W. and H.X. analysed the data; and H.Y. and H.X. wrote the paper.

Additional information
Accession codes: The DNA microarray and high-throughput sequencing data have been deposited in the NCBI GEO library under accession numbers GSE70597 and GSE70592.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Yang, H. et al. Activation of a dormant replication origin is essential for Haloferax mediterranei lacking the primary origins. Nat. Commun. 6:8321 doi: 10.1038/ncomms9321 (2015).

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