Published online 17 February 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 7* **2087–2095**
doi:10.1093/nar/gkp065

The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication

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Received December 27, 2008; Revised January 23, 2009; Accepted January 24, 2009

ABSTRACT

The GINS complex, originally discovered in *Saccharomyces cerevisiae* and *Xenopus laevis*, binds to DNA replication origins shortly before the onset of S phase and travels with the replication forks after initiation. In this study we present a detailed characterization of the human GINS (hGINS) homolog. Using new antibodies that allow the detection of endogenous hGINS in cells and tissues, we have examined its expression, abundance, subcellular localization and association with other DNA replication proteins. Expression of hGINS is restricted to actively proliferating cells. During the S phase, hGINS becomes part of a Cdc45–MCM–GINS (CMG) complex that is assembled on chromatin. Down-regulation of hGINS destabilizes CMG, causes a G1–S arrest and slows down ongoing DNA replication, effectively blocking cell proliferation. Our data support the notion that hGINS is an essential component of the human replisome.

INTRODUCTION

Eukaryotic DNA replication depends on multi-protein complexes that are assembled at the origins in two steps. The first one is referred to as 'origin licensing' and takes place at the M/G1 transition, when the combined action of the origin recognition complex (ORC), Cdc6 and Cdt1 proteins engage the Mini-chromosome maintenance complex (Mcm2-7; referred through the text as MCM) with the DNA. The second step occurs upon activation of the S phase-promoting cyclin-dependent kinases (CDK) and Dbf4-dependent kinase (DDK), which trigger the recruitment of additional initiator proteins. In *Saccharomyces cerevisiae*, phosphorylation of Mcm4 by DDK facilitates the binding of Cdc45 (1), which interacts with Sld3 (2). Phosphorylation of Sld3 and its related protein Sld2 by CDK creates a binding site for Dpb11 protein (3,4), which in turn serves as an anchor for DNA polymerases, RPA and the GINS complex. GINS is formed by proteins Sld5 (synthetic lethal with Dpb11), Psf1, Psf2 and Psf3 [partners of Sld-five; (5–7)]. Some of these factors, e.g. MCM, Cdc45 and GINS are involved in the initiation reaction and later become part of the replisome machinery (8–10); reviewed by (11–14). In mammalian cells, oncogenes such as Cyclin E interfere with this pathway of origin activation, causing an inefficient S phase and genomic damage (15); reviewed by (16).

Most of the proteins involved in the licensing step (ORC, Cdc6 and MCM) are conserved in eukaryotic organisms and have recognizable ancestors in archaea. This is not always the case for the initiators acting at the G1–S transition: yeast Dpb11 and Sld2 proteins are related to mammalian TopBP1 and RecQ4L, respectively, but the latter are much larger and contain additional domains that could serve other cellular functions. Sld3 has no evident homologs outside fungi, at least at the amino acid sequence level. In contrast, the GINS complex is highly conserved and the mammalian paralogues of its four subunits have been identified by sequence homology (6,7). We have recently cloned the four subunits of human GINS (hGINS), reconstituted the recombinant protein complex and proposed its 3D volume based on electron microscopy imaging (17). In this report we turn to the characterization of endogenous hGINS, analyzing its expression, abundance, regulation in the cell division cycle and interactions with other DNA replication proteins.

MATERIALS AND METHODS

Protein purification and antibody production

Recombinant hGINS, purified as described (17), was used to immunize Balb/c mice. Three hybridoma cell lines were selected that produced monoclonal antibodies against Psf1

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(clone 192B), Psf2 (clone 78C) and Psf3 (clone 40F). Polyclonal antibodies against Psf1 and Psf3 were generated using synthetic peptides conjugated to KLH protein (Pierce): N-CQITASNLVQNYKKRKF-C (Psf1); N-LL KKNSQHFLPRWCK-C (Psf3). Polyclonal antibodies against Sld5, Psf2 and Cdc45 were raised in rabbits injected with recombinant Sld5, Psf3 and Cdc45 (amino acids 388–566) purified from *Escherichia coli* as GST fusions. Other antibodies used in this study were: Orc2, Mcm2-7 and PCNA, kindly provided by B. Stillman (Cold Spring Harbor Laboratory, NY, USA), CDC6 and γ-H2AX (DCS-180 and JBW301; Upstate Biotechnology), DNA polymerase δ and Cyclin B1 (H-300 and GNS1; Santa Cruz Biotechnology), Mek2, p27 and FITC-BrdU (BD-610235, BD-554069 and BD-556028, BD Sciences Pharmingen), T7 (69522, Novagen), Chk1 P-S345 (133D3, Cell Signaling Technology), α-tubulin (DM1A, Sigma), H3 (Ab1791, Abcam), GAPDH (CNIO Monoclonal Antibody Unit). Secondary antibodies for immunoblot and immunofluorescence (IF) were from Amersham and Jackson Immunoresearch Inc., respectively.

Cell culture and manipulations

IMR90, Wi-38, BJ-hTERT, HeLa, U2OS and 293T cell lines were grown in DMEM–10% FBS with penicillin and streptomycin. K562 and Jurkat were grown in RPMI 1630-10% FBS plus antibiotics. To drive BJ-hTERT cells into a quiescent state by contact inhibition, cells were grown to confluency and kept for 72 h before collection. G0 cells were driven back into S phase by re-plating the confluent culture (1:2 split). Cells were collected 24 h later.

Transfection of plasmidic DNA into HeLa cells was carried out with Lipofectamine 2000 (Invitrogen). Stealth siRNA duplexes directed to *SLD5*, *PSF1*, *PSF2* or *PSF3* (sequences available upon request) were transfected twice at a final concentration of 100 nM, using oligofectamine (Invitrogen). Cell cycle synchronization was achieved by thymidine block and release (18). The biochemical fractionation to separate soluble and chromatin-associated proteins was performed as described (19). For the detection of DNA replication foci, cells were incubated for 10 min with 10 μM EdU (20) prior to fixation with 2% PFA. EdU incorporation was visualized using Click-IT EdU AF647 (Invitrogen).

Immunological detection of proteins in cells and tissues

Standard protocols for immunoblotting, immunoprecipitation (IP), indirect IF and immunohistochemistry (IHC) were followed. When indicated, immunoblotting signals were quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Prior to IP, whole cell extracts were prepared in 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM NaF, 5 mM 2-glycerol phosphate, 1 mM $NaVO_4$, 1 mM 1,4-dithiothreitol, 1 mM PMSF and a protease inhibitor cocktail (Roche). Cell lysis proceeded for 20 min on ice and was followed by brief sonication. For soluble extracts and digested chromatin extracts, cells were lysed in 10 mM HEPES pH 7.9, 0.2 M KOAc, 0.1% Triton X-100, 0.34 M sucrose, 10% glycerol, 1 mM 1,4-dithiothreitol, protease and phosphatase inhibitors as above. After a 5 min spin at 800 *g*, the fraction corresponding to the soluble (cytosol/extracted nucleoplasm) proteins was recovered. The chromatin fraction in the pellet was incubated with micrococcal nuclease (Sigma N3755; 0.33 U/10^6 cells) for 20 min at 24°C. Insoluble material was removed from all fractions by centrifugation at 16 000*g* for 30 min.

Flow cytometry analyses

Cells were fixed in 70% ethanol and washed in PBS-0.05% Tween-20. To measure DNA content, cells were treated with 20 μg/ml propidium iodide and 100 μg/ml RNaseA. For BrdU detection, 10 μM BrdU was added to the medium for 60 min prior to cell collection. Cells were fixed and washed as above, treated with 4 M HCl/0.5% Triton X-100 for 20 min and incubated with FITC-conjugated anti-BrdU antibody. Cells were processed in a FACScalibur cytometer (BD Biosciences).

RESULTS

Immunodetection of endogenous hGINS in cells and tissues

Recombinant hGINS complex was purified through affinity, ion-exchange and gel-filtration chromatographic steps (Figure 1A), as previously described (17) and used as antigen to generate monoclonal antibodies in mice. In addition, polyclonal antibodies were raised using recombinant Sld5 and Psf2 proteins or synthetic peptides corresponding to Psf1 and Psf3 (see Materials and methods section). All the new antibodies detected the corresponding endogenous hGINS subunits from whole cell extracts in immunoblot assays (Figure 1B), and some of them were suitable for IP, IF and IHC studies.

The four subunits of hGINS form a stable complex that can be recovered from cell extracts by IP with a Psf2 monoclonal antibody (Figure 1C, lane 3). Virtually the entire amounts of the four hGINS subunits were precipitated (Figure 1C, compare lanes 3 and 5). Furthermore, the complex was detected at all stages of the cell division cycle (Supplementary Figure 1). DNA replication proteins Mcm2 or DNA polymerase δ did not co-precipitate with hGINS in a whole cell extract, but an interaction between hGINS and MCM was observed on chromatin (see subsequently). hGINS is a nuclear protein, as revealed by indirect IF in HeLa cells (Figure 1D, panels i–ii). The specificity of the IF staining was confirmed by down-regulation of *PSF2* expression by RNAi (Figure 1D, panels iii–iv). The effects of hGINS down-regulation are discussed in a later section.

hGINS could also be detected in human tissues by IHC with a Psf1 monoclonal antibody. In a tonsil sample, Psf1 was more abundant in the proliferating lymphocytes of the germinal center, compared with the interfollicular tissue surrounding it (Figure 1E, left panel). This staining pattern, similar to that of Mcm3 protein (Figure 1E, right panel), indicates that hGINS is a useful marker of cell proliferation.

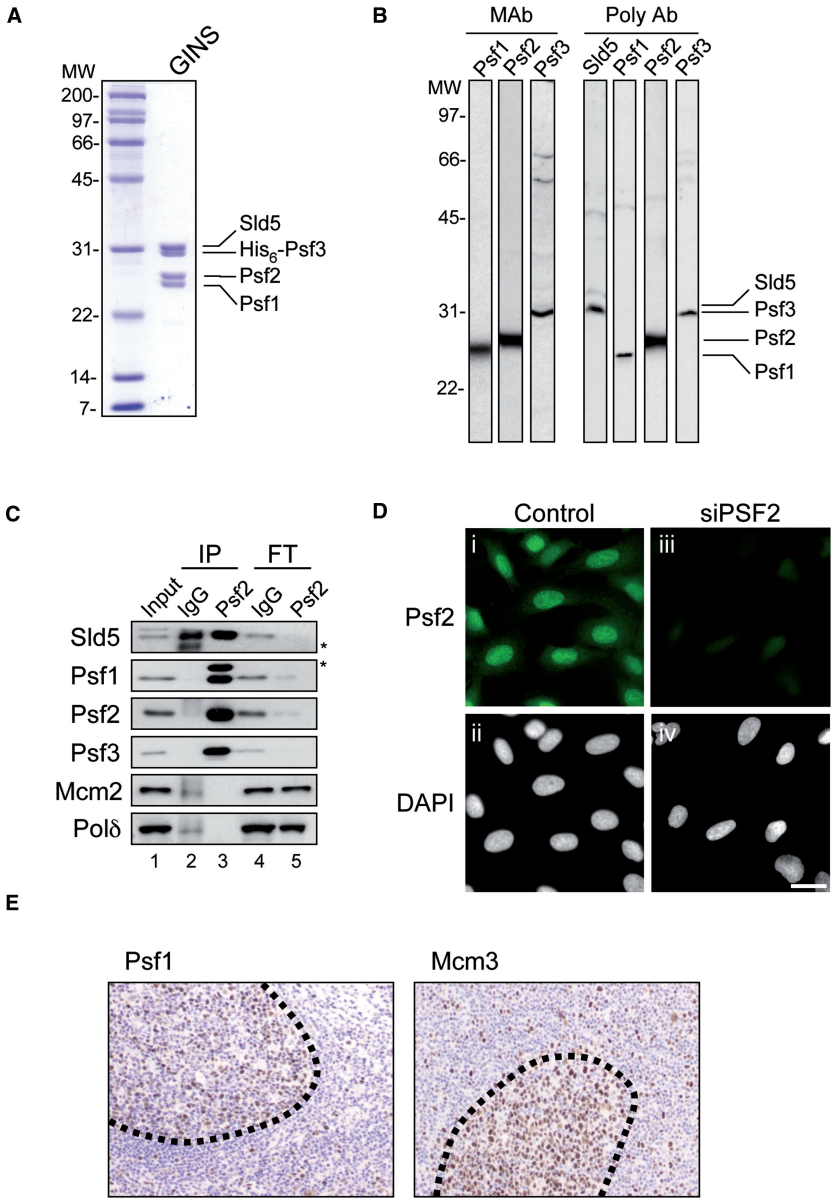

Figure 1. Immunodetection of endogenous hGINS in cells and tissues. (**A**) Coomassie staining of purified recombinant hGINS complex. (**B**) Immunoblot detection of hGINS subunits in 293T whole cell extracts with the indicated antibodies. (**C**) IP of the endogenous hGINS complex from HeLa whole cell extracts. Immunoblot detection of Sld5, Psf1, Psf2 and Psf3 after IPs with control IgG or anti-Psf2 monoclonal antibody. Input lane contains 2% of the extract amount used in each IP. FT, flow through (2% of the amount used in each IP). Asterisks indicate cross-reactions with the IgG light chain. (**D**) IF detection of Psf2 (green) and DNA staining (gray) in cells treated with control or *PSF2* siRNA molecules. Bar, 20 μm. (**E**) Immunohistochemical detection of Psf1 and Mcm3 proteins in biopsies of human tonsil. Brown peroxidase staining indicates the presence of the target protein. Dashed lines mark the tonsil germinal centers of proliferating lymphocytes.

hGINS expression correlates with cell proliferation

Using defined amounts of recombinant hGINS as reference, we estimated that diploid IMR90 and Wi-38 lines contain between 85 000 and 130 000 molecules of hGINS per cell. hGINS is more abundant in immortalized BJ-hTERT fibroblasts (220 000 molecules/cell) and tumor-derived cell lines such as Jurkat, K562, HeLa, U2OS and 293T (500 000–1 100 000 molecules/cell; Figure 2A). The promoter regions of hGINS genes include binding sites for multiple transcription factors, notably E2F-1 and Myc-Max (21), which are frequently deregulated in cancer (Supplementary Figure 2A).

In diploid fibroblasts driven into G0 by contact inhibition, the four hGINS subunits were virtually undetectable (Figure 2B). As expected, the levels of DNA replication factors Cdc6, MCM and Cdc45 and mitotic cyclin B1 were also reduced in the quiescent state, whereas the concentration of CDK inhibitor p27 was increased. Other DNA replication factors such as Orc2 or PCNA were not degraded after 3 days in G0 (Figure 2B). The strong

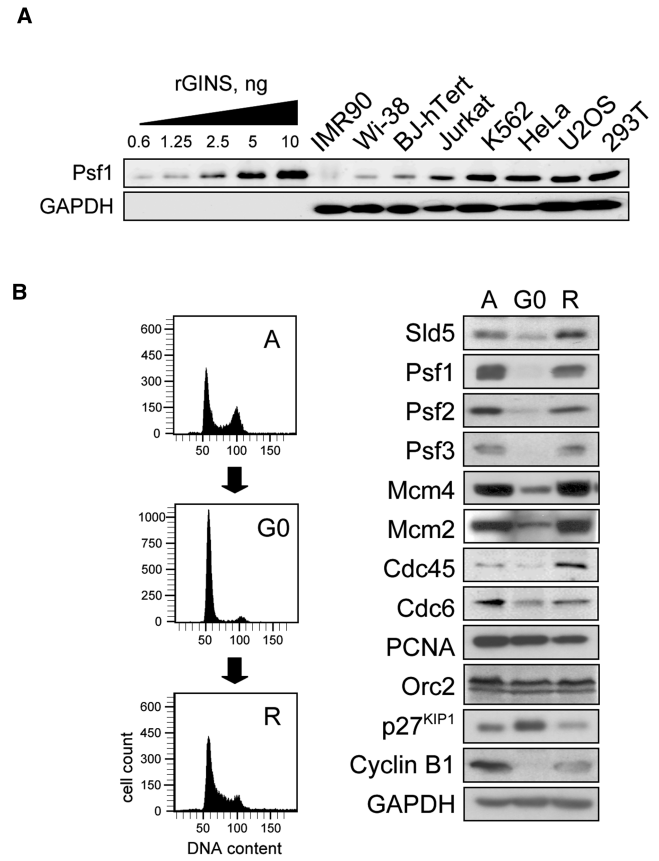

Figure 2. hGINS expression is linked to proliferation. (**A**) Abundance of hGINS in different cell lines. Immunoblot detection of Psf1 in whole cell extracts prepared from 50 000 cells of each cell line. The indicated amounts of recombinant GINS were used as reference. Levels of GAPDH are shown as loading control. (**B**) Immunoblot detection of hGINS subunits and the indicated proteins in asynchronously proliferating (A), quiescent (G0) and re-plated (R) BJ-hTERT fibroblasts.

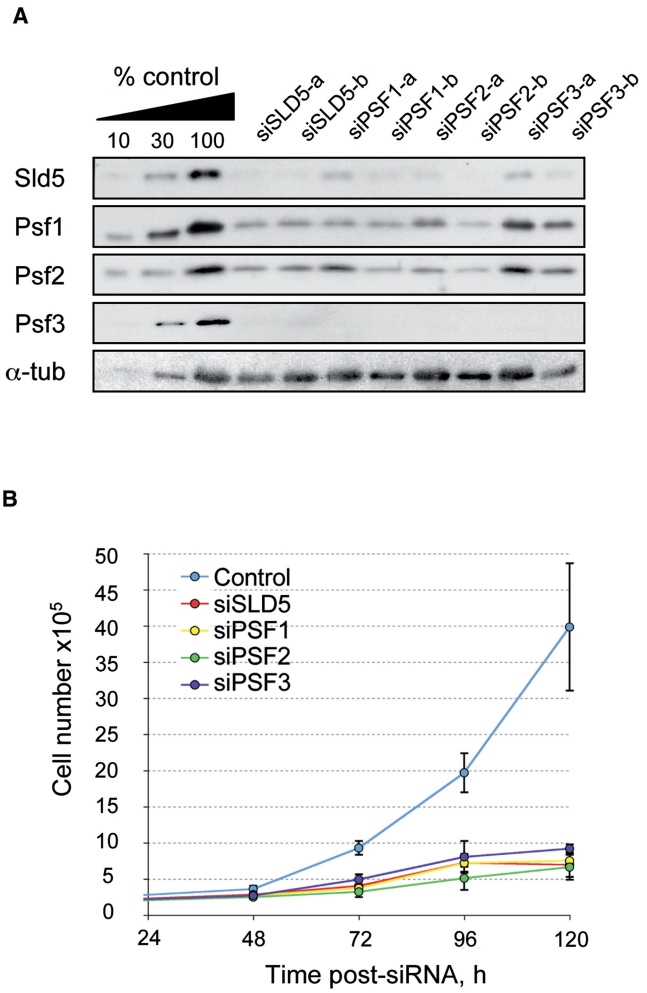

Figure 3. hGINS is essential for cell proliferation. (**A**) Immunoblot detection of hGINS subunits in HeLa cell extracts prepared 72 h after transfection of control or two independent (a/b) siRNA oligonucleotides for each subunit. Different amounts of control HeLa cell extracts were loaded as standard. α-Tubulin is shown as loading control. (**B**) Proliferation curves after hGINS down-regulation. HeLa cells transfected with control or hGINS siRNAs were collected at the indicated time points to measure cell density. Curves represent the average of three independent experiments.

down-regulation of hGINS was likely the result of transcriptional repression and the proteolytic degradation of existing hGINS complexes. Protein degradation may be relatively rapid because the half-life of hGINS subunits is in the 6–8 h range, similar to that of cyclins (Supplementary Figure 2B). Interestingly, 24 h after re-plating the contact-inhibited cell population at a lower density, the four components of hGINS were expressed again, as well as Cdc6, MCM and Cdc45 (Figure 2B). This result confirms that hGINS expression is linked to cell proliferation.

hGINS is essential for initiation of DNA replication and S phase progression

We next evaluated whether hGINS is directly involved in cellular processes necessary for proliferation and viability. Small interference RNA (siRNA) molecules designed against each one of its subunits reduced the amount of hGINS to <10% of its normal levels in HeLa cells (Figure 3A). Silencing the expression of any individual subunit triggered the concomitant degradation of the others, indicating that the stability of hGINS components, particularly Sld5 and Psf3, depends on the formation of the full complex. Indeed, hGINS down-regulation prevented cell proliferation (Figure 3B). Because no cell death was observed for the duration of the experiment, the failure to proliferate suggests a block in cell cycle progression.

The efficiency of DNA replication in cells with reduced levels of hGINS was first estimated by IF after the incorporation of a nucleotide analog onto nuclear DNA. After a pulse of 10 min with EdU (20), 28% of the cells in the control population scored positive for replication compared with ~4% of the cells treated with *SLD5* or *PSF1* siRNA molecules (Figure 4A). The incorporation of nucleotide analogues was also analyzed by flow cytometry to visualize the different phases of the cell cycle (Figure 4B). In the control population, the intensity of

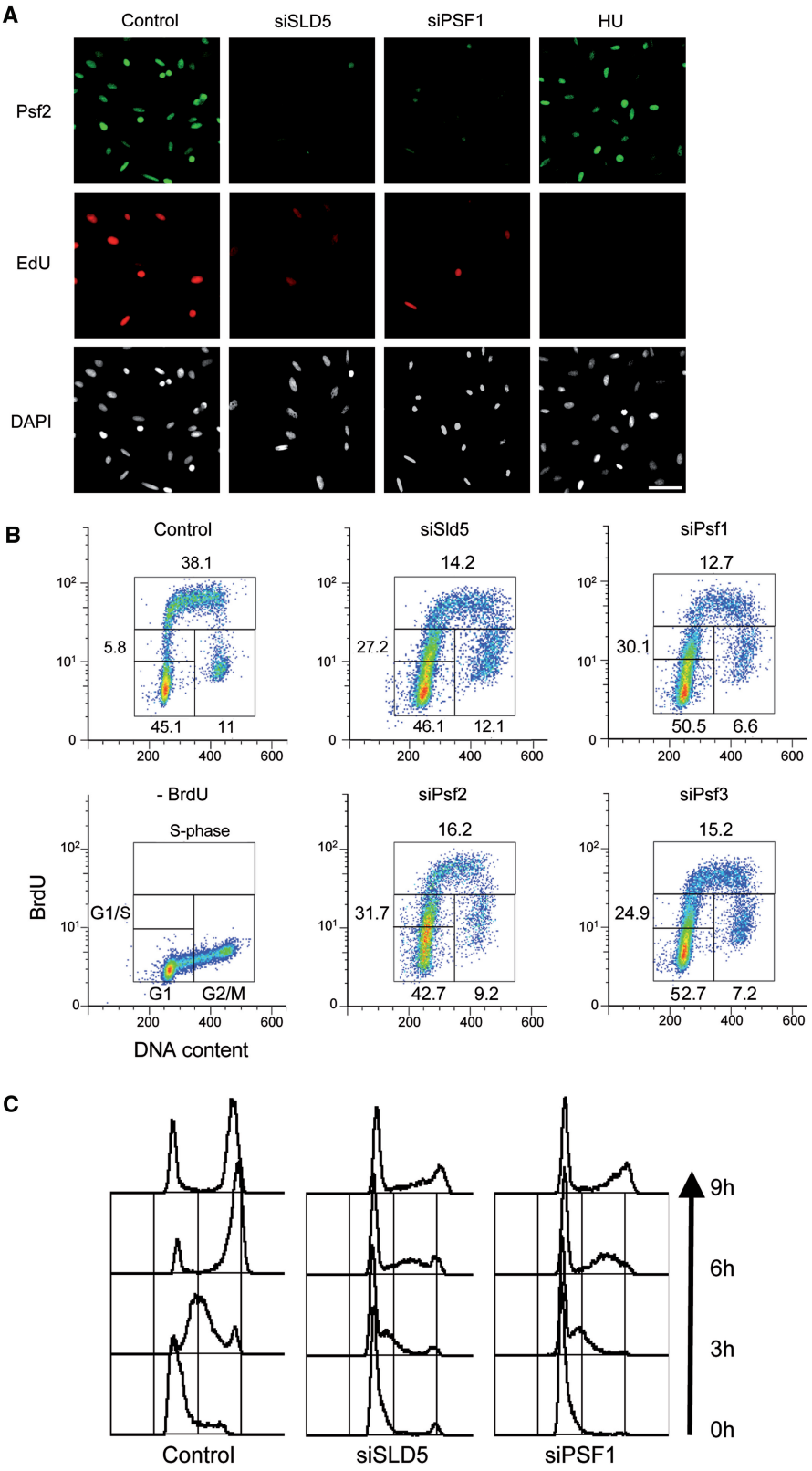

Figure 4. hGINS is required for initiation and elongation of DNA replication. (**A**) EdU incorporation assay. HeLa cells, transfected with control, *SLD5* or *PSF1* siRNAs, were labeled for 10 min with EdU. Psf2 was visualized by indirect IF (green), EdU foci by an alkyl-azide reaction (red) and DNA by DAPI staining (gray). As a negative control, cells were treated for 4 h with 4 mM hydroxyurea (HU) prior to the EdU pulse. Bar, 50 μm. (**B**) Flow cytometry detection of BrdU incorporation. HeLa cells transfected with the indicated siRNAs were labeled with BrdU for 60 min prior to fixation. BrdU intensity is represented in the logarithmic *y*-axis and DNA content on the linear *x*-axis. Gates define the percentage of cells in G1, G1/S, S (BrdU positive) and G2/M. Similar analysis of a control cell population not labeled with BrdU is shown. (**C**) S-phase progression assay. Cells treated with control, *SLD5* or *PSF1* siRNAs were synchronized at the G1/S transition and released for the indicated times. S-phase progression was monitored by the analysis of DNA content by flow cytometry.

BrdU incorporation in cells with 1C DNA content (left part of the horseshoe shape) reflects a rapid transition from G1 into S phase. In contrast, hGINS down-regulation caused 25–32% of the cells to accumulate at the G1/S transition, compared with 5.8% of the control population. As a consequence, a reduced percentage of cells (13–16%) progressed into S phase, compared with 38% in the control (Figure 4B).

To analyze whether hGINS was also required during S phase progression, control or siRNA-treated cells were synchronized at G1/S. Upon release from the block, control cells initiated and completed S phase in a period of 6 h (Figure 4C, left panel). In contrast, the majority of cells depleted of hGINS did not progress into S phase and the fraction of cells that started DNA replication took longer than 9 h to complete it (Figure 4C, mid and right panels). This result is consistent with hGINS also participating in the elongation phase of DNA replication. Alternatively, the delay in S phase completion could be caused by a reduction in the number of active origins. We have also observed that cells treated with hGINS RNAi displayed γ-H2AX foci and activated checkpoint kinase Chk1 (Supplementary Figure 3), suggesting an accumulation of DNA damage as a consequence of defective DNA replication.

hGINS associates with chromatin during S phase and binds to Cdc45/MCM

The cellular levels of hGINS remained constant in the cell cycle, as determined by immunoblots in cells synchronized in G1, S or G2/M (Figure 5A, WCE panel). However, a biochemical fractionation that separates soluble from chromatin-bound proteins (19) revealed that a fraction of hGINS associated with chromatin specifically during S phase (Figure 5A). This pattern of chromatin association was also observed with DNA polymerase processivity factor PCNA (Figure 5A) and suggests the participation of hGINS in the replisomes.

In *Drosophila*, GINS associates with Cdc45 and MCM proteins in the so-called CMG (Cdc45–MCM–GINS) complex, which displays DNA helicase activity *in vitro* (22). Despite the relevance of this finding, the formation of such a CMG complex in mammalian cells remained to be confirmed. Using cells synchronized in S phase, we observed that the IP of Mcm3 co-precipitated Cdc45 and hGINS subunits from nuclease-treated chromatin extracts, but not from soluble cytosol/nucleoplasm extracts (Figure 5B). This result is consistent with the existence of a human CMG complex, which appears to be assembled on chromatin. In contrast, hGINS antibodies failed to co-precipitate MCM or Cdc45 (data not shown). Because the same antibodies precipitated soluble hGINS (Figure 1C), this negative result could be explained if the recognition of hGINS epitopes disrupted the interactions with other components of hCMG.

hGINS stabilizes the Cdc45–MCM interaction

As shown above, Cdc45 and GINS were detected after the IP of MCM in a nuclease-digested chromatin extract. This result might reflect the formation of a ternary CMG

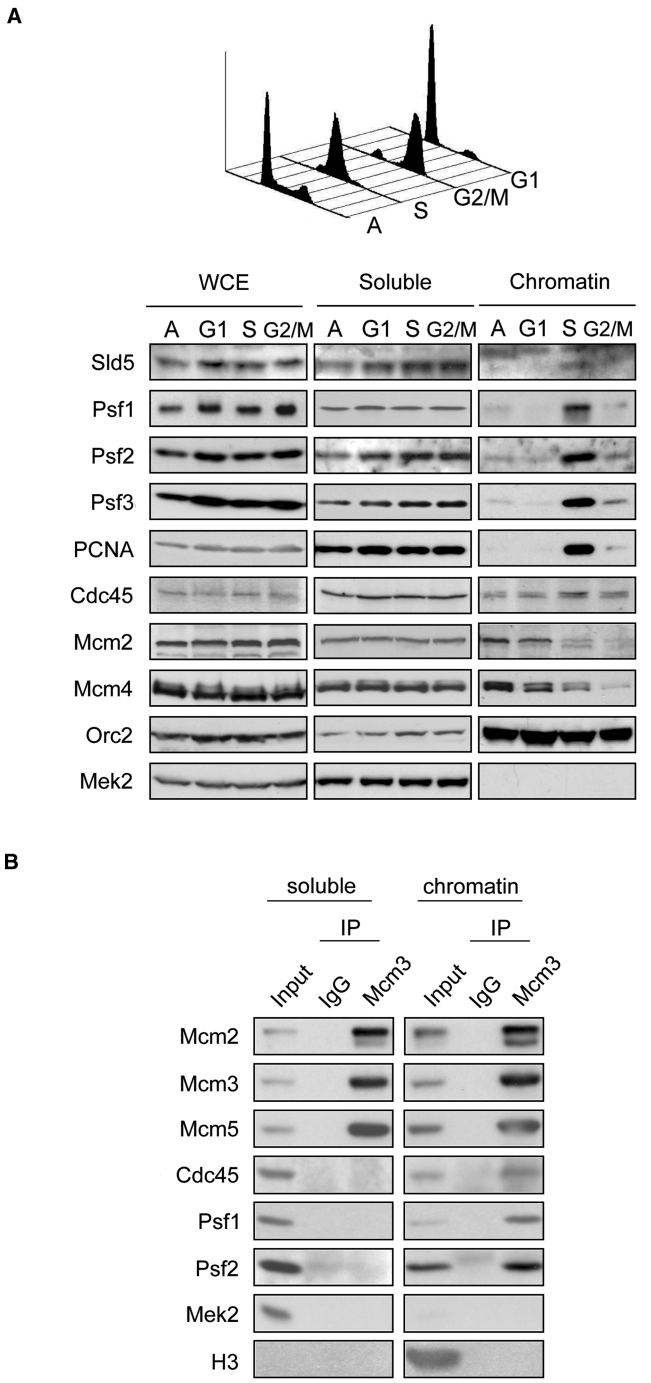

Figure 5. hGINS associates with MCM and CDC45 proteins on chromatin. (**A**) hGINS binds to chromatin during S phase. The indicated proteins were detected by immunoblots in whole cell extracts (WCE), soluble and chromatin fractions from cells synchronized at different stages of the cell cycle. The DNA content of the synchronized fractions is shown. Mek2, a cytosolic kinase, and Orc2, a chromatin-associated protein, are shown as controls of the subcellular fractionation protocol. (**B**) Detection of the human CMG complex. IPs with control or Mcm3 antibodies were carried out in a soluble (cytosol/extracted nucleoplasm) extract or a nuclease-treated chromatin extract from HeLa cells synchronized in S phase. The indicated proteins were detected by immunoblot. Input represents 0.6% of the amount of extract used in the IP.

complex but could also be explained by the formation of independent MCM–Cdc45 and MCM–GINS complexes. In the latter case, the cellular abundance of GINS proteins should not affect the MCM–Cdc45 interaction. Therefore, an experiment was designed to check the integrity of the MCM–Cdc45 interaction after GINS down-regulation. A T7-tagged version of Cdc45 was transfected into HeLa cells treated with control or Psf1 siRNAs (Figure 6A). In control cells, an antibody directed to T7 co-precipitated subunits of MCM and GINS from solubilized chromatin (Figure 6B, lane 5), consistent with the result shown in Figure 5B. The efficiency of co-precipitation of GINS and MCM was significantly reduced after a partial down-regulation of Psf1 (Figure 6B, lane 6; see quantification in bar graph). This result suggests that hGINS may act as a molecular bridge between Cdc45 and MCM, or at least stabilize their interaction.

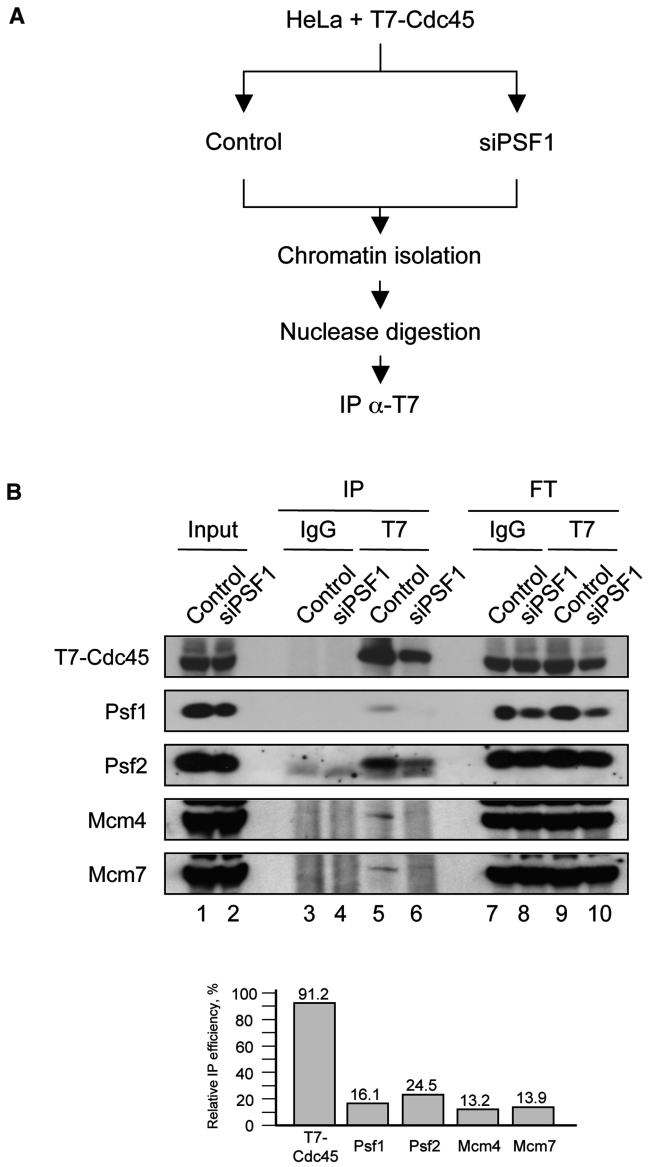

Figure 6. hGINS is required for the stability of the CMG complex. (**A**) Experimental design. (**B**) Immunoblot detection of components of the CMG complex after the IP with anti-T7 antibody in cells treated with control or hPsf1 siRNA. Input and FT (flow through) lanes contain 5% of the extract used in the IP. The bar graph represents the 'relative IP efficiency' for the indicated proteins. Absolute IP efficiency was calculated in each case as the ratio between the amount of protein detected in the immunoprecipitate and the total amount in the input. For a given protein, 'relative IP efficiency' was estimated as the ratio between the IP efficiencies in cells treated with Psf1 siRNA and control cells.

DISCUSSION

The GINS complex was discovered in 2003 as an essential DNA replication factor in yeast and *Xenopus*, and its human homolog has remained largely uncharacterized except for the elucidation of its 3D structure (17,23–25). In this study, we have gained new insights into the function and regulation of endogenous hGINS. First, we observed a correlation between hGINS abundance and cell proliferation. In cultured cells, the four components of hGINS are expressed during mitotic cycles, degraded shortly after the cells are driven into G0, and resynthesized upon re-entry in the cell cycle. In tissue sections, hGINS antibodies stain preferentially the areas with active proliferation, such as the germinal centers of the tonsil. In addition, hGINS is more abundant in cancer cell lines than in primary, diploid lines. Consistent with this observation, hGINS is up-regulated in gene-expression profiles of intrahepatic cholangiocarcinomas (26) and melanomas (27). Therefore, hGINS is a good proliferation marker and its immunodetection may become useful in cancer diagnosis.

In yeast, GINS participates in origin activation and then becomes part of the 'replisome progression complex' (RPC) traveling with the forks (8,28,29). In human cells, down-regulation of hGINS expression impaired entry into S phase as well as S phase progression, suggesting that hGINS participates in both initiation and elongation of DNA replication.

hGINS interacts with DNA replication factors MCM and Cdc45 in chromatin during S phase. This is relevant because previous work in *Drosophila* has suggested that a complex formed by Cdc45, MCM and GINS (CMG) could constitute the replicative DNA helicase (22). MCM2-7 may hydrolyze ATP to provide the energy required for DNA melting, with Cdc45 and GINS playing structural roles. Actually, hGINS is required to maintain the stability of the MCM–Cdc45 interaction, a 'bridge' function that seems to be evolutionary conserved from yeast to human (8). Binary interactions between human Cdc45–MCM and Cdc45–GINS have been recently reported (30), but we provide new data that strongly support the existence of a CMG complex in human cells.

An additional insight into the possible function of GINS at the fork comes from studies in the archaea *Sulfolobus solfataricus*. In this organism, GINS associates with the archaeal MCM and primase complexes, suggesting that it might somehow coordinate the DNA helicase tracking along the leading strand with the DNA primase

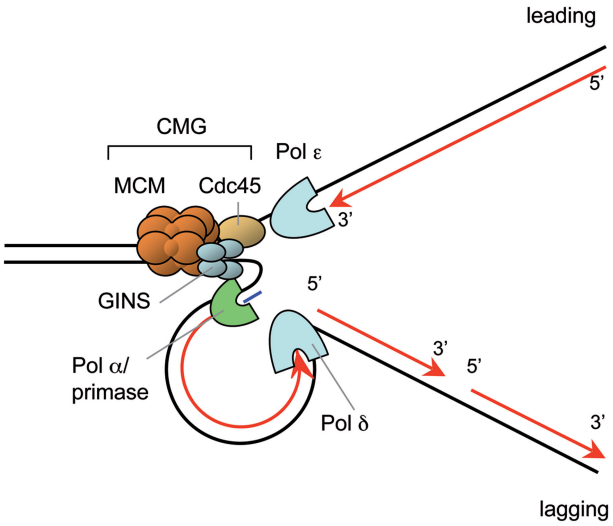

Figure 7. A model for hGINS at the replication fork. The hGINS tetramer is part of the CMG DNA helicase, probably acting as a molecular bridge between the MCM hexamer and the Cdc45 monomer and stabilizing their interaction. hGINS may also interact with ssDNA during DNA unwinding. At the same time, it may assist in the recruitment and/or function of the DNA polymerase α/primase acting at the lagging strand. See text for details.

that synthesizes short RNA primers at the lagging strand (31). Interestingly, a physical interaction between hGINS and DNA polymerase α/primase has been revealed by surface plasmon resonance and hGINS seems to stimulate DNA polymerase α/primase activity *in vitro* (32). Finally, it should also be considered that hGINS can bind to single-stranded DNA (ssDNA) or ss/double-stranded DNA (ss/dsDNA) duplexes and may participate actively in the DNA unwinding reaction, possibly acting as a 'molecular ploughshare' (11,17,33). Combining these observations, we support a model (Figure 7) in which GINS is an essential component of the eukaryotic replisome, on one hand as a key element of the DNA helicase complex that generates the two template strands, and on the other hand facilitating the recruitment and/or activity of the DNA polymerase α/primase that operates at the lagging strand.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Ana Losada for useful comments on the manuscript. We are indebted to Mónica López (DNA Replication Group); Diego Megías (CNIO Flow Cytometry and Confocal Microscopy Unit); Giovanna Roncador (CNIO Monoclonal Antibody Unit) and Lydia Sánchez (CNIO Immunohistochemistry Unit) for technical support.

FUNDING

Spanish Ministry of Science and Innovation (BFU-04886, CSD2007-0015 and a 'Ramón y Cajal' contract to J.M., BFU2008-01344 to G.M., PhD studentships to T.A. and J.C.); European Union (MC IRG FP6-031129 to J.M., 3D-Repertoire LSHG-CT-2005-512028 to G.M.); Fundación Caja Madrid; postdoctoral fellowship from the Fondation Recherche Médicale, France (to E.G.). Funding for open access charge: Spanish Ministry of Science and Innovation.

Conflict of interest statement. None declared.

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