

# Ciz1 promotes mammalian DNA replication

Dawn Coverley<sup>1,2,\*</sup>, Jackie Marr<sup>2</sup> and Justin Ainscough<sup>3</sup>

<sup>1</sup>Department of Biology (Area 9), University of York, York, YO10 5YW, UK

<sup>2</sup>Hutchison/MRC Research Centre, MRC Cancer Cell Unit, Hills Road, Cambridge, CB2 2XZ, UK

<sup>3</sup>Heart Research Centre and CRISTAL, Leeds University, Leeds, LS2 9JT, UK

\*Author for correspondence (e-mail: dc17@york.ac.uk)

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## Summary

Using a cell-free system that reconstitutes initiation of mammalian DNA replication, we identified a cyclin A-responsive protein, p21<sup>Cip1</sup>-interacting zinc finger protein 1 (Ciz1). In cell-free experiments, Ciz1 protein increases the number of nuclei that initiate DNA replication, and in intact cells GFP-tagged Ciz1 stimulates DNA synthesis, in both a wild-type and a p21<sup>Cip1</sup> null background. Furthermore, mutation of a putative cyclin-dependent kinase phosphorylation site at threonines 191/2 alters Ciz1 activity *in vitro*, indicating that this site plays a role in regulating Ciz1. Consistent with a role in DNA replication, endogenous Ciz1 is present in nuclear foci that co-localize with PCNA during S phase, and targeted depletion of Ciz1 transcripts restrains cell proliferation by inhibiting entry

to S phase. Ciz1-depleted cells accumulate with chromatin bound Mcm3 and PCNA but fail to synthesize DNA efficiently. These cell-based and cell-free experiments suggest that Ciz1 functions to promote DNA replication after replication complex formation. Finally, alternatively spliced forms of Ciz1 occur in embryonic cells from mouse and man, raising the possibility that Ciz1 splicing contributes to the regulation of DNA replication during development.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/118/1/101/DC1>

Key words: DNA replication, Cell cycle, Ciz1, Alternative splicing

## Introduction

Initiation of DNA replication is a major control point in the mammalian cell cycle and the point of action of many gene products that are mis-regulated in cancer (Hanahan and Weinberg, 2000). The initiation process involves assembly of pre-replication complex proteins, which include the origin recognition complex (ORC), Cdc6, Cdt1 and Mcm proteins, at replication origins during G1 phase of the cell cycle. This is followed by the action of a second group of proteins, which facilitate loading of DNA polymerases and their accessory factors, including PCNA, and the transition to S phase. The initiation process is regulated by cyclin-dependent protein kinase 2 (Cdk2), Cdc7-dbf4 and the Cdt1 inhibitor geminin (Bell and Dutta, 2002; Li and Blow, 2004). However, the temporal and spatial organisation of DNA replication within the S phase nucleus is less well understood, particularly in multicellular organisms that must organise the genome to facilitate changing patterns of gene expression (Gilbert, 2002). In the nucleus of S phase cells, replication forks cluster together to form hundreds of immobilised replication factories (Cook, 1999; Leonhardt et al., 2000) that appear to be linked to a structural framework, however, the molecules that link replication factories with the nucleoskeleton are unknown.

Identification of proteins involved in eukaryotic DNA replication and analysis of the basic pathways that regulate their activity during the cell cycle has been driven largely by yeast genetics. These proteins and pathways are generally conserved from yeast to man. However, in multicellular

organisms that differentiate down diverse developmental pathways, additional layers of complexity are being uncovered. For example, in vertebrates several proteins involved in neuronal differentiation also regulate the G1 to S phase transition (Ohnuma et al., 2001). These include the cdk inhibitor p21<sup>CIP1/WAF1/SDI1</sup>, which has been implicated in oligodendrocyte differentiation following growth arrest (Zezula et al., 2001), and in the terminal differentiation of other cell types (Parker et al., 1995). p21<sup>Cip1</sup> has both positive and negative effects during G1 phase (Sherr and Roberts, 1999). It promotes cyclin-cdk complex formation, and it also functions to restrain the onset of S phase by two well characterized routes: the amino-terminal domain binds and inhibits cyclin-cdk2 complexes, and the carboxyl-terminal domain binds and inhibits the DNA replication activity of PCNA. The pathways by which p21<sup>Cip1</sup> regulates cell proliferation during differentiation have not been established, therefore proteins that interact with p21<sup>Cip1</sup>, particularly those that have a role in DNA replication, are of considerable interest.

Initiation of DNA replication can be reconstituted *in vitro* with isolated nuclei and cytosolic extracts from mammalian cells (Krude, 2000; Krude et al., 1997; Laman et al., 2001; Stoeber et al., 1998). Furthermore, using recombinant Cdk2 complexed with either cyclin E or A, replication complex assembly and activation of DNA synthesis can be reconstituted independently (Coverley et al., 2002). Here, we focus on the activation step, catalysed *in vitro* by cyclin A-cdk2, and present evidence that a relatively unstudied protein, p21-Cip1

interacting zinc-finger protein (Ciz1) is involved in this stage. Human Ciz1 was previously identified using a modified yeast two-hybrid screen with cyclin E-p21, and biochemical analysis supported an interaction with p21<sup>Cip1</sup> (Mitsui et al., 1999). A potential role in transcription was proposed but not demonstrated, and no other function has been assigned to Ciz1. More recently the Ciz1 gene was isolated from a human medulloblastoma-derived cDNA library using an in vivo tumorigenesis model (Warder and Keherly, 2003). These two investigations used very different approaches to implicate Ciz1 in proliferation control in mammalian cells. Our analysis takes these observations further to show that Ciz1 plays a positive role in DNA replication.

## Materials and Methods

### Cloning

Ciz1 was cloned from a  $\lambda$  TriPLEX full-length enriched cDNA expression library derived from day 11 mouse embryos (Clontech ML5015t). Antibody V was applied to approximately  $3 \times 10^6$  plaques, as recommended, and 43 independent plaques were picked. Only two clones survived a further three rounds of screening. One encoded mouse Cdc6 (clone P) and the other (clone L) encoded an uncharacterized protein, with strong homology to human Ciz1. We refer to this as embryonic Ciz1 (ECiz1) and it was submitted to EMBL under the accession number AJ575057.

### Bacterial expression

pGEX-based bacterial expression constructs (Amersham) were used to produce ECiz1 proteins for in vitro analysis. pGEX-ECiz1 was generated by inserting a 2.3 kb *SmaI-XbaI* (blunt ended) fragment from clone L into the *SmaI* site of pGEX-6P-3. pGEX-Nterm442 was generated by inserting the 1.35 kb *XmaI-XhoI* fragment into *XmaI-XhoI*-digested pGEX-6P-3, and pGEX-Cterm274 by inserting the 0.95 kb *XhoI* fragment into *XhoI*-digested pGEX-6P-3. pGEX-T(191/2)A was generated from pGEX-ECiz1 by site directed mutagenesis (Stratagene Quikchange) using primers AACCCCCTCTTCCGCGCCCAATCGCAAGA and TCTTGCGATTGGGGCGGCGGAAGAGGGGGTT. pGEX-T(293)A was generated from pGEX-ECiz1 using primers AAGCAGACACAGGCCCGGATCGGCTGCCT and AGGCAGCCGATCCGGGGCCTGTGTCTGCTT. Integrity and reading frame of all clones were sequence verified. Alignments were performed with Multialin (Corpet, 1998).

Recombinant GST-tagged proteins were produced in BL21-pLysS (Stratagene) and purified from sonicated cleared lysates by binding to glutathione Sepharose 4B (Amersham). Protein was eluted by cleavage from the GST tag using precision protease (Amersham), into buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM DTT) yielding protein preparations between 0.2 and 2.0 mg/ml. Cyclin A-cdk2 was produced in baculovirus-infected SF9 insect cells as described previously (Laman et al., 2001). Activity in clarified insect cell lysates was measured using histone H1 or pRb as a template, then calibrated for initiation activity by titrating into replication assays as shown previously (Coverley et al., 2002). Peak cyclin A-cdk2 activity corresponds to  $3.5 \times 10^5$  Rb phosphorylating units per  $\mu$ l of replication reaction, and has the same replication specific activity as 1 nM purified kinase (Coverley et al., 2002).

### Mammalian expression

Full-length mouse Ciz1 cDNA was obtained from UK HGMP Resource Centre (MGC clone 27988). A 2.8 kb *SmaI-XbaI* (blunt ended) fragment from this clone and a 2.3 kb *SmaI-XbaI* (blunt ended) ECiz1 fragment from pTriplex-clone L were ligated into the *SmaI* site

of pEGFP-C3 (Clontech). pEGFP-C3 was used as control. Constructs were delivered into cells using *TransIT-293* (Mirus) or Amaxa nucleofactor kit R, program U-30.

### Anti-Ciz1 antibodies

Rabbit polyclonal antibody V (Coverley et al., 2000; Stoeber et al., 1998; Williams et al., 1998) was raised against an internal fragment of bacterially expressed human Cdc6 corresponding to amino-acids 145-360 and affinity purified by standard procedures (Harlow and Lane, 1988). This antibody reacts strongly with endogenous mouse p100-Ciz1 and also with ECiz1 Nterm442 fragment. Alignment of Nterm442 with human Cdc6 amino-acids 145-360 suggest that the shared epitope could be at 294-298 or 304-312 in mouse Ciz1. Recombinant Nterm442 was used to generate Ciz1-specific polyclonal anti-serum designated 1793 (Abcam). Its specificity was verified by reciprocal immunoprecipitation and western blot analysis with antibody V, by inclusion of Nterm 442 (25  $\mu$ g/ml in antibody buffer: 10 mg/ml BSA, 0.02% SDS, 0.1% Triton X-100 in PBS), which blocked reactivity with endogenous epitopes (not shown), by reactivity with GFP-ECiz1 and by siRNA-mediated depletion of endogenous and GFP-Ciz1, that specifically reduced the reactivity of 1793.

### Immunoprecipitation

Asynchronously growing 3T3 cells were washed in PBS, rinsed in extraction buffer (20 mM Hepes pH 7.8, 5 mM potassium acetate, 0.5 mM magnesium chloride) supplemented with EDTA-free protease inhibitor cocktail (Roche) and scrape harvested as for replication extracts. Cells were lysed with 0.1% Triton X-100 and the detergent-resistant pellet fraction extracted with 0.3 M NaCl in extraction buffer. 5  $\mu$ l of 1793 or 2  $\mu$ l of antibody V were used per 100  $\mu$ l of extract and incubated for 1 hour at 4°C. Antigen-antibody complexes were extracted with 100  $\mu$ l of protein G-Sepharose (Sigma) and beads were washed five times with 50 mM Tris pH 7.8, 1 mM EDTA, 0.1% NP40, 150 mM NaCl. Complexes were boiled in loading buffer (100 mM DTT, 2% SDS, 60 mM Tris pH 6.8, 0.001% bromophenol blue) and resolved by 6.5% SDS-polyacrylamide gel electrophoresis.

### Western blots

Ciz1 protein was divided into soluble and insoluble fractions by mixing scrape-harvested cells (in hypotonic buffer plus protease inhibitor cocktail) with an equal volume of 0.1% Triton X-100, followed by centrifugation to gently pellet the insoluble nuclear-bound fraction. 1793 was used at a 1/1000 dilution, according to standard procedures and western blots were developed using enhanced chemiluminescence (ECL) and pre-flashed chemiluminescence film (Amersham).

### Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde, with or without brief pre-exposure to 0.05% Triton X-100 in PBS. Endogenous Ciz1 was detected with 1793 serum diluted 1/2000 in antibody buffer following standard procedures. Mcm3 was detected with monoclonal antibody sc9850 (1/1000), Cdc6 with monoclonal sc9964 (1/100) and PCNA with monoclonal antibody PC10 (1/100; all from Santa Cruz Biotechnology). Actin was detected with monoclonal A4700 antibody (1/500; Sigma). Co-localisation analysis of dual stained fluorescent confocal images was carried out as described previously (Rubbi and Milner, 2000; van Steensel et al., 1996).

### Cell culture, synchrony and replication materials

Mouse 3T3 cells were synchronized by release from quiescence as

previously described (Coverley et al., 2002). Cell-free replication materials were prepared as described previously (Coverley et al., 2002; Krude et al., 1997), except that nuclei were frozen in two times their packed cell volume of hypotonic buffer without additional wash steps. Nuclei prepared from cells harvested 17 hours after release (referred to as 'late-G1') were used in all cell-free replication experiments described here. Recipient, mid-G1 3T3 extracts were prepared at 15 hours. In the series of replication experiments that test the activity of Ciz1 and derived fragments and mutants, a large amount of standardized extract was required, therefore HeLa cells were used because they are more easily synchronized in bulk. S phase HeLa extracts were prepared from cells released for 2 hours from two sequential thymidine-induced S phase blocks, as described previously (Krude et al., 1997).

Mouse embryo fibroblasts from p21 null mice were kindly provided by M. Roussel and cultured as described previously (Zindy et al., 1997). Transfection experiments were carried out between passages 8-11, one day after plating for cycling cells and four days after plating for confluent cells.

### Cell-free DNA replication

DNA replication assays were performed as described previously (Coverley et al., 2002; Krude et al., 1997). Briefly, 10  $\mu$ l of mid G1 or S phase extract (supplemented with energy regenerating system, nucleotides and biotinylated dUTP) and  $5 \times 10^4$  late G1 phase nuclei were incubated for 60 minutes at 37°C. Reactions were supplemented with recombinant proteins that were serially diluted in 100 mM Hepes pH 7.8, 1 mM DTT, 50% glycerol, so that not more than 1  $\mu$ l was added to 10  $\mu$ l replication assays, generating the concentrations indicated. Reactions were stopped with 50  $\mu$ l of 0.5% Triton X-100 and fixed by the addition of 50  $\mu$ l of 8% paraformaldehyde, for 5 minutes. After transfer to coverslips, nuclei were stained with streptavidin-FITC (Amersham) and counterstained with TOTO-3 iodide (Molecular Probes). The proportion of labelled nuclei was quantified by inspection at 1000 $\times$  magnification, and all nuclei with fluorescent foci or intense uniform labelling were scored positive. For analysis of nuclear proteins, nuclei were re-isolated after 15 minutes exposure to initiating conditions, by diluting reactions twofold with cold PBS and gentle centrifugation.

### Analysis of cell-free replication data

Prior to use in replication experiments each preparation of synchronized G1 phase nuclei was tested to determine the proportion of nuclei already in S phase ('%S'), by incubating in non-initiating mid-G1 phase extract which only supports elongation DNA synthesis. This fraction is uninformative in studies of initiation, and is usually around 20% in 3T3 populations harvested at 17 hours after release from quiescence.

When 3T3 cells are released from quiescence by the protocol used here, no more than 70% of the total population enters S phase (Coverley et al., 2002). However, the highest observed replication frequency in vitro is nearer 50%; usually obtained by incubation with ECiz1. For the G1 population of 3T3 nuclei used, 17% were in S phase (%S) and the maximum number that replicated in any assay in vitro was 51%. Therefore, 34% of this population is *competent* to initiate replication in vitro (%C). Thus, for each data point, % initiation = (% replication - %S)/%C  $\times$  100, where '% replication' is variable and depends on the conditions of the reaction.

### RNA interference

Endogenous Ciz1 was targeted in proliferating NIH3T3 cells using in vitro transcribed siRNAs (Ambion Silencer kit), directed against two regions of mouse Ciz1 that are present in all known Ciz1 splice

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variants. Oligonucleotide sequences used to generate siRNAs using the T7 transcription-based Silencer Kit are:

AA-GCACAGTCACAGGAGCAGA-CCTGTCTC

and AA-TCTGCTCCTGTGACTGTGC-CCTGTCTC for siRNA 4,

and AA-TCTGTACAAGTTCTACGA-CCTGTCTC

and AA-TCGTAGAACTTGTGACAGA-CCTGTCTC for siRNA 8.

Negative controls were untreated, mock treated (transfection reagents but no siRNA) and cells treated with GAPDH siRNA (Ambion). In northern blot experiments, GAPDH siRNA caused a specific 50% reduction in GAPDH, relative to  $\beta$ -actin, at 24 hours (not shown). Cy3-labelled siRNAs (Ambion) were used to estimate transfection efficiency, which was found to be greater than 95% (not shown). RNA interference experiments were performed in 24-well plates, starting with  $2 \times 10^4$  cells per well in 500  $\mu$ l medium (DMEM with glutamax supplemented with 4% FCS), or scaled up fivefold. siRNAs were added 12 hours after plating using oligofectamine reagent for delivery (Invitrogen). siRNAs were delivered individually at 3 nM or 4 and 8 were used as a mixture at 2 nM total concentration, with control GAPDH siRNA also used at 2 nM. Where results were analysed at 48 hours cells received two doses separated by 24 hours. For synchronous siRNA delivery we used an Amaxa nucleofector (kit R, program U-30) with approximately  $1 \times 10^6$  quiescent cells, or  $1 \times 10^6$  cycling cells. For cell cycle re-entry experiments, 40 ng/ml nocodazole was added to the culture medium to prevent passage through mitosis. The effect of Ciz1 siRNAs on endogenous Ciz1 protein was assessed by immunostaining detergent-resistant and detergent-soluble protein fractions using antibody 1793. Band intensities were quantified using Image Quant TL software (v2002) and expressed in arbitrary units, relative to actin levels. Prevention of new Ciz1 synthesis was verified by co-expressing GFP-ECiz1 with Ciz1 siRNA4/8 or GAPDH siRNA. Results were assessed by western blotting total protein from approximately  $5 \times 10^5$  cells with anti-Ciz1 1793, and by imaging GFP expression at 22 hours after transfection (not shown).

### S phase labelling

The fraction of cells engaged in DNA synthesis was monitored by supplementing culture medium with 20  $\mu$ M BrdU (Sigma), for 17 hours or for 30 minute pulse. Incorporated BrdU was visualised with monoclonal RPN20EZ (Amersham) and anti-mouse Alexa 568 (Alexis Biochemicals).

### Flow cytometry

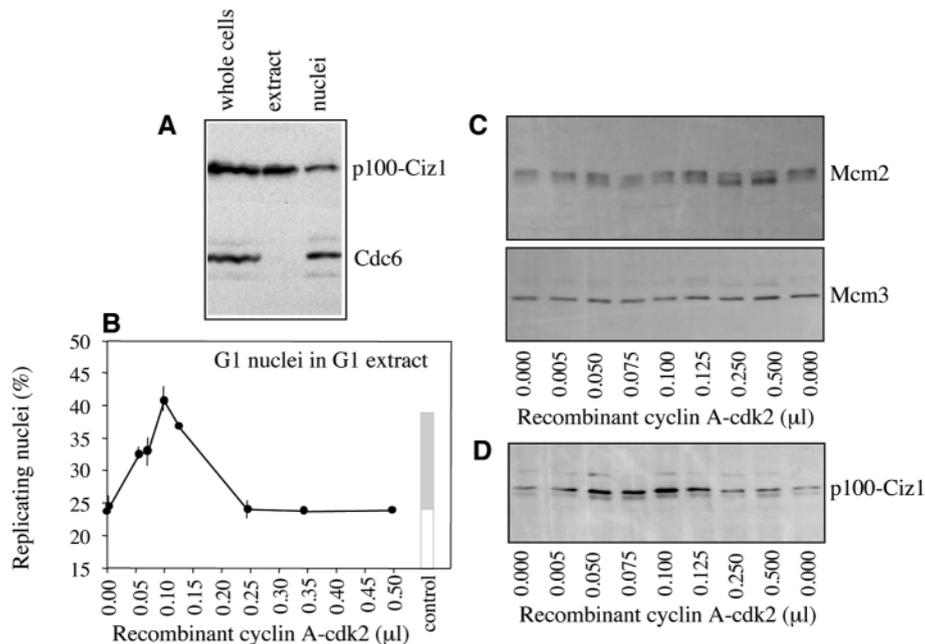
siRNA-treated cells were harvested with trypsin 18 and 36 hours after treatment and fixed in cold 80% ethanol. Cells were resuspended in PBS, and incubated with 0.1% Triton X-100 and 0.1 mg/ml propidium iodide before profiling DNA content using a Dako Cytomation CyAn flow cytometer and Summit ver3.1 software.

## Results

### Identification of Ciz1

We have exploited a polyclonal antibody (antibody V) that was raised against recombinant human Cdc6 (Coverley et al., 2000; Stoeber et al., 1998; Williams et al., 1998) to identify and study an unknown cross-reacting antigen whose behaviour correlates with initiation of DNA replication in vitro. The antigen has an apparent molecular mass of 100 kDa and is readily detectable in extracts from mouse 3T3 cells (Fig. 1A).

DNA synthesis can be activated in cell-free replication reactions containing 'replication competent' late G1 phase nuclei and G1 extracts, using recombinant cyclin A-cdk2



**Fig. 1.** p100-Ciz1 is co-regulated with cyclin A-cdk2-induced DNA replication. (A) Anti-Cdc6 antibody V detects mouse Cdc6 and a second antigen in western blots of 3T3 whole cell extract, which migrates with an approximate molecular mass of 100 kDa. p100 is present in both the soluble fraction and insoluble nuclear fraction (prepared under *in vitro* replication conditions). (B) Initiation of DNA synthesis in 'replication competent' late G1 phase nuclei by G1 extract supplemented with recombinant cyclin A-cdk2. Control bar shows the proportion of nuclei already in S phase (unshaded), and those that initiated replication in extract from S phase cells (shaded). (C) Late G1 nuclei re-isolated after incubation in parallel reactions with recombinant cyclin A-cdk2 and G1 extract, probed for Mcm2 and Mcm3. (D) p100 antigen is more abundant in nuclei exposed to initiation-inducing concentrations of cyclin A-cdk2, revealed when the same nuclei are probed with antibody V.

(Coverley et al., 2002). Under these conditions nuclei will incorporate labelled nucleotides into nascent DNA, in a manner strictly dependent on the concentration of active protein kinase (Fig. 1B). Above and below the optimum concentration no initiation of DNA replication takes place. However, other events occur which inversely correlate with initiation. We used activation of DNA synthesis (Fig. 1B) and Mcm2 phosphorylation (which results in increased mobility; Fig. 1C) to calibrate the effects of recombinant cyclin A-cdk2 in cell-free replication experiments, and correlate the behaviour of p100 with activation of DNA synthesis.

In G1 nuclei re-isolated from reactions containing initiation-inducing concentrations of cyclin A-cdk2, p100 antigen is more prevalent than in nuclei exposed to lower or higher concentrations of kinase (Fig. 1D). This suggests that p100 is regulated at some level by cyclin A-cdk2, in a manner coincident with activation of DNA synthesis. Interestingly, increase in p100 may be due to alterations in antigenicity rather than altered protein level, as a second anti-p100 antibody (1793, see below) does not reveal a similar change (not shown). As the behaviour of p100 correlates so closely with *in vitro* activation of DNA synthesis, we used antibody V to clone the gene for mouse p100.

When applied to a cDNA expression library derived from 11-day mouse embryos antibody V identified two clones (see Materials and Methods). One encoded mouse Cdc6, and the other encoded 716 amino acids of the murine homologue of human Ciz1 (Mitsui et al., 1999). Full-length human and mouse Ciz1 have approximately 70% overall homology at the amino-acid level, with greatest (>80%) homology in the N- and C-terminal regions (not shown). Ciz1 is conserved among vertebrates as homologues exist in rat and fugu, but no proteins with a high degree of homology or similar domain structure could be identified in lower eukaryotes, raising the possibility that Ciz1 evolved to perform a specialised role in vertebrate development.

#### Multiple Ciz1 isoforms

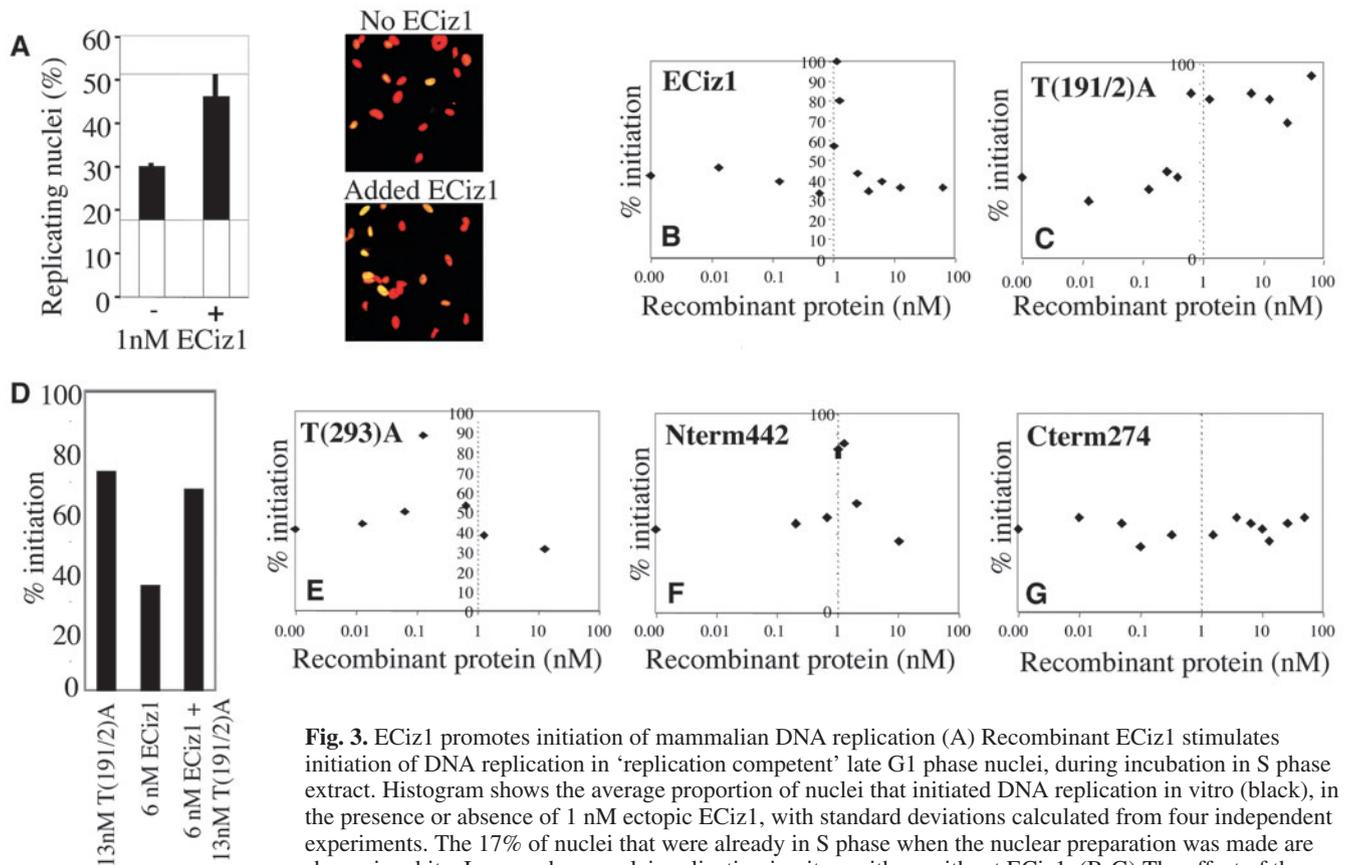
The predicted mouse Ciz1 open reading frame and a cDNA derived from a mouse mammary tumour library (BC018483) contain three regions that are not present in our embryonic clone (AJ575057), hereafter referred to as ECiz1 (Fig. 2A). The three variable regions in ECiz1 are generated by alternative splicing of exons 2/3, 6 and 8 (Fig. 2B). Mouse melanoma clone AK089986 lacks two of the same three regions as ECiz1 (Fig. 2A), while the third encodes an N-terminal glutamine-rich stretch that is also absent from human medulloblastoma-derived clones. In fact, all known variations in mouse Ciz1 cDNAs have close human parallels, some of which are identical at the amino-acid level. This suggests that differences between Ciz1 isoforms have functional significance.

The regions that are consistently present in all known isoforms of human and mouse Ciz1 contain several sequence features that suggest possible functions (Fig. 2C). These include predicted cyclin-dependent kinase phosphorylation sites, a putative nuclear localisation sequence (NLS), three C2H2-type zinc fingers and a C-terminal region with homology to the nuclear matrix protein matrin 3 (Nakayasu and Berezney, 1991). In order to investigate the functional significance of these domains we generated a series of protein expression constructs, derived from ECiz1, that disrupt several of these features (Fig. 2D).

#### ECiz1 stimulates DNA replication *in vitro*

Upon exposure to cytosolic extract from S phase cells, late G1 phase nuclei initiate DNA replication and begin synthesizing nascent DNA (Krude et al., 1997). We used this cell-free assay to test the effect of ECiz1 and derived recombinant fragments on DNA synthesis (Fig. 3; see also supplementary material). Full-length ECiz1 protein consistently increased the number of nuclei that replicated *in vitro*, from 30% ( $\pm 0.9\%$ ) to 46%





**Fig. 3.** ECiz1 promotes initiation of mammalian DNA replication (A) Recombinant ECiz1 stimulates initiation of DNA replication in 'replication competent' late G1 phase nuclei, during incubation in S phase extract. Histogram shows the average proportion of nuclei that initiated DNA replication in vitro (black), in the presence or absence of 1 nM ectopic ECiz1, with standard deviations calculated from four independent experiments. The 17% of nuclei that were already in S phase when the nuclear preparation was made are shown in white. Images show nuclei replicating in vitro, with or without ECiz1. (B-G) The effect of the various recombinant proteins on initiation of DNA replication. (B) The effect of ECiz1 is concentration dependent, with a sharp optimum around 1 nM. (C) Mutation of the predicted cdk phosphorylation site at 191/2 alters the activity profile of ECiz1, so that T(191/2)A mutant remains capable of stimulating initiation even at high concentrations. (D) T(191/2)A (13 nM) functions in the presence of inactive (6 nM) concentrations of ECiz1. (E) Cdk site mutant T(293)A stimulates initiation with a similar profile to ECiz1. (F) Truncated ECiz1 (Nterm 442) lacks C-terminal sequences, but stimulates in vitro initiation to a similar extent as ECiz1. (G) Cterm 274 retains no DNA replication activity in this assay.

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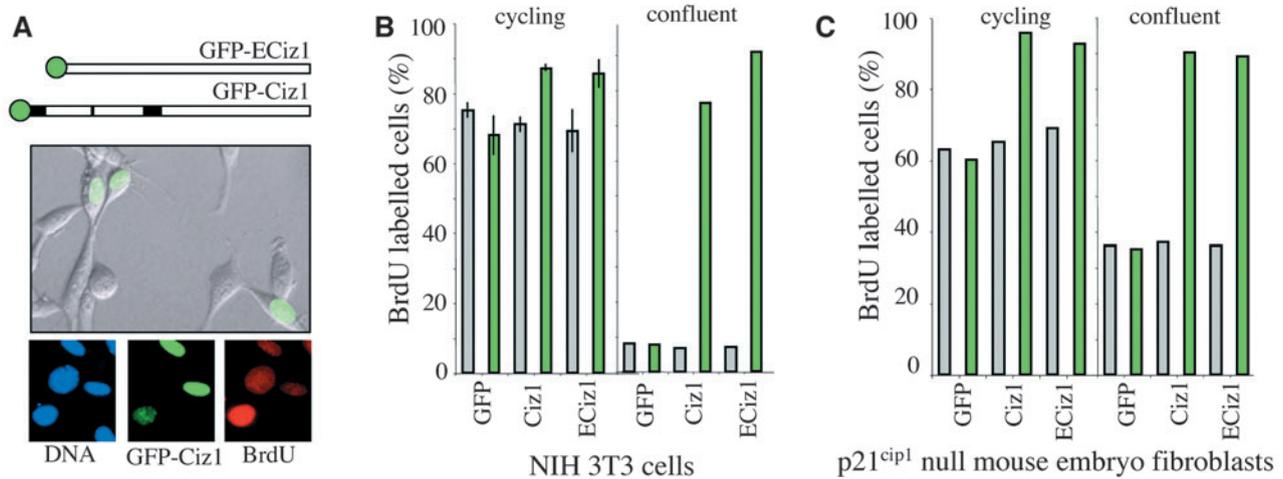
the N-terminal fragment of ECiz1 that contains in vitro replication activity (see below) and one of these is adjacent to the site at which exon 6 is alternatively spliced to exclude a short DSSSQ sequence motif (Fig. 2A,C). As this motif is 100% identical and alternatively spliced in both mouse and man we reasoned that conditional inclusion might serve to regulate Ciz1 activity, identifying this region of the protein as potentially important. We therefore chose to focus on the cdk site that is four residues upstream and which is also conserved in mouse and man, by combining a genetic approach with cell-free replication assays. Starting with ECiz1, two threonines at 191 and 192 were changed to two alanines, generating ECiz1T(191/2)A (Fig. 2D). When tested in vitro for DNA replication activity, ECiz1T(191/2)A stimulated initiation in late G1 nuclei to a similar extent as ECiz1 (Fig. 3C). However, unlike ECiz1, stimulation of initiation was maintained over a broad range of concentrations that extended over at least three orders of magnitude. Therefore, a mechanism to restrict the activity of excess ECiz1 exists and operates in a cell-free environment. Furthermore, in mixed assays with ECiz1 in the high, inactivated concentration range (6 nM) and ECiz1T(191/2)A (13 nM) the unphosphorylatable mutant is dominant (Fig. 3D). These results demonstrate that down-

regulation of ECiz1 activity involves threonine 191/2, and suggest it may be caused by cyclin-dependent kinase-mediated phosphorylation at this site.

In a separate construct, the threonine at position 293 was also changed to alanine generating ECiz1T(293)A (Fig. 2D). This increased the potency of ECiz1 so that peak stimulation of initiation occurred around 0.1 nM (Fig. 3E), and is also consistent with an inhibitory role for cyclin-dependent kinase-mediated phosphorylation in the regulation of Ciz1.

#### In vitro replication activity resides in the N-terminal portion of ECiz1

Ciz1 possesses several C-terminal features that may anchor the protein within the nucleus. The matrin 3 domain suggests possible interaction with the nuclear architecture and the three zinc fingers imply interaction with nucleic acids. Indeed, recent evidence suggests that human Ciz1 binds DNA in a weakly sequence-specific manner (Warder and Keherley, 2003). To determine whether C-terminal domains are important for ECiz1 replication activity we divided the protein into two fragments (Fig. 2D). Nterm442 (which contains the NLS, four cdk sites, one zinc finger and all known sites where variable



**Fig. 4.** Ciz1 and ECiz1 stimulate S phase entry in wild-type and p21 null cells. (A) ECiz1 and the full-length variant, Ciz1, were tagged with green fluorescent protein to allow identification of expressing cells (green), within transfected populations. Examples of Ciz1-expressing and -non-expressing cells that have or have not engaged in DNA synthesis (and incorporated BrdU into DNA, red) are shown. Nuclei are counterstained with Hoechst 33258 (blue). (B) Expression of ectopic GFP-Ciz1 or ECiz1 in the presence of BrdU increases the number of NIH 3T3 cells that undergo DNA synthesis in rapidly cycling and newly confluent populations, during the 17 hour period following transfection. Cells expressing GFP alone are not stimulated to engage in DNA synthesis. (C) Similar results were obtained with p21<sup>Cip1</sup> null mouse embryo fibroblasts. Histograms show GFP-expressing cells as green bars and non-expressing cells from the same populations as grey bars. Representative results from three independent experiments for each of the conditions are shown. All experiments gave the same effect, but the background fraction of labelled cells in the untransfected population varied according to the density and growth rate of the cells.

splicing has been observed) stimulates initiation to a similar extent and at the same concentration as ECiz1 (Fig. 3F). In contrast, the C-terminal portion (Cterm274) contains no residual replication activity (Fig. 3G). Therefore, the matrin 3 domain, one of the cdk sites and two of the zinc-fingers are not required for activity, when assayed *in vitro*. It should be noted, however, that this analysis measures ECiz1 activity *in trans* under conditions where the consequences of mis-localisation are unlikely to be detected and it remains possible that these features normally act to direct Ciz1 to specific sites, thus limiting the scope of its activity.

#### Ciz1 and ECiz1 stimulate DNA synthesis in intact cells

We have been unable to produce full-length recombinant mouse Ciz1 protein in soluble form (including the three alternatively spliced regions), which precludes direct comparison with ECiz1 in cell-free DNA replication experiments. Therefore, to make comparisons between Ciz1 and ECiz1 and also to test the effect of exogenous Ciz1/ECiz1 on intact cells, we transfected GFP-tagged constructs into cycling NIH 3T3 cells, in the presence of the nucleotide analogue bromodeoxyuridine (BrdU; Fig. 4A). GFP-tagged proteins were clearly detectable around 12 hours after transfection, reaching maximal levels by 22 hours. In cell populations exposed to BrdU continuously between 0 and 17 hours those transfected with Ciz1 or ECiz1 incorporated BrdU in greater numbers than untransfected cells in the same population, rising from 70% ( $\pm 4.6\%$ ), to 87% ( $\pm 0.7\%$ ) and 86% ( $\pm 3.5\%$ ), respectively (Fig. 4B). Cells transfected with GFP control showed no difference. This data is drawn from three experiments, in which the effects of Ciz1 and ECiz1 are indistinguishable, but differ from non-expressing cells by 16-

17% ( $P < 0.0005$ ). More dramatically, in newly confluent cells that had recently exited the cell cycle, Ciz1 and ECiz1 increased the number of BrdU-labelled cells more than tenfold (Fig. 4B). Using this strategy the effect of Ciz1/ECiz1 was measured against a lower background of replication in the untransfected fraction (less than 10% rather than the 70% mentioned above). BrdU labelling for the whole period between transfection and expression, rather than pulse labelling at intervals, rules out the possibility that Ciz1/ECiz1 increase the labelled fraction by delaying *exit* from S phase, rather than promoting *entry* to S phase. It should be noted that deeply quiescent cells did not respond to Ciz1/ECiz1 (not shown). These experiments indicate that both Ciz1 and ECiz1 stimulate DNA replication in intact cells, and are consistent with our cell-free analysis that demonstrates a positive effect on DNA replication in isolated nuclei.

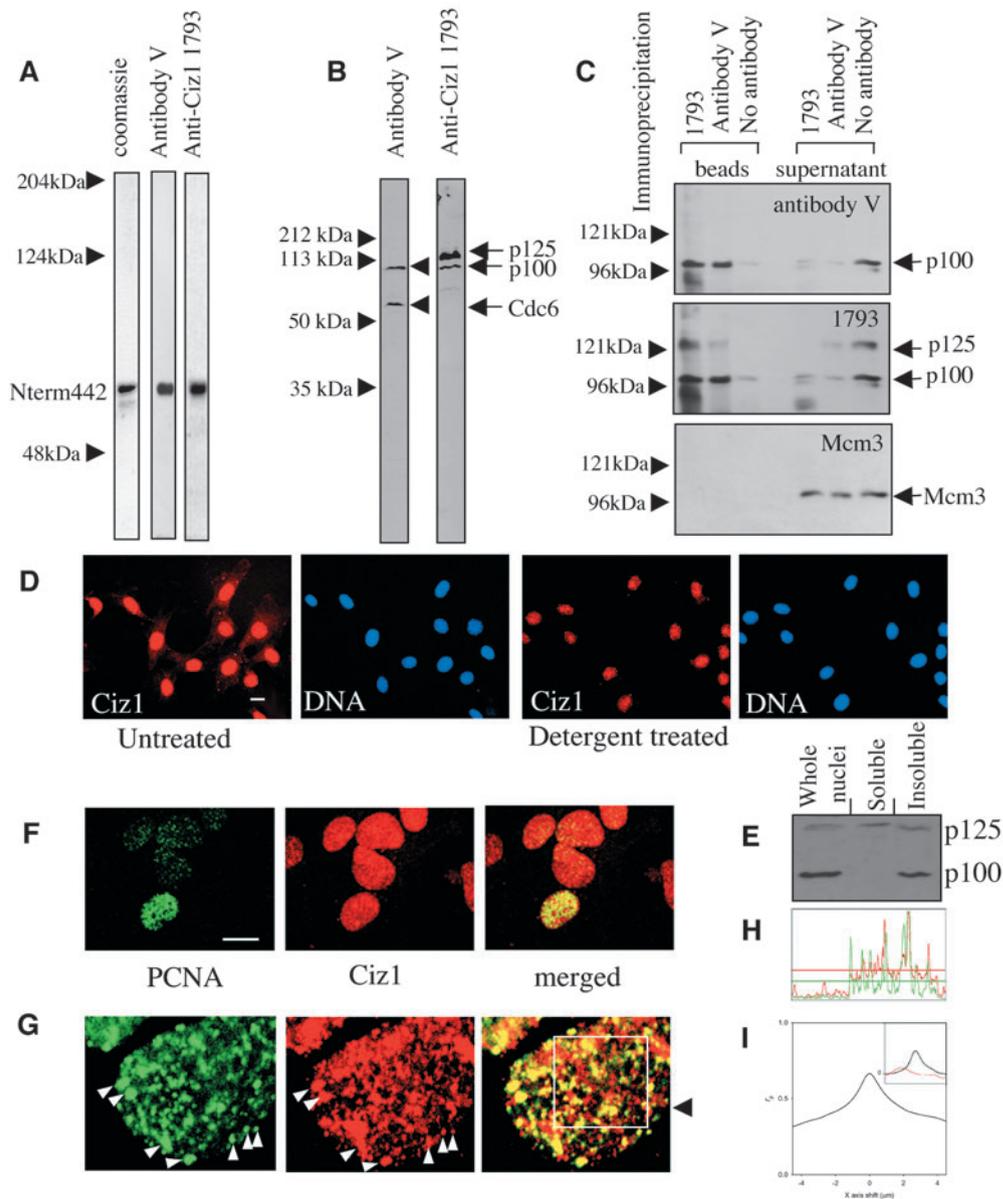
#### Ciz1 and ECiz1 stimulate DNA synthesis in p21<sup>Cip1</sup> null cells

The stimulatory effect that we observed in cell-free and cell-based replication experiments could occur in several ways. Ciz1 could act directly to stimulate the replication machinery, or indirectly by neutralizing factors that normally restrain the replication machinery. Ciz1 was previously identified because of its interaction with p21<sup>Cip1</sup> (Mitsui et al., 1999) and might therefore relieve p21-mediated inhibition of cdk2 in a manner similar to the E7 oncoprotein (Jones et al., 1997). Consistent with this possibility Ciz1 binds to the cdk-interacting N-terminal part of p21<sup>Cip1</sup> (Mitsui et al., 1999). To test whether Ciz1 stimulates DNA replication through p21<sup>Cip1</sup> we looked at the ability of Ciz1/ECiz1 to stimulate DNA replication in p21<sup>Cip1</sup> null cells (Fig. 4C). In both cycling and newly

**Fig. 5.** Anti-Ciz1 antibody detects endogenous Ciz1 in sub-nuclear foci that overlap with sites of DNA replication. (A) Coomassie Blue-stained SDS-polyacrylamide gel showing purified recombinant ECiz1 fragment Nterm442, and western blots of recombinant Nterm442 using anti-Cdc6 antibody V, and anti-Ciz1 antibody 1793. (B) Western blot of 3T3 whole cell extract. Of the two bands detected by anti-Ciz1 antibody 1793 one has the same mobility as p100-Ciz1 recognized by antibody V and the other has an apparent molecular mass of 125 kDa.

(C) Immunoprecipitation from 3T3 nuclear extract, using antibody V or anti-Ciz1 1793. Both antibodies precipitate p100, which is recognized by the reciprocal antibody in western blots. p125 is precipitated by antibody 1793, and to a lesser extent by antibody V and these are recognized by 1793 in western blots. Mcm3 is shown as a control. (D) Endogenous Ciz1 (red) in 3T3 cells fixed before (untreated) or after (detergent treated) exposure to TritonX100, detected with anti-Ciz1 antibody 1793. Nuclei are counterstained with Hoechst 33258 (blue).

(E) Nuclei isolated for replication experiments contain detergent resistant and detergent soluble Ciz1 protein, detected with anti-Ciz1 1793. (F) Detergent-resistant Ciz1 (red) is present in all nuclei in cycling populations, while detergent resistant PCNA (green) persists only in S phase nuclei. (G) High magnification confocal section showing detergent resistant Ciz1 and PCNA foci, and merged image showing co-localising foci (yellow). White arrows indicate some foci common to both antigens. (H) Line plot of red and green fluorescence across the merged image in G, at the position indicated by an arrow. (I) Cross-correlation plot (Rubbi and Milner, 2000; van Steensel et al., 1996) for green foci compared to red over the whole merged image in G, and (inset) for the marked section after thresh-holding fluorescence at the levels shown in H. The red line in the inset shows loss of correlation when the Ciz1 image is rotated 90° with respect to PCNA. Bar is 10 μM.



confluent p21<sup>Cip1</sup> null mouse embryo fibroblasts, Ciz1/ECiz1 significantly increased the number engaged in DNA synthesis, while GFP control had no effect. Thus the positive effect of Ciz1/ECiz1 on DNA replication is direct and is not only a result of modulating the inhibitory activity of p21<sup>Cip1</sup>.

### Endogenous Ciz1

Antibody V recognises Cdc6 as well as p100-Ciz1 (Fig. 1A), so it is not suitable for immunofluorescence experiments aimed at visualizing the sub-cellular localization of endogenous Ciz1. We therefore generated a new rabbit polyclonal anti-serum against recombinant ECiz1 fragment Nterm442, designated

anti-Ciz1 1793. As expected, purified Nterm442 is recognised by anti-Ciz1 1793 in western blots and it is also recognised by antibody V (Fig. 5A), supporting the conclusion that p100 is Ciz1. Consistent with this, anti-Ciz1 1793 also recognised GFP-ECiz1 in whole cell extracts of transfected cells (see Fig. 6G).

When applied to protein extracts derived from growing 3T3 cells, anti-Ciz1 1793 recognised two endogenous antigens, with molecular masses of 125 and 100 kDa (Fig. 5B), whose relative proportions vary between preparations. p100 comigrates with the cyclin-A-responsive antigen that is recognised by antibody V (Fig. 1 and Fig. 5B), which suggests that both antibodies recognise the same protein in vivo. We

confirmed, by immunoprecipitation, that the p100-Ciz1 bands recognised by antibody V and 1793 are the same protein (Fig. 5C). Antibody V precipitated a p100 band that was recognised in western blots by 1793, and vice versa. Furthermore, in the same experiment, 1793, and to a lesser extent, antibody V, precipitated a 125 kDa antigen that was recognised in western blots by 1793. Taken together our observations indicate that p100 is indeed Ciz1, and they suggest that Ciz1 protein exists in at least two forms in cycling cells.

Our results are strikingly similar to those of Mitsui et al. (Mitsui et al., 1999) whose anti-human Ciz1 monoclonal antibody detected two antigens with apparent molecular masses of 120 and 95 kDa in HEK293 cells. They proposed that the 120 kDa form of human Ciz1 protein is processed to produce the 95 kDa form and our results are consistent with this proposal. However, it is also possible that p125 is a modified form of p100 or that p100 and p125 are alternatively spliced forms of Ciz1.

#### Sub-cellular distribution of Ciz1

Anti-Ciz1 1793 was used to visualise the sub-cellular distribution of Ciz1 protein (p100 and p125) in 3T3 cells (Fig. 5D) and in HeLa cells (not shown). In both cell types 1793 reacted with a nuclear-specific antigen and this was blocked by inclusion of recombinant Nterm442 fragment (not shown).

Ciz1 is present in the nuclei of all 3T3 cells in cycling populations and remains clearly detectable in all nuclei after detergent treatment, but at lower levels (Fig. 5D). This suggests that Ciz1 is present in the nucleus throughout interphase, although variations in isoform would not be detected by this method. Reduced staining in detergent-treated nuclei is consistent with western blot data (Fig. 5E) and suggests that Ciz1 is present in the nucleus as both a soluble fraction and also bound to insoluble nuclear structures.

When soluble protein is washed away, the insoluble, immobilised Ciz1 antigen resolves into a punctate sub-nuclear pattern at high magnification (Fig. 5F,G). Ciz1 speckles have a similar size range and distribution as replication 'foci' or 'factories', the sites at which DNA synthesis takes place in S phase. To determine whether Ciz1 is coincident with sites of replication factories, we compared the position of Ciz1 speckles with the position of PCNA, a component of replication complexes in S phase nuclei. In confocal sections, PCNA foci are less abundant than Ciz1 foci, but they are almost all co-incident with Ciz1 (Fig. 5G-I). In merged images, overlap between the positions of PCNA and Ciz1 foci results in yellow spots, while the remaining Ciz1 foci that are not co-incident with PCNA are red. Green (PCNA alone) foci are infrequent, which suggests that Ciz1 is present at almost all the sites where DNA replication factories have formed.

Ciz1 is also present at sites that do not contain PCNA (Fig. 5G) and, unlike PCNA, Ciz1 foci persist throughout interphase. One interpretation of these observations is that Ciz1 marks the positions in the nucleus at which PCNA-containing replication complexes come together into factories, but that not all of these sites are used at the same time. It remains to be determined whether different Ciz1 foci become active sites of DNA replication at different times in S phase, or whether other nuclear activities also occur at sites where Ciz1 is bound. Indeed, it also remains possible that the p100 and p125 forms

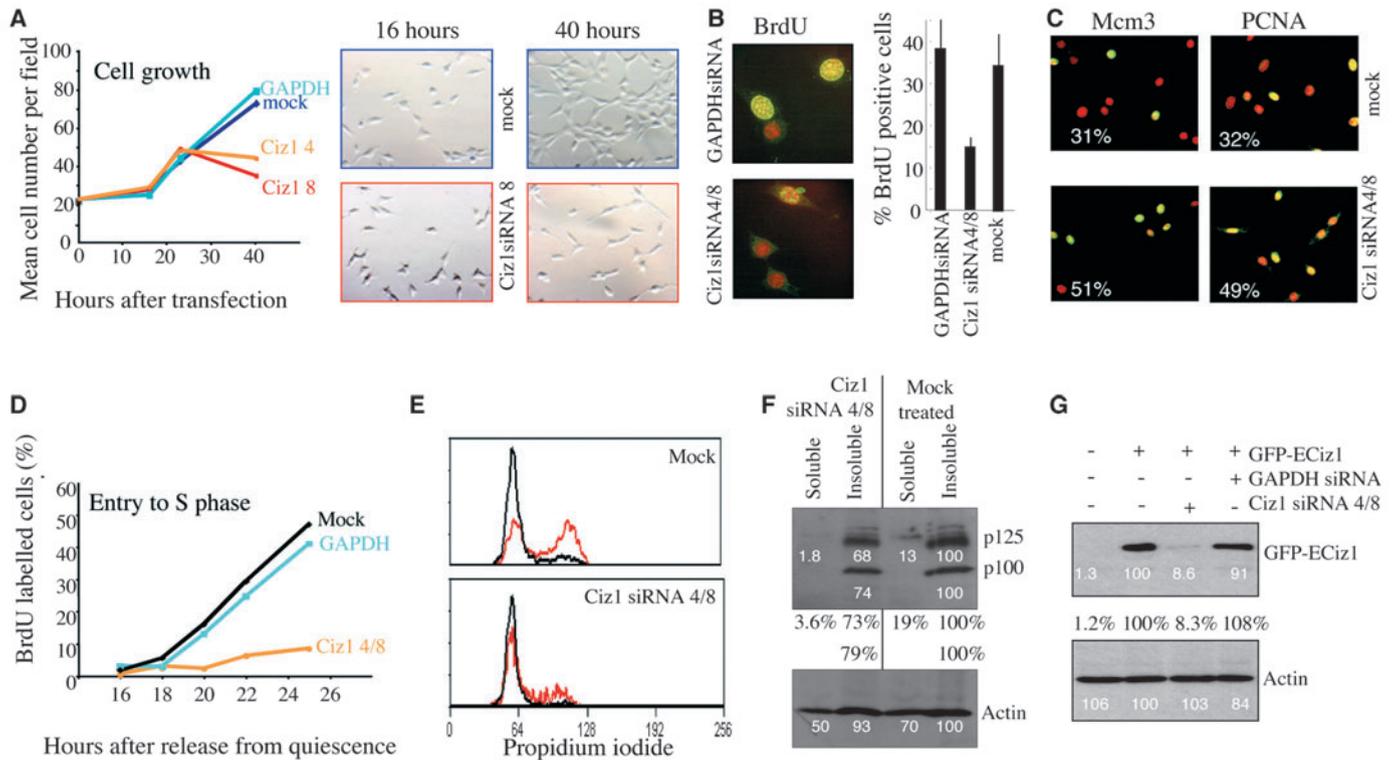
of Ciz1 have different activities and that they reside at nuclear sites with different functions.

Recently, it was reported that human Ciz1 does not co-localise with newly synthesized DNA (Warder and Keherly, 2003), which might appear to contradict our own observations. However, there were differences in the way the two studies were conducted. We used confocal microscopy of PCNA to visualise the fixed sites where DNA replication factories reside and compared those with the insoluble fraction of endogenous Ciz1, while the former study used a long pulse with BrdU to visualise nascent DNA and compared that with over-expressed human Ciz1 in Cos-7 cells. Based on our experiments with over-expressed mouse Ciz1 and derived fragments in mouse cells we would not expect significant co-localisation under those circumstances (H. Sercombe, J.A. and D.C., unpublished), and even for endogenous Ciz1 the soluble pool must be removed before detection of individual Ciz1 foci is possible.

#### Inhibition of Ciz1 synthesis restrains cell proliferation

So far we have shown that the behaviour of p100-Ciz1 correlates with initiation of DNA replication in cell-free assays, that ECiz1 stimulates the frequency of initiation in vitro, that Ciz1 and ECiz1 stimulate DNA replication in normal and p21<sup>Cip1</sup> null cells and that Ciz1 is present at the same nuclear sites as components of the DNA replication machinery. However, these data do not show that Ciz1 is *required* for cell proliferation. To test this we used RNA interference (RNAi) to selectively reduce Ciz1 transcript levels in NIH3T3 cells. Short interfering (si)RNA molecules (Elbashir et al., 2001) were produced in vitro against two target sequences within Ciz1 (see Fig. 2A). Both anti-Ciz1 siRNAs restricted growth when applied to cells, taking effect between 23 and 40 hours post-transfection (Fig. 6A). Consistent with this, fewer cells in the Ciz1-depleted population labelled with BrdU during a pulse at 48 hours, and the remainder labelled less intensely than untreated populations (Fig. 6B). This suggests that in some cells DNA synthesis is slowed down and in others it is inhibited completely, possibly because of varying levels of depletion of Ciz1. Inhibition of DNA synthesis is consistent with a primary function for Ciz1 in DNA replication, however it could also be the result of general disruption of nuclear function. Therefore, we looked in more detail at a range of replication proteins whose levels are regulated in the cell cycle, to ask whether depleted cells accumulate before or after replication complex assembly.

During initiation of eukaryotic DNA replication, Mcm complex proteins assemble at replication origins in late G1, in a Cdc6-dependent manner. Later, DNA polymerases and their accessory factors (including PCNA) become bound to chromatin and origins are activated. This is associated with nuclear export and proteolysis of the majority of Cdc6 and, as DNA synthesis proceeds, gradual displacement of the Mcm complex from chromatin (Bell and Dutta, 2002). In order to identify the point of action of Ciz1 we used immunofluorescence to monitor Mcm3 and PCNA. In Ciz1-depleted cells (Fig. 6C) both proteins were detectable within the nucleus, bound to detergent resistant nuclear structures. Therefore, these factors are unlikely to bind directly to Ciz1, or to be dependent upon Ciz1 for their initial assembly on



**Fig. 6.** Inhibition of new Ciz1 synthesis restrains entry to S phase. (A) Left: siRNAs that target Ciz1 transcripts at two sites (see Fig. 2A) were individually applied to cycling 3T3 cells and cell number was monitored at the indicated times. Right: images of cell populations at 16 and 40 hours after transfection with siRNA 8 (red outline) or mock treated cells (blue outline). (B) The proportion of cells that incorporate BrdU into DNA (green) is significantly decreased in cells treated with Ciz1 siRNAs 4 and 8, compared to GAPDH siRNA, 48 hours after treatment. Histogram shows average results from four independent experiments. (C) The number of nuclei with detergent-resistant Mcm3 and PCNA (green) increases in populations treated with Ciz1 siRNA 4/8, compared with mock-treated controls. All nuclei were counterstained and are shown in pseudocolour (red). (D) Quiescent 3T3 cells were stimulated to re-enter the cell cycle (Coverley et al., 2002) with and without exposure to Ciz1 siRNA 4/8 or GAPDH siRNA, and pulse labelled with BrdU at the indicated times to reveal the proportion of cells in S phase. (E) Quiescent 3T3 cells were stimulated to re-enter the cell cycle and harvested at the G1-S transition (18 hours, black) or after S phase (36 hours in the presence of nocodazole, red) with and without exposure to Ciz1 siRNA 4/8. (F) Detergent-soluble and -insoluble fractions of endogenous Ciz1 protein in approximately  $1 \times 10^5$  cells after mock treatment or treatment with Ciz1 siRNAs 4/8, detected with anti-Ciz1 1793. (G) To focus on newly synthesized Ciz1, expression of ectopic GFP-ECiz1 was monitored in whole cell extracts made from approximately  $8 \times 10^3$  cells, in the presence and absence of Ciz1 siRNA 4/8 using anti-Ciz1 1793. Expression levels in extracts prepared 22 hours after transfection are shown. For F and G band intensities were quantified (shown in white), normalised against actin levels and expressed as a percentage.

chromatin. In fact, in four independent experiments the average number of cells with detergent-resistant chromatin-bound Mcm3 and PCNA actually *increased* from 31% ( $\pm 6\%$ ) to 51% ( $\pm 5\%$ ) and from 32% ( $\pm 5\%$ ) to 49% ( $\pm 6\%$ ), respectively. This suggests that Ciz1 acts after replication complex assembly but before completion of S phase, while failure to act inhibits progression through S phase, leaving Mcm3 and PCNA in place.

Consistent with this, when quiescent cells are stimulated to re-enter the cell cycle significantly fewer undergo S phase if they are prevented from producing Ciz1. Under normal circumstances DNA synthesis is first detected 19–20 hours after 3T3 cells are released from quiescence, when 50–70% of the total population enter S phase (Coverley et al., 2002). In cells treated with Ciz1 siRNA at the time of release, four- to fivefold fewer entered S phase compared with untreated cells or cells treated with GAPDH siRNA (Fig. 6D). Similarly, analysis of DNA content in cells taken at the normal point of entry to S phase, and cells allowed to grow for a further 18 hours (in the

presence of nocodazole to prevent passage through mitosis) revealed a significant inhibition in progression from late G1 through S phase and G2 in Ciz1-depleted populations (Fig. 6E). These analyses with synchronised populations of cells strongly suggest that synthesis of new Ciz1 is required for S phase entry.

To demonstrate the effectiveness of Ciz1 siRNAs on Ciz1 production we first looked at endogenous Ciz1 protein. Cells treated with siRNAs 4 and 8 displayed a fivefold reduction in the soluble p125-Ciz1 fraction, relative to actin, but only a 27% and 21% drop in the much more abundant detergent-resistant p125-Ciz1 and p100-Ciz1 forms, respectively (Fig. 6F). The difference between the effect of Ciz1 siRNAs on the soluble and insoluble pools suggests that insoluble Ciz1 is relatively stable. Therefore, cell division may be required for dilution of this pool to be observed. Our data indicate that prevention of *new* Ciz1 synthesis inhibits S phase, which would make dilution difficult to achieve. Therefore, to clearly demonstrate the efficiency of our siRNA treatment we looked specifically

at its effect on newly synthesized Ciz1 protein by co-transfecting GFP-ECiz1 at the same time as siRNAs 4 and 8 (Fig. 6G). In three western blot experiments, Ciz1 siRNAs inhibited expression of recombinant Ciz1 protein by between 87-95% at 6 hours, and 80-92% at 22 hours, compared to controls. These results are consistent with the interpretation that new Ciz1 protein is required for continued cell proliferation, despite stability of the existing pool.

## Discussion

As a first step towards understanding the function of Ciz1, we asked when in the cell cycle does Ciz1 act. Several of our lines of investigation suggest that Ciz1 is required during a late stage in the replication initiation process, after pre-replication complex formation. First, p100-Ciz1 antigen is most prevalent in nuclei exposed to cyclin A-cdk2 concentrations that activate DNA synthesis, implying that Ciz1 may be involved in this step, rather than during earlier replication complex assembly steps (Coverley et al., 2002). Second, functional studies with late G1 nuclei show that recombinant ECiz1 increases the number of nuclei that incorporate labelled nucleotides *in vitro*. Therefore, Ciz1 is active in a step that converts nuclei that are poised to begin DNA synthesis into nuclei that are actively synthesizing DNA. These results are supported by findings in intact cells where ectopic Ciz1 stimulates cells to engage in DNA synthesis. Third, RNA interference studies in both synchronized and cycling cells point to a Ciz1-dependent step during S phase entry, after Mcm complex formation and after PCNA has become assembled onto DNA. Taken together, these distinct lines of investigation suggest that Ciz1 acts to promote DNA replication at a step following, or independent from, replication complex formation.

Most pre-replication complex proteins and many replication fork proteins are phosphorylated *in vivo*, often by cyclin-dependent kinases (Bell and Dutta, 2002; Fujita, 1999). Our data show that the behaviour of p100-Ciz1 antigen is regulated (directly or indirectly) by cyclin A-cdk2, and functional studies implicate a putative cdk phosphorylation site at threonine 191/192 in control of Ciz1 activity. When this site is made unphosphorylatable activity is maintained over a broader range of concentrations in cell-free assays, indicating that Ciz1 is normally down regulated at this site. At this stage we don't know whether these two observations relate to the same phosphorylation event because the function(s) of the other conserved cdk phosphorylation sites remain to be determined. Conditional inclusion of a cyclin-binding motif in an alternatively spliced N-terminal portion of Ciz1 may also influence the relationship between Ciz1 and cdks. Thus, the simple negative relationship between Ciz1 activity and cdk-dependent phosphorylation uncovered by our mutagenesis experiments, is unlikely to be the whole story.

Like cdk-phosphorylation, the relationship between Ciz1 and p21<sup>Cip1</sup> is likely to be important for the regulation of Ciz1. We showed that Ciz1 stimulates DNA replication even in p21<sup>Cip1</sup> null cells, strongly supporting the conclusion that Ciz1 acts directly in DNA replication, rather than by modulating the inhibitory effects of p21<sup>Cip1</sup>. This raises important questions about the role of the Ciz1-p21<sup>Cip1</sup> interaction reported previously (Mitsui et al., 1999). None of the evidence reported so far argues against the possibility that p21<sup>Cip1</sup> might exert

effects on the cell-cycle by influencing Ciz1 activity, in addition to its more well characterized targets. Indeed, co-expression of p21<sup>Cip1</sup> with Ciz1 caused ectopic Ciz1 to become predominantly cytoplasmic (Mitsui et al., 1999), a potential mechanism by which endogenous Ciz1 function might be restrained.

In summary, we have described the application of a recently developed cdk-dependent cell-free DNA replication system (Coverley et al., 2002), to the identification of a new factor involved in mammalian DNA replication. Ciz1 has been implicated in tumorigenesis (Warder and Keherly, 2003) and has been linked to the mammalian cell cycle machinery, both by its interaction with p21<sup>Cip1</sup> and our cell free experiments. It contains sequences that are homologous to nuclear matrix proteins and we find that it is present at sites of DNA replication. These observations place Ciz1 at the heart of proliferation control in mammalian cells, and raise the possibility that Ciz1 may form a regulatable link between DNA replication factories and the nuclear structures upon which they are mounted.

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**Supplementary Tables Showing Raw Data For Cell-Free Initiation Experiments**

Late G1 nuclei preparation 1 (used in experiments 1-10) contained 17% S phase nuclei and 34% 'replication competent' nuclei. Preparation 2 (used in experiments 11 and 12) contained 12% S phase nuclei and 32% 'replication competent' nuclei. '% initiation' is calculated from '% replication' for each data point by subtracting '% S phase' and dividing by '% replication competent'. For the first data point in the table below this is  $(31-17)/34 \times 100 = 41\%$ .

**T(191/2)A** (data for Fig. 3C)

Concentration of recombinant Ciz1 in replication assays (nm)	Labelled nuclei/total (nuclei prep.1)			% Replication *	% Initiation **
	Expt. 6	Expt. 7	Expt. 9		
0.00	39/132	57/172		31***	41
0.01			54/200	27	29
0.1		24/84		29	35
0.25		49/154		32	44
0.4			49/156	31	41
0.6	61/137			45	82
1.3	53/142	28/62	74/152	44	79
6.3	89/196	53/122		45	82
12.7		61/148	44/100	42	74
25.4	48/125	44/105		40	68
63.5			55/115	48	91

**T(293)A** (data for Fig. 3E)

Concentration of recombinant Ciz1 in replication assays (nm)	Labelled nuclei/total (nuclei prep.2)		% Replication *	% Initiation **
	Expt. 11	Expt. 12		
0.00	24/104	38/142	25	41
0.01		24/93	26	44
0.06		28/100	28	50
0.13	60/146	29/75	40	88
0.6		30/105	29	53
1.3	32/135		24	38
12.7	30/134		22	31

**Nterm442 (data for Fig. 3F)**

Concentration of recombinant Ciz1 in replication assays (nm)	Labelled nuclei/total (nuclei prep.1)				% Replication *	% Initiation **
	Expt. 1	Expt. 2	Expt. 3	Expt. 4		
0.0	54/181	24/75	38/121	41/137	31***	41
0.2		30/94			32	44
0.7			19/57		33	47
1.0	76/175	102/242	62/152	51/93	44	79
1.3				44/97	45	82
2.1		45/117	20/70		35	53
10.3	53/183				29	35
103.3	37/107				35	53
1033.0	29/87				33	47

**Cterm274 (data for Fig. 3G)**

Concentration of recombinant Ciz1 in replication assays (nm)	Labelled nuclei/total (nuclei prep.1)				% Replication *	% Initiation **
	Expt. 1	Expt. 2	Expt. 8	Expt. 5		
0.00	54/181	24/75	47/150		31***	41
0.01				53/163	33	47
0.05				54/168	32	44
0.1				39/138	28	32
0.3		30/100	21/69		30	38
1.6		46/162	33/99		30	38
5.0	36/108				33	47
6.5			24/75		32	44
10.0	30/98				31	41
13.0			20/68		29	35
26.0			44/136		32	44
50.0	40/122				33	47
165.0		36/112			32	44
1653		31/90			34	50
4958	30/96				31	41

**ECiz1 (data for Fig. 3B)**

Concentration of recombinant Ciz1 in replication assays (nm)	Labelled nuclei/total (nuclei prep.1)		% replication *	% initiation **	Labelled nuclei/total (nuclei prep.2)		% replication *	% initiation **	Mean % initiation
	Expt. 9	Expt 5			Expt. 11	Expt. 12			
0.00			31 ***	41	24/104	38/142	25.2	41.2	41
0.01	38/113		33.6	48.8		37/149	24.8	40.0	44
0.13	33/111		29.7	37.3					37
0.63	34/116	27/98	28.5	33.8					34
1.02		44/138	31.9	43.8	39/120	35/96	34.3	69.7	57
1.14						55/125	44.0	100	100
1.3	114/264	54/136	42.0	73.5	64/169	50/120	39.5	85.9	80
2.5					26/102		25.5	42.2	42
3.8		30/111	27.0	29.4	32/132		24.2	38.1	34
6.3	56/188		29.8	37.6					38
12.7	30/105		28.6	34.1					34
63.0	30/102		29.4	36.4					36

\*Proportion of nuclei that incorporated labelled nucleotides in vitro.

\*\*Proportion of 'initiation competent' nuclei that initiated in vitro.

\*\*\*Mean % replication, calculated from control samples from experiments 1-10.