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CycA is involved in the control of endoreplication dynamics in the *Drosophila* bristle lineage

Jérémy Sallé[1,2], Shelagh D. Campbell[3], Michel Gho[1,2,*] and Agnès Audibert[1,2,*]

SUMMARY

Endocycles, which are characterised by repeated rounds of DNA replication without intervening mitosis, are involved in developmental processes associated with an increase in metabolic cell activity and are part of terminal differentiation. Endocycles are currently viewed as a restriction of the canonical cell cycle. As such, mitotic cyclins have been omitted from the endocycle mechanism and their role in this process has not been specifically analysed. In order to study such a role, we focused on CycA, which has been described to function exclusively during mitosis in *Drosophila.* Using developing mechanosensory organs as model system and PCNA::GFP to follow endocycle dynamics, we show that (1) CycA proteins accumulate during the last period of endoreplication, (2) both *CycA* loss and gain of function induce changes in endoreplication dynamics and reduce the number of endocycles, and (3) heterochromatin localisation of ORC2, a member of the Pre-RC complex, depends on CycA. These results show for the first time that CycA is involved in endocycle dynamics in *Drosophila*. As such, CycA controls the final ploidy that cells reached during terminal differentiation. Furthermore, our data suggest that the control of endocycles by CycA involves the subnuclear relocalisation of pre-RC complex members. Our work therefore sheds new light on the mechanism underlying endocycles, implicating a process that involves remodelling of the entire cell cycle network rather than simply a restriction of the canonical cell cycle.

Key words: Endocycle, Cyclin A, ORC2, Cdk1, Neural development, *Drosophila*

INTRODUCTION

Endocycles arise from drastic modifications of the canonical G1-S-G2-M cell cycle that result in successive rounds of DNA replication without intervening mitoses, leading to polyploid cells. Multiple genomic copies are associated with an increase in transcriptional activity that results in a high metabolic activity and is important for terminal differentiation in several developmental processes (Lee et al., 2009). In spite of their terminal commitment, it has been observed that polyploid cells can undergo mitotic divisions and that these divisions are error-prone (Fox et al., 2010). Interestingly, polyploid cells are observed in a wide range of pathological situations such as hypertension, oxidative stress, genomic instability and ultimately tumourigenesis (Storchova and Pellman, 2004). As such, unravelling the mechanism that drives endocycles is fundamental to understanding the role of endocycles in normal and pathological cellular processes.

The establishment of endocycles, where mitosis is skipped, involves modifications in the dynamics of cell cycle regulators, reflecting the inherent flexibility of the archetypal mitotic cell cycle. Progression through both endocycles and mitotic cycles is driven by periodic activation and inactivation of Cdk/cyclin complexes, which are largely conserved throughout evolution. These complexes are composed of a kinase called Cdk (cyclin-dependent kinase), and are active only when they are associated with a cyclin protein. During mitotic cycles, cyclin A, cyclin B and cyclin B3, which are associated with Cdk1 (cdc2), control mitosis, whereas cyclin E, which is associated with Cdk2, controls the G1/S transition (Budirahardja and Gönczy, 2009). In mammals, the CycA/Cdk2 complex is also involved in S-phase progression (Budirahardja and Gönczy, 2009). By contrast, *Drosophila* CycA seems to be able to bind only Cdk1 and as such is considered to be an exclusive mitotic regulator (Knoblich et al., 1994). The activity of cyclin/Cdk complexes is regulated by multiple processes, including cyclin/Cdk dimerisation, Cdk protein phosphorylation and degradation of cyclins (Jin et al., 2008; Edgar and O'Farrell, 1989).

In *Drosophila,* Cdk2/CycE is the major complex required for progression through endocycles. The periodic activation and inactivation of this complex results in alternating S and G phases. In *CycE* mutants, DNA synthesis is blocked and endocycles are absent (Knoblich et al., 1994). Moreover, current data indicate that oscillation of Cdk2/CycE activity drives successive endocycles, as sustained CycE overexpression results in endocycle block (Follette et al., 1998). One important target of Cdk2/CycE is the anaphase-promoting complex (APC/C). Cdk2/CycE inhibits Fzr, a Cdh1-related positive regulator of APC/C (Narbonne-Reveau et al., 2008; Zielke et al., 2008; Sigrist and Lehner, 1997; Sigrist et al., 1995). As such, the oscillatory activity of Cdk2/CycE generates anti-parallel oscillations of APC/C-Fzr that are essential for both entry and progression through successive endocycles (Shcherbata et al., 2004). The oscillatory activity of APC/C-Fzr generates alternating permissive and refractory periods for pre-replication complex (pre-RC) assembly. In particular, the levels of Orc1 and Geminin, a member of the pre-RC and a pre-RC assembly inhibitor, respectively, are periodically reduced in response to APC/C-Fzr complex activity (Narbonne-Reveau et al., 2008; Zielke et al., 2008). Interestingly in *orc1/2* mutants, endoreplication occurs, but the level of ploidy is reduced, suggesting that ORC proteins are required but are not essential for endoreplication (Landis et al., 1997; Park and Asano, 2008).

[1]Université Pierre et Marie Curie-Paris 6, UMR 7622, Paris, France. [2]CNRS, UMR 7622, Paris, France. [3]Biological Sciences, CW405, University of Alberta, Edmonton-Alberta, T6G 2E9 Canada.

*Authors for correspondence (michel.gho@snv.jussieu.fr; agnes.audibert@snv.jussieu.fr)

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The status of the mitotic cyclins during endocycles remains unclear. It has been shown that during the endocycles, transcription of almost all mitotic regulators is severely reduced when compared with proliferating cells (Maqbool et al., 2010). Indeed, it has been observed that mitotic regulators such as CycA and CycB accumulate in *fzr* (*rap* – FlyBase) mutants during endocycles (Schaeffer et al., 2004; Shcherbata et al., 2004). Moreover, overexpression of CycA but not CycB or CycB3 during endocycles in salivary glands results in low ploidy in apparently randomly distributed cells (Weiss et al., 1998). Thus, *CycA* is transcribed during endocycles and must be actively post-transcriptionally downregulated to allow successive endocycles. Other experimental data also support the idea that CycA has a role during the S phase in *Drosophila*. For example, in the embryo it has been shown that ectopic expression of CycA triggers the G1/S transition in wild-type and *CycE* mutant backgrounds (Sprenger et al., 1997). Similarly, CycA overexpression in imaginal eye discs leads to ectopic S phase in differentiating photoreceptors (Dong et al., 1997). The function of CycA on S-phase appears to be rather cryptic, however. Under physiological conditions, the CycA/Cdk1 complex is inhibited by active degradation, phosphorylation and physical interaction with the inhibitory factor Roughex (Avedisov et al., 2000; Sprenger et al., 1997). Thus, a direct role for endogenous CycA in the S phase, and in particular during endocycles, has yet to be demonstrated in *Drosophila.*

In order to elucidate whether mitotic cyclins regulate endoreplication, we focused our analysis on the role of CycA in endocycles of *Drosophila*. In this system, endoreplication occurs in almost all larval tissues and in several adult tissues, such as nurse cells, follicular cells in ovaries and external cells of mechanosensory organs (Hartenstein and Posakony, 1989; Audibert et al., 2005; Shcherbata et al., 2004). Two populations of mechanosensory bristles can be distinguished on the dorsal thorax (notum): microchaetes and macrochaetes, which are short and long bristles, respectively. Each bristle is composed of four cells: two outer cells (the socket and the shaft cells) and two inner cells (the neuron and the sheath cell) (Gho et al., 1999). These four cells arise from a succession of four asymmetric divisions of a single precursor cell pI during pupal development (Gho et al., 1999). After the completion of the cell lineage, shaft and socket cells undergo endocycles associated with the acquisition of a specific morphology that give rise to the external cuticular structures of the sensory organs (Hartenstein and Posakony, 1989; Audibert et al., 2005). As such, the size of the external bristle can be used as readout of the number of endocycles that the shaft cell undergoes (Szuplewski et al., 2009). In addition, this system offers the opportunity to study the dynamics of endocycles at the cellular level using in vivo techniques, in contrast to other models.

Using a combination of in vivo experiments to follow endocycle dynamics, clonal analysis and RNAi expression, we have shown that: (1) endoreplication present the classical distinction between early and late S phases observed in proliferative cells; (2) endocycle dynamics are regulated in a cell-specific way; (3) CycA proteins levels oscillate and these proteins accumulate during the transition between early and late S phase (4) in agreement with its expression pattern, the duration of early and late S phases are modified in *CycA* loss and gain of function leading to a reduction in ploidy; and (5) CycA is required for relocalisation of ORC2 protein, a member of the pre-RC complex, to the heterochromatin. Altogether, our data show for the first time that endocycle dynamics are regulated by oscillations of CycA, which are required for the completion of the early S phase and the duration of the late S phase.

MATERIALS AND METHODS

Fly strains

In mechanosensory bristle cells, the *neuralized*^{P72}-GAL4 (*neur*>) driver was used to express specifically the following constructions: *UAS-histoneH2B::yfp* (H2B::YFP) (Bellaiche et al., 2001), *UAS-pcna::gfp* (a gift from M. Cardoso, Max Delbrück Center for Molecular Medicine, Germany) (Easwaran et al., 2007), *UAS-CycA* (Bloomington Stock Center), *UAS-CycE* (a gift from H. Richardson, Peter MacCallum Cancer Centre, Australia) and *UAS-CycARNAi* (VDRC) using the GAL4/*UAS* expression system (Brand and Perrimon, 1993), *EP-orc2*^{EY04752} (Bloomington Stock Center). We used the *tub-Gal80*^{ts}, *neuralized*^{P72}*-Gal4*/SM5CyO, TM6Tb as an inducible driver specific to the bristle cell lineage. Fly crosses, embryonic and larval development were carried out at 18°C, and white pupae (0 hours after pupae formation, APF) were transferred to 30°C to allow the expression of GAL4. *CycA*^{C8LR1} and *CycE*^{AR95} (Bloomington Stock Center) null alleles were recombined with FRT80B and FRT40A chromosomes, respectively, for clonal analysis.

Immunostaining

Dissected nota from pupae at 15-35 hours APF were processed as described previously (Gho et al., 1996). The following primary antibodies were used: mouse anti-Cut (DSHB, 1:500); rabbit anti-GFP (Santa-Cruz, 1:500); mouse anti-GFP (Roche, 1:500); rat anti-CycE (a gift from H. Richardson, 1:1000); rat anti-ELAV (DSHB, 1:100); rat anti-Su(H) [a gift from F. Schweisguth (Institut Pasteur, Paris, France), 1:500]; rabbit anti-CycA [a gift from P. O'Farrell (UCSF, CA, USA), 1:500]; rabbit anti-γH2Av (Rockland Immunochemicals, 1:500); rabbit anti-β-Galactosidase (Cappel, 1:500); rabbit anti-ORC2 [a gift from I. Chesnokov (University of Alabama, Birmingham, AL, USA), 1:1000]; rabbit anti-Pdm1 [a gift from T. Préat (ESPCI, France), 1:200]; rabbit anti-MCM5 (a gift from P. O'Farrell, 1:50); guinea-pig anti-E2F1 [a gift from T. Orr-Weaver (Whitehead Institute, Cambridge, MA, USA), 1:1000]; and mouse anti-HP1 (DSHB, 1:500). DNA was stained using Hoechst 33345 (Sigma) solution (10 μg/ml), incubated for 20 minutes and washed three times for 30 minutes in PBS. Alexa 488-, 568- (1:1000) and Cy5- (1:2000) conjugated secondary antibodies were purchased from Molecular Probes and Promega. For S-phase detection, dissected nota were incubated with 10 mM EdU or 25 ng/μl BrdU in Schneider culture medium complemented with foetal bovine serum (2%), insulin (0.1 U/ml) and ecdyson (0.5 μg/ml), for 1 hour at 25°C. Immediately afterwards, nota were fixed for 20 minutes in 4% paraformaldehyde. Detection was realised using a Click-iT EdU kit according to the manufacturer's instructions (Invitrogen) or with mouse anti-BrdU antibodies (Becton Dickinson, 1:50). Image acquisition was performed using a Leica SP5 confocal microsocope (63×, NA 1.4 objective) driven by LAS-AF (Leica) software or using a spinning disc coupled to an Olympus BX-41 microscope (60×, NA 1.25 objective and 40×, NA 0.75 objective) associated with a CoolSnapHQ2 camera (Ropert Scientific, France), driven by Metamorph software (Universal Imaging).

Clonal analysis

Intra-lineage clones

CycA and *CycE*-null mitotic clones were generated during the bristle lineage using the FLP/FRT recombination system (Theodosiou and Xu, 1998). *UAS-FLP* associated with the *scabrous*-Gal4 and *neur*-Gal4 drivers promote recombination only in bristle lineage cells. Nota were then dissected at 36 or 72 hours APF and processed as above. *CycE* and *CycA* intralineage clones were obtained by crossing the following fly lines: *UAS-FLP*; *FRT40A GFP*/*CyO* and *FRT40A CycE*^{AR95}*; neuralized*^{P72}*-Gal4/SM5CyO, TM6Tb* and *UAS-FLP; FRT80B GFP/TM6Tb* and *scabrous-Gal4*; *FRT80B CycA*^{C8LR1}*/SM5CyO, TM6Tb.*

Salivary gland clones

Clonal analysis in salivary glands was performed using the FLP/FRT system with a heat-shock inducible Flipase (hs-FLP). Clones were generated after a 1-hour heat-shock at 37°C in embryos. Salivary glands were dissected from third instar larvae in PBS and fixed in 4% paraformaldehyde solution. Fixed salivary glands were processed using the same method as for nota. All images were processed using ImageJ software.

Ploidy measurements

Stack acquisition was performed using a Leica SP5 confocal microsocope. Acquisition settings were: 16 bits image depth, 1024×1024 pixels, with no saturation. Stacks were processed using a 3D object counter ImageJ plug-in in order to quantify DNA staining fluorescence intensity in 3D nuclei. For each nucleus, ploidy measurements were normalised using the average value of epithelial nuclei. The number of endocycles was determined by $\log_2$ (DNA staining intensity in polyploid nuclei/DNA staining intensity of epithelial nuclei).

Time lapse microscopy

Live imaging was carried out using a spinning disc (see Immunostaining). *Z*-stacks of images were acquired every 3-6 minutes and assembled using ImageJ software. At the end of the movie, pupae dissection was carried out as described previously (Gho et al., 1996). The temperature of the recording chamber was carefully controlled (±0.1°C) using a homemade temperature controller containing a Peltier device fixed to the microscope stage. *tub*-Gal80^{ts}; *neur>UAS*-PCNA::GFP flies were maintained at 30°C from 0 hours APF until the end of the time lapse acquisition. In the movies, cells were unambiguously identified by their relative position, nuclear size and order of birth.

RESULTS

Endocycles present cell type-specific dynamics in the microchaete lineage

In order to characterise endocycles in the bristle lineage, we monitored endoreplication in vivo in shaft and socket cells of microcheates. Using the *UAS*/Gal4 system (Brand and Perrimon, 1993), we expressed a PCNA::GFP fusion protein under control of the *neuralized*^{p72}-Gal4 driver (Bellaiche et al., 2001) specifically in developing bristle cells. The PCNA protein is a component of the replication machinery and was used to mark sites of active DNA replication (Easwaran et al., 2007). During the gap-phase, PCNA::GFP fluorescence was diffuse throughout the nucleus (Fig. 1A, left panel). Then, numerous foci simultaneously emerged in the nucleus corresponding to the start of replication. Later, as DNA replication progressed, the number of foci decreased as one single bright spot associated with perinucleolar heterochromatin appeared (Fig. 1A, open arrowheads and data not shown). By combining time-lapse imaging and subsequent BrdU incorporation, we observed that no BrdU incorporation occurred when PCNA::GFP was homogenously distributed throughout the nucleus (Fig. 1B, gap phase). Thereafter, BrdU incorporation followed the PCNA::GFP profile (Fig. 1B, S phase). As such, in accordance with BrdU incorporation, cells with numerous PCNA::GFP foci were considered to be in early S phase and those with a the final single dot were in late S phase. During the late S phase, PCNA::GFP and BrdU staining remained at the periphery of the heterochromatin, in accordance with evidence showing that heterochromatin is under-replicated during endocycles (see Fig. 5) (Lilly and Spradling, 1996). This is in contrast with the late PCNA::GFP foci observed in mitotic cells which overlapped with de-condensed heterochromatic domains (Shermoen et al., 2010).

In microchaetes, PCNA::GFP recordings showed that shaft and socket cells undergo three and two rounds of endoreplication, respectively, between 21 and 36 hours APF (Fig. 1C). This corresponds to the complete set of endoreplication cycles, as DNA content measurements later than 36 hours APF showed that the ploidy was unchanged in these cells (Fig. 1C). These data establish that endocycles occur during a well-defined developmental time window.

Quantitative analysis of 122 cells revealed that shaft cells began endoreplication prior to socket cells (Fig. 1A, black arrowheads, lower panel; see also supplementary material Movie 1 and Fig. S1 for complete data and statistical analysis). The duration of the early and late S phases were also quantified during the first and the second replication cycles in shaft and socket cells. The timing of

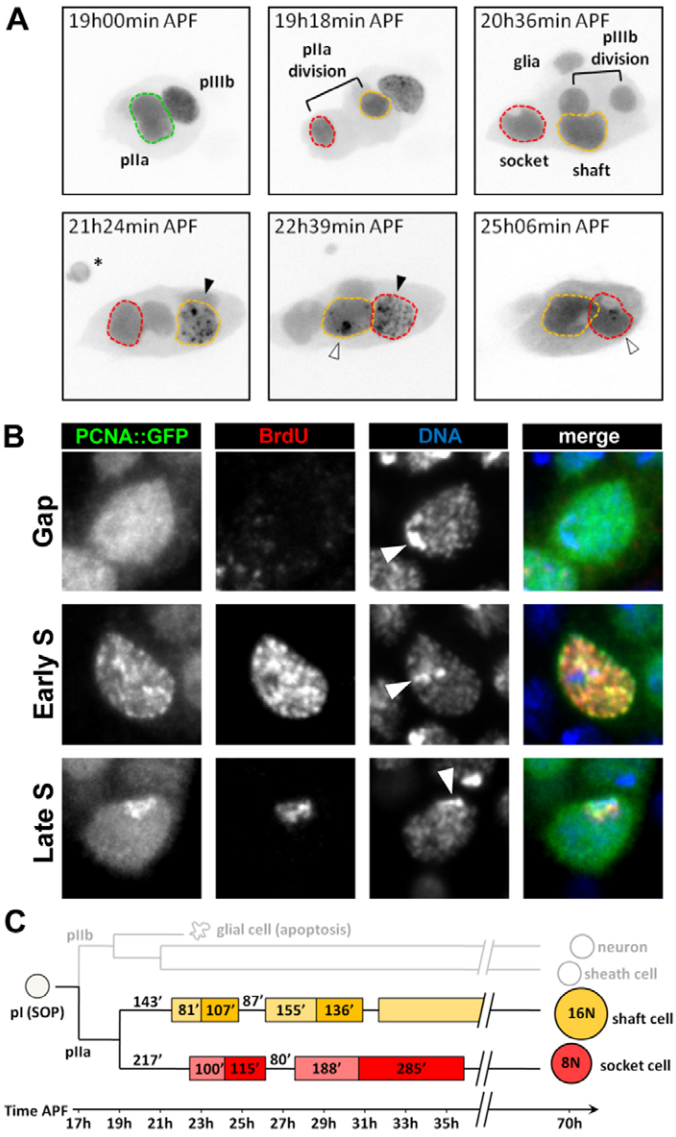

Fig. 1. Endocycles in the mechanosensory bristle cell lineage. (**A**) In vivo acquisition of *tub-Gal80^{ts}, neur>UAS-pcna::gfp* pupae shifted to 30°C at 0 hours APF. Representative frames from a time-lapse recording are shown for one cluster in inverted fluorescence. The pIIa cell is outlined in green. The two daughter cells, the shaft and the socket cell, are outlined in yellow and red, respectively. pIIa and pIIIb divisions are indicated by the brackets. An asterisk indicates glial cell apoptosis. Numerous PCNA::GFP foci correspond to the early S phase (black arrowheads) and a single PCNA::GFP focus corresponds to late replication (open arrowheads). Time APF is indicated in the top left-hand corner. (**B**) BrdU incorporation (red) in PCNA::GFP endocycling cells (green). DNA is stained using DAPI staining (blue). Heterochromatin is indicated by arrowheads. (**C**) Schematic view of the endocycle dynamics measured by in vivo recording with the PCNA::GFP construct. Endoreplication times are average values established from data obtained from several movies (*n*=122 cells). Yellow and red boxes indicate endoreplication periods in shaft and socket cell, respectively. Early S phases are indicated by light tones and late S phases are indicated by dark colours.

the third endocycle in the shaft cell was not analysed owing to the low number of recordings obtained at this late pupal stage. In all cases, the first round of endoreplication was the shortest in both shaft and socket cells, and the duration of the subsequent endoreplication increased, probably owing to an increase in the DNA content (Fig. 1C; supplementary material Fig. S1). Endoreplication always took longer to complete in the socket cell than in the shaft cell, however (Fig. 1C; supplementary material Fig. S1). The increase in the S-phase duration occurred during both early and late S phases (Fig. 1C; supplementary material Fig. S1).

These data indicate that endocycles are regulated in a cell-type specific manner, which leads to a different DNA content in shaft and socket cells. In addition, by using PCNA::GFP recordings, we were able to distinguish for the first time in endocycling cells, the classical distinction of early and late S phases previously described in proliferating cells (Shermoen et al., 2010).

Cyclin A levels oscillate during endocycles

In order to identify a potential role of mitotic cyclins, in particular CycA, in endocycles, we first determined whether CycA could be detected in endocycling cells of the bristle lineage. Using CycA immunostaining, a weak but reproducible CycA protein accumulation was observed mainly in the cytoplasm of some external cells of the lineage (Fig. 2A, arrowheads). As endocycles are not perfectly synchronous from one cluster to another, the irregular accumulation of CycA among clusters suggested that CycA protein levels were oscillating during endocycles.

To characterise the dynamics of CycA with respect to endoreplication, we performed CycA immunostainings in *neur*>PCNA::GFP flies. In order to identify more precisely the timing of the early S phase, and in agreement with the in vivo recording (see supplementary material Movie 1), we subdivided the early S phase into two stages: an initial early-1 where numerous and coalescent PCNA::GFP foci were present in the nucleus followed by an early-2 where a reduced number of discrete PCNA::GFP foci were visible. The late S phase was characterised, as before, by the presence of single PCNA::GFP foci. CycA protein was undetectable in gap and early-1 S phases (Fig. 2B, first and second columns). By contrast, CycA protein accumulated in early-2 and late S phases (Fig. 2B, open arrowheads, third and fourth columns). Quantification showed a slight accumulation of CycA in external cells during the beginning of the replication (18% of CycA-positive cells during gap phase and 32% in early-1) followed by a dramatic increase at the end of the DNA replication (73% of Cyc A-positive cells during early-2 and 82% during late S phase, Fig. 2C).

These results show that CycA is specifically expressed and displays a dynamic pattern during endocycles in external cells of developing mechanosensory organs. More precisely, we found that CycA accumulates during the last period of the S phase (early-2 and late S phase), suggesting that CycA play a specific role during this stage of the endoreplication cycle.

Cyclin A is required to reach final ploidy in endoreplicating cells

In order to determine whether the oscillations of CycA protein affect endocycle progression, we studied the $CycA^{c8lr1}$ null mutant in bristle external cells (shaft and/or socket cells) by clonal analysis using the classical FLP/FRT system. As we did not expect clones of more than two cells due to the lack of the mitotic CycA

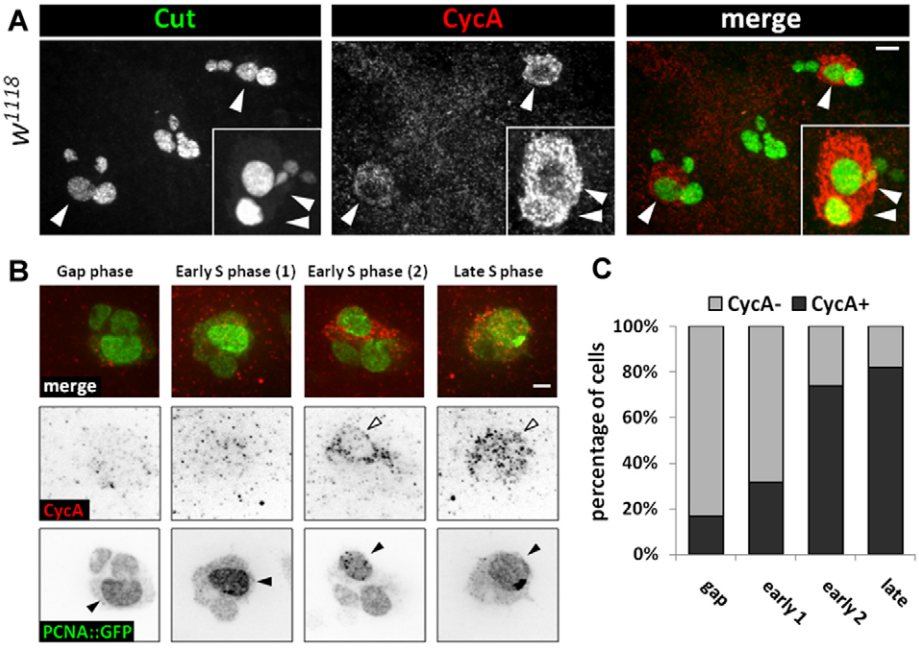

Fig. 2. Cyclin A protein levels oscillate during endocycles. (**A**) Immunostaining against CycA protein (red) in microchaetes and macrochaetes (inset) at 27 hours APF. Lineage cells are revealed by Cut staining (green). Arrowheads indicate CycA accumulation in external cells. Scale bar: 4 μm. (**B**) Immunostaining against CycA (red) in cells expressing the PCNA::GFP fusion protein (green) at 25 hours APF during the gap phase and endoreplication progression. Individual panels are displayed in inverted fluorescence. Open arrowheads show CycA accumulation in endoreplicating external cells. Black arrowheads indicate cells with a specific PCNA::GFP pattern. From left to right: nuclear and diffuse signals correspond to the gap phase; multiple coalescent PCNA::GFP foci to the early-1 S phase; a reduced number of PCNA::GFP foci to the early-2 S phase; and one single large spot to the late S phase. Scale bar: 2 μm. (**C**) The proportion of CycA-positive cells (black boxes) and CycA-negative cells (grey boxes) observed during each step of endoreplication (*n*=66).

function, recombination was specifically targeted to mitoses during the bristle cells lineage (intra-lineage clones, supplementary material Fig. S2). As a positive control for intra-lineage clones, we initially analysed null $CycE^{AR95}$ clones. Under the conditions used in our experiments, endocycles were completely abolished in null *CycE* clonal external cells (supplementary material Fig. S3), validating this approach. In $CycA^{c8lr1}$ intra-lineage clones, we did not observed obvious endoreplication phenotypes in microchaetes with respect to bristle size or DNA content (n=43 clusters observed). By contrast, in $CycA^{c8lr1}$ macrochaetes, we observed short bristles, suggesting a reduced ploidy in these external cells (supplementary material Fig. S2B, white arrowhead). Quantification of DNA content was accomplished by comparing equivalent clonal and control dorsocentral macrochaetes on the same notum at 72 hours APF (Fig. 3A,A′). Fig. 3A′ shows one example, in which both external cells were $CycA^{c8lr1}$ mutant. A consistent reduction in DNA content was observed in mutant cells, compared with the external cells from the contralateral macrochaete (Fig. 3B). The magnitude of this effect observed in null $CycA^{c8lr1}$ mutant cells may reflect the low number of endocycles that macrocheate cells undergo (six rounds). To test this idea, we generated $CycA^{c8lr1}$ mutant clones in larval salivary glands, a highly polyploid tissue (exhibiting ~12 rounds of endocycles). Nuclear size in all $CycA^{c8lr1}$ clonal cells was reduced by 30% compared with control heterozygous nuclei (Fig. 3C). Consistently, DNA staining measurements revealed that ploidy in $CycA^{c8lr1}$ mutant cells was half that of control cells (Fig. 3E). As salivary gland cells proliferate during embryogenesis, maternal *CycA* mRNA could account for the high number of cells present in salivary glands $CycA^{c8lr1}$ mutant clones. Indeed, we expected that CycA protein would be degraded during mitoses.

Taken together, these results indicate that CycA, although not essential, is required during endocycles for cells to reach their proper final ploidy. We hypothesised that under a $CycA^{c8lr1}$ loss-of-function condition, a cumulative slight delay in endocycle dynamics could reduce the final ploidy that cells reach.

CycA controls the early to late S-phase transition and late S-phase progression

To test the hypothesis that CycA is involved in endocycle dynamics, we performed in vivo analysis of endoreplication under conditions of CycA downregulation in microchaetes. Although CycA downregulation does not have an obvious effect on microchaete size, we chose these organs because this is the only system in which the required long-lasting in vivo recordings may be obtained. We expected that CycA downregulation would induce only slight modifications in the endocycle dynamics of this system, but that the predicted modifications would be sufficiently revealed by in vivo analysis. To this end, we generated a fly line *neur>UAS*-PCNA::GFP, UAS-*CycA*-RNAi, $tub\text{-}gal80^{ts}$. As in the previous experiments, PCNA::GFP was used as the S-phase reporter. The RNAi-hairpin targeting *CycA* (RNAi) allowed us to decrease the level of CycA. $tub\text{-}gal80^{ts}$ was used to decrease CycA level in a time- and tissue-dependent manner (0 hours APF pupae were shifted to 30°C to allow for PCNA::GFP and *CycA* RNAi expression). Movies were monitored from 18 to 35 hours APF at 30°C. In 70 microchaetes studied, two types of lineages were observed. In 71%, cell divisions in the lineage were obviously impaired, confirming that the *CycA*-RNAi was effective (supplementary material Fig. S4). In the remaining 29%, apparently wild-type lineages generated the normal set of terminal cells (Fig. 4A,A′; supplementary material Fig. S4, Movie 2).

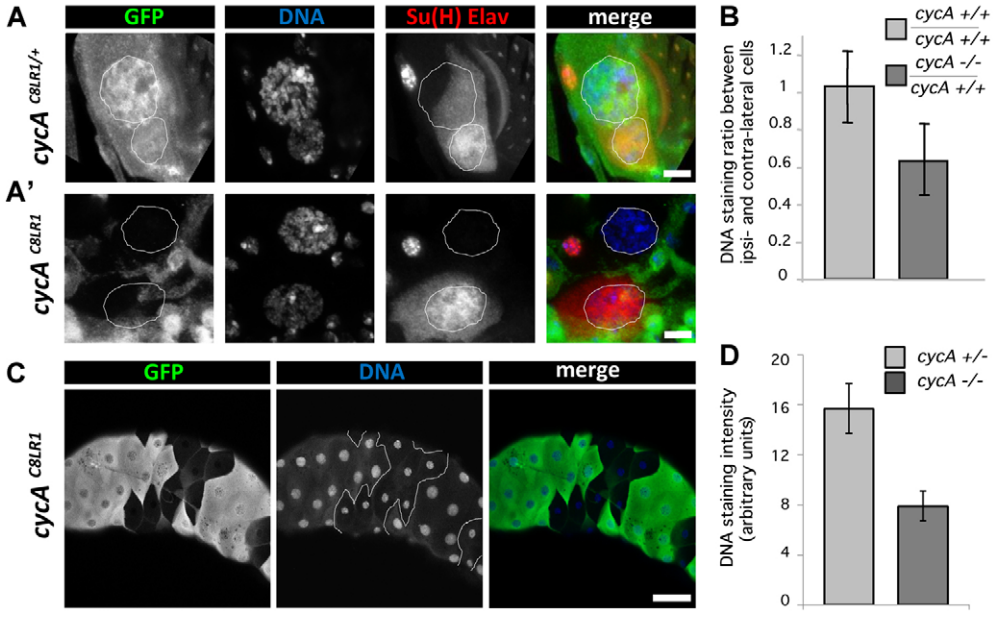

Fig. 3. *cyclin A* loss of function leads to ploidy reduction in endoreplicating cells. (**A**,**A'**) Control and $CycA^{C8LR1}$-null mutant shaft and socket cells from the same notum and at the same position (centro-dorsal posterior macrochaete). Nuclei are outlined. In the merge, Su(H) and Elav staining (red) allow the identification of the socket cell (large nucleus) and the neuron (small nucleus), respectively. Hoechst 33345 (blue) was used for DNA staining. Scale bars: 4 μm. (**B**) Ratio of DNA staining intensity measured between ipsi- and contralateral wild-type cells (grey boxes) and between $CycA^{C8LR1}$ clonal cell and contralateral wild-type cell (dark boxes). Mean and standard deviation (n=4 and 5, respectively). (**C**) Salivary gland from a third instar larvae containing a $CycA^{C8LR1}$-null mutant clones revealed by the lack of GFP (white line). Hoechst 33345 (blue) was used for DNA staining. Scale bar: 100 μm. (**D**) DNA staining intensity measured in control (grey box) and $CycA^{C8LR1}$-null mutant salivary gland cells (dark box) (n=79).

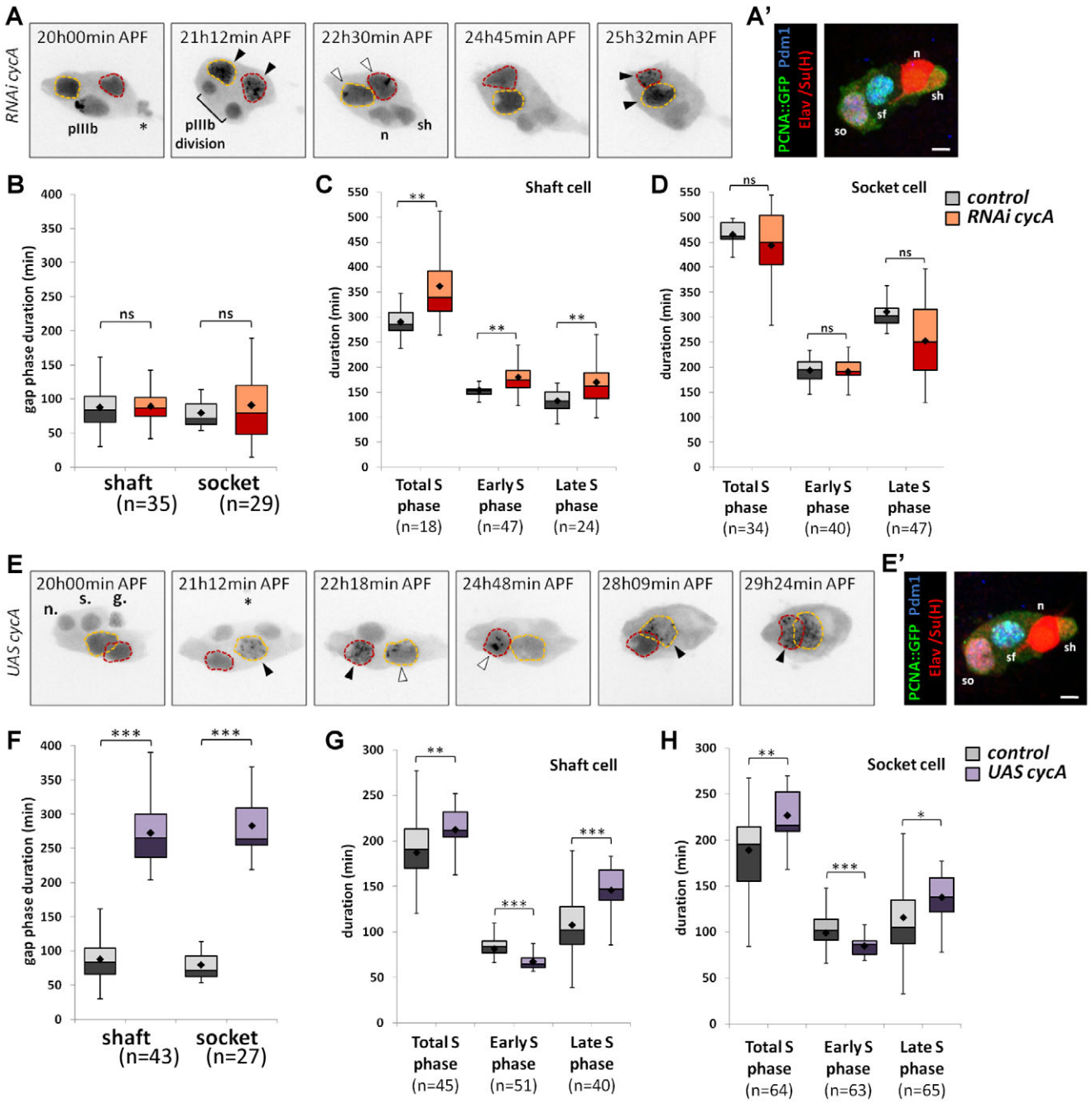

Fig. 4. *cyclin A* loss and gain of function impairs endocycle dynamics in microchaetes. Endocycle dynamics after RNAi-mediated CycA downregulation (A-D) and overexpression (E-H). (**A**,**E**) Representative frames (inverted fluorescence) from in vivo recordings of *tub-Gal80^{ts}, neur>UAS-pcna::gfp, UAS-CycA*-RNAi (A) and of *tub-Gal80^{ts}, neur>UAS-pcna::gfp, UAS-CycA* (E) pupae. Pupae were shifted from 18°C to 30°C at 0 hours APF. The socket and the shaft cells are outlined in red and yellow, respectively. pIIIb precursor cell division is marked with a bracket and glial cell apoptosis with an asterisk. Black arrowheads indicate nuclei in the early S phase and open arrowheads those in late S phase. Time APF is indicated in the left upper corner. (**A′**,**E′**) Socket and shaft cells identified by Pdm1 (blue), socket cells and neurons are detected by Su(H) and Elav, respectively (red). (B-D,F-H) Box plot representation of endocycles dynamics in *CycA*-RNAi (B-D) as in A and in CycA overexpression (F-H) as in E. (**B**,**F**) Duration of the gap phase between first and second rounds of endoreplication in shaft and socket cells under *CycA*-RNAi (B) and CycA overexpression (F) conditions. (**C**,**D**,**G**,**H**) Duration of the total, early and late S-phase during the second round of endoreplication in shaft (C,G), socket cells (D,H) and in control (grey boxes), CycA downregulation (C,D, red boxes) and overexpression (G,H, blue boxes) conditions. *P* values of two-tailed Mann-Whitney test are indicated as follows: ns=*P*>0.05, *P<0.05, **P<0,01, ***P<0.001. so, socket cells; sf, shaft cells; n, neurons; sh, sheath cells.

In order to quantify endocycles dynamics, we analysed the wild-type-like lineages (Fig. 4A). Endocycles were followed by in vivo recordings of both pIIa daughter cells. To increase sensitivity, we focused the analysis on the second round of endoreplication and the previous gap phase. The result of statistical analyses, comparing control versus RNAi conditions, are depicted in boxplots on Fig.

4B-D. The data show two main features. (1) In both cells, the duration of the gap phase (between the first and the second round of endoreplication) was not affected by *CycA* downregulation (Fig. 4B). (2) We did observe changes in the duration of endoreplication, however. As expected these changes were small, but reproducible. Although differences were not statistically significant among socket cells (Fig. 4D), a significant increase in the duration of endoreplication was observed in shaft cells. This increment was ~25% (Fig. 4C) and involved augmentation in the duration of both early and late S phase (Fig. 4C). As CycA protein starts to accumulate late during the early S phase (Fig. 2), these data suggests that CycA regulates the early to late S-phase transition and progression through late S phase.

Reciprocal *CycA* over-expression experiments were also performed. Specific overexpression of CycA in the sensory organs resulted in short macrochaetes (supplementary material Fig. S2). After CycA overexpression, 85% of the lineages were wild-type-like (n=43), whereas the remainder showed a supplementary division (Fig. 4E, supplementary material Movie 3 and data not shown). Only the wild-type-like lineages were analysed. In these lineages, the duration of the gap phase between the first and the second round of endoreplication was dramatically increased (Fig. 4F). Quantitative data were obtained from the first round of endoreplication, as the increase in gap-phase duration prevented reliable measurements from the second round. Unexpectedly, we observed an increase in the total replication time in both shaft and socket cells. Detailed analysis showed that this increase was exclusively due to augmentation of the late S-phase duration, as the duration of the early S-phase decreased (Fig. 4G,H). These results are in agreement with our hypothesis involving CycA in the early-to-late S-phase transition.

As endocycles occurred during a defined time window, increases in the duration of the entire S phase observed with both CycA down- and upregulation correlated well with the reduction in ploidy associated with bristle shortening. Collectively, our data show that although CycA is not required for promoting endoreplication, it appears to be involved in controlling the timing of early to late S-phase transition and late S-phase progression and/or exit.

Endogenous CycA levels are not sufficient for heterochromatin replication

We have shown that CycA accumulates and regulates the timing of the last stage of the S phase. The late S phase corresponds to the period in which one single PCNA dot is associated with under-replicated perinucleolar heterochromatin during endocycles (Follette et al., 1998; Andreyeva et al., 2008). In order to determine whether CycA has an influence on replication of heterochromatin, we analysed heterochromatin replication under CycA down- and upregulation conditions. DNA replication was visualised by the incorporation of 5-ethyl-2-deoxyuridine (EdU). In wild-type pupae, EdU incorporation was never observed inside the heterochromatin (identified by the brightest spot of Hoechst DNA staining, Fig. 5A, open arrowhead). Similar EdU profiles were observed after RNAi-mediated CycA depletion (Fig. 5B), showing that heterochromatin is also not replicated under these conditions. By contrast, EdU incorporation strongly co-localised with the highest spot of Hoechst DNA staining under conditions of CycA upregulation (white and open arrowheads, Fig. 5C), indicating that the heterochromatin was being effectively replicated.

To confirm these data, we analysed γH2Av staining under similar CycA down- and upregulation conditions. Under-replication of heterochromatin creates double strand breaks (DSBs), which are recognised by γH2Av immunoreactivity (Andreyeva et al., 2008). In control pupae, γH2Av immunoreactivity was observed as foci that were tightly associated with perinucleolar heterochromatin (Fig. 5D). Similar γH2Av foci around heterochromatin were observed in perinucleolar heterochromatin after RNAi-mediated

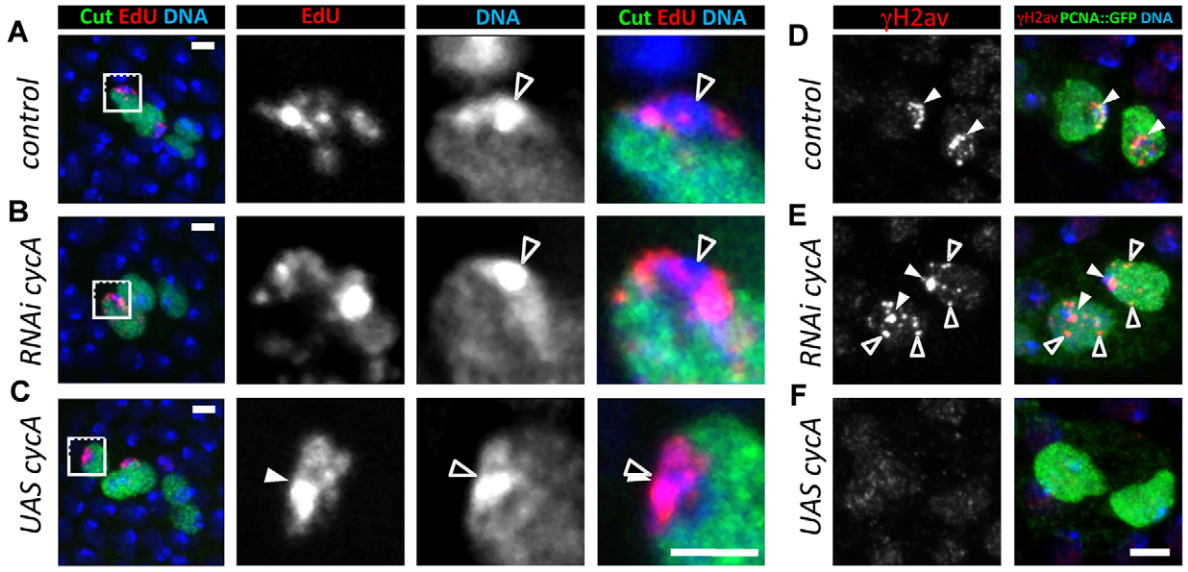

Fig. 5. Endogenous CycA level is not sufficient for heterochromatin replication. (**A-C**) Heterochromatin replication was monitored by EdU incorporation under control (A), CycA downregulation (B) and upregulation (C) conditions. Incorporated EdU was detected in pupae 26 hours APF (white arrowheads, red). Heterochromatin was identified by the strong spot of Hoechst DNA staining (blue, open arrowheads). Lineages cells were detected by Cut immunoreactivity (green). Each image corresponds to one confocal plane at the level of the heterochromatin. Scale bar: 2 μm. (**D-F**) *neur>UAS-pcna::gfp* pupae. γH2Av staining revealed DNA DSBs in control (D), under CycA down- (E) and upregulation (F) conditions. In the merge, sensory cells are identified by specific expression of PCNA (green), DNA in blue and γH2Av in red. White arrowheads indicate DSBs localised around peri-nucleolar heterochromatin, whereas open arrowheads show foci dispersed in the nucleoplasm after CycA downregulation. Scale bar: 2 μm.

CycA depletion (white arrowheads, Fig. 5E). In addition, we also observed foci dispersed in the nucleoplasm of both shaft and socket cells (open arrowheads, Fig. 5E), after RNAi-mediated CycA depletion. This observation suggests that DSBs were also being induced in euchromatin in a *CycA* loss-of-function background. Confirming our previous data showing that heterochromatin underwent replication after CycA overexpression, γH2Av foci were absent under these conditions (Fig. 5F). By showing that heterochromatin under-replication was not due to the presence of CycA, and that CycA overexpression induced heterochromatic replication, these results establish that low endogenous levels of CycA are not sufficient to promote replication of heterochromatin during endocycles.

CycA regulates ORC2 localisation

ORC2 belongs to the ORC family of proteins, which are required for forming the pre-RC (Bell and Dutta, 2002). In addition, ORC2 is involved in the establishment and maintenance of heterochromatin (Loupart et al., 2000; Leatherwood and Vas, 2003; Prasanth et al., 2004; Wallace and Orr-Weaver, 2005). Given this dual role, we analysed whether modulation of the early to late S-phase transition by CycA might be correlated with modifications in ORC2 distribution. In control *neur*>PCNA::GFP pupae, ORC2 was excluded from the heterochromatin region during early S phase (open arrowhead, Fig. 6A), colocalised with the heterochromatin during the late S phase (open and white arrowheads, Fig. 6B) and disappeared from heterochromatin during the subsequent gap phase (Fig. 6C). After CycA depletion, specific accumulation of ORC2 on the heterochromatin during late S phase was never observed, and ORC2 remained dispersed in the nucleus (Fig. 6D). By contrast, CycA overexpression led to a dramatic accumulation of ORC2 on heterochromatin in late S phase that persisted during the following gap phase (white arrowheads, Fig. 6E,F). Similar re-localisation of ORC2 proteins was observed after CycA down- and upregulation in salivary glands (supplementary material Fig. S5). Collectively, these results showed that, in controls, ORC2 proteins and PCNA::GFP normally shuttle between euchromatin and heterochromatin in the early to late S-phase transition. Moreover, they demonstrate for the first time that ORC2, but not PCNA::GFP relocalises to heterochromatin in a CycA-dependent manner.

Euchromatin replication is impaired by both CycA depletion and ORC2 upregulation

The previous experiments showed that RNAi-mediated CycA depletion induced both DSBs and ORC2 maintenance in euchromatin. As ORC2 has already been implicated in heterochromatin formation, we wondered whether the observed DSBs represented ectopic foci of under-replicated heterochromatic DNA. In order to test this possibility, we analysed whether DSBs colocalised with heterochromatin detected by HP1 immunoreactivity under CycA downregulation conditions. In control cells, a unique HP1-positive mass was detected that overlapped with nucleolar heterochromatin marked by Hoechst staining. As expected, γH2Av immunoreactivity was only observed as foci located around this HP1 mass (Fig. 7A). After CycA depletion conditions, HP1 staining was not modified demonstrating that the ectopic γH2Av foci were not associated with novel HP1-associated domains (Fig. 7B). These data strongly suggest that ectopic DSBs result from impairment of euchromatin replication per se, rather than replication failure due to ectopic heterochromatin boundaries.

Finally, we wondered whether ectopic γH2Av foci observed under conditions of CycA depletion were due to the persistence of ORC2 in the euchromatic region (Fig. 6D). Consistent with this hypothesis, ectopic γH2Av foci were also observed when ORC2 was overexpressed (Fig. 7C). Similar to conditions of CycA depletion, these ectopic DSBs were not associated with novel HP1 foci (Fig. 7C). These data show that upregulation of ORC2 mimic the phenotype observed under CycA downregulation in which ORC2 localisation was impaired.

Altogether, our data show that euchromatin located DSBs were not associated with ectopic heterochromatic foci under condition of either *CycA* downregulation or ORC2 overexpression. As such,

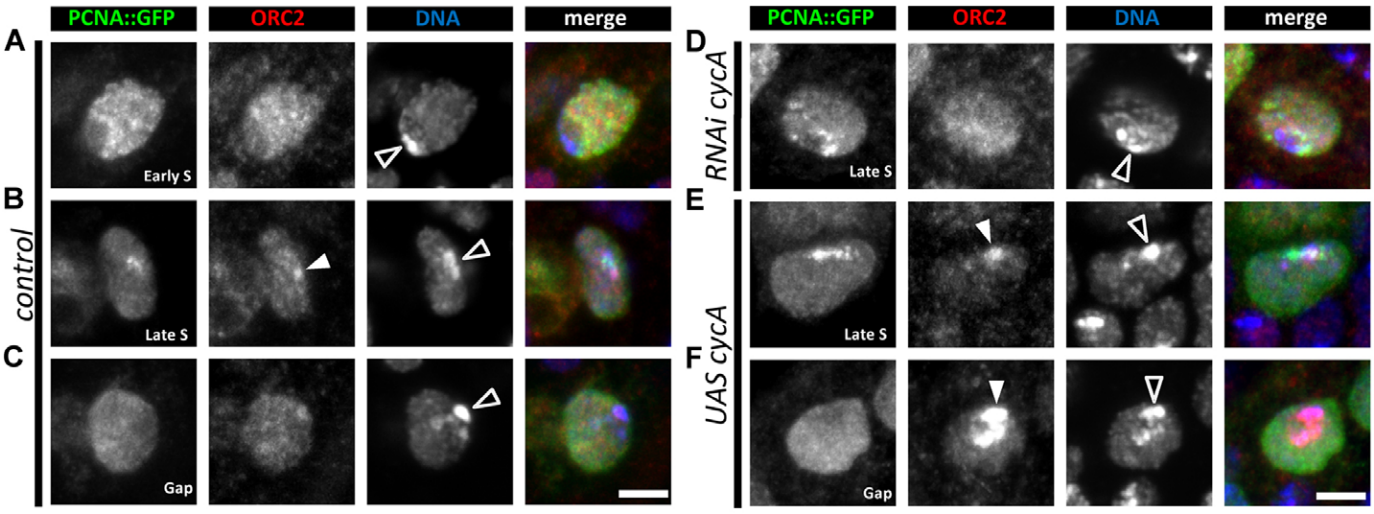

Fig. 6. ORC2 localisation to the heterochromatin depends on CycA. (**A-F**) Orc2 localisation in shaft and socket cells from *neur>UAS-pcna::gfp* at 25 hours APF pupae under control (A-C), CycA down- (D) and upregulation (E,F) conditions. Endocycle phases were determined as previously described using PCNA::GFP (green): early S (A), late S (B,D,E) and gap (C,F) phases. Heterochromatin was identified as the strongest spot of Hoechst DNA staining (open arrowheads, blue). ORC2 immunoreactivity is shown in red. White arrowheads indicate localisation of ORC2 on the heterochromatin. Scale bars: 2 μm.

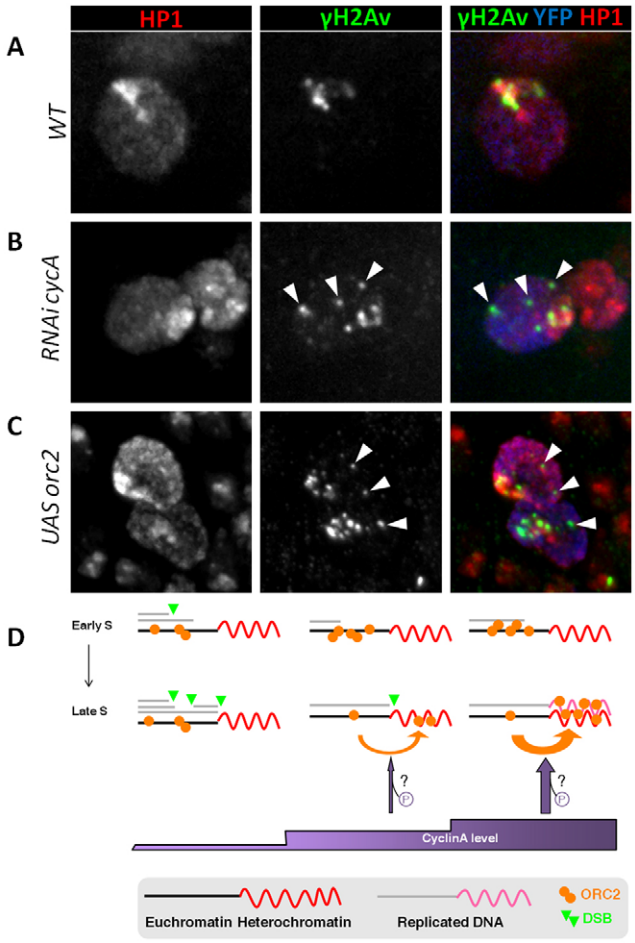

Fig. 7. Euchromatin located DSBs are not associated with ectopic HP1 foci. (**A-C**) HP1 (red) and γH2Av (green) immunostaining from control (A), *tub-Gal80^{ts}, neur>UAS-H2B::YFP, UAS-CycA*-RNAi (B) and *tub-Gal80^{ts}, neur>UAS-H2B::YFP,* EP-*ORC2* (C) pupae at 28 hours APF. Arrowheads indicate DSBs foci dispersed in the nucleoplasm. (**D**) Working model of endoreplication control by CycA via ORC2 relocalisation. ORC2 localisation between eu- and heterochromatin depends on the level of CycA and occurs at the early to late S-phase transition. At low CycA levels conditions, ORC2 remains on euchromatin and together with the low Cdk activity, partial replication occurs inducing DSBs. At normal CycA levels conditions, during the transition between early and late S-phase, ORC2 re-localises toward the heterochromatin but does not replicate. At high CycA levels conditions, ORC2 localises at high levels on the heterochromatin and, together with the high Cdk activity, allows full heterochromatin replication during late S phase. Question marks depict possible modes of action of CycA on ORC2.

these data suggest that euchromatin replication failures observed after CycA depletion were induced by a mechanism involving ORC2 delocalisation.

DISCUSSION

Our data reveal for the first time a role for CycA in the control of S phase in *Drosophila*. Until now, CycA has been considered exclusively as a mitotic cyclin in this organism. We observed that both up- and downregulation of CycA levels reduces the number of endocycles. In particular, the transition between early and late S phase is either advanced or delayed after CycA up- or downregulation respectively. In addition, the duration of the late S phase is increased under both conditions. The combination of these effects leads to a reduction in ploidy. Finally, we showed that CycA controls the localisation of the pre-replicative factor ORC2, an observation that may explain the effects of CycA on endocycle dynamics. All of these observations were performed under *CycA* hypomorphic conditions that do not affect mitoses. Collectively, these and our results show that the mechanism underlying endocycles requires a very low level expression (even undetectable) of major cell cycle regulators such as CycE and CycA (Audibert et al., 2005; Maqbool et al., 2010).

In *Drosophila*, CycA has been found in exclusive association with Cdk1, rather than with both Cdk1 and Cdk2 as in mammals (Knoblich et al., 1994; Budirahardja and Gönczy, 2009). As such, CycA has been always considered as a mitotic cyclin. Several observations suggest that CycA is implicated in DNA replication, however. CycA overexpression can trigger S phase, even in a *CycE* mutant background (Sprenger et al., 1997). Here, we show that CycA has a bona fide function that influences the progression of the S phase, and that more precisely involves the control of late S-phase timing during endocycles. Two possible mechanisms could account for our results: either CycA affects components required for overall S-phase progression or it modulates the activity of specific members of the core replication machinery. The former hypothesis is supported by data showing that in *Drosophila* Cdk1 and CycA cooperatively inhibit transcriptional activation by affecting the essential S-phase regulator E2F1 (Hayashi and Yamaguchi, 1999). In addition, degradation of E2F1 is related to its CycA-dependent phosphorylation in mammalian cells (Koseoglu et al., 2010). However, during endocycles in the bristle lineage, neither overexpression nor downregulation of CycA had a significant effect on the level of E2F1 accumulation (data not shown). Hence, our results favour a mechanism where CycA regulates specific components of the replication machinery. Indeed, we have shown that CycA is required for the relocalisation of the pre-RC protein ORC2 during the early-to-late S-phase transition.

Classically, ORC2 is considered to be part of the pre-RC (Shermoen et al., 2010). However, the pre-RC component MCM5 (not shown) and the replication factor PCNA::GFP (Fig. 6D,F) do not undergo CycA-dependant control of their localisation, as was observed for ORC2. Although we have not ruled out the possibility that localisation of Cdc6 or Cdt1 (or other components) might depend on CycA activity, these data suggest that ORC2 acts independently of the pre-RC. Indeed, apart from its well-known role in DNA replication, ORC2 has been shown to participate in heterochromatin maintenance (Loupart et al., 2000; Leatherwood and Vas, 2003; Prasanth et al., 2004; Wallace and Orr-Weaver, 2005). Moreover, ORC proteins localise to heterochromatin in vivo and interact directly with the essential heterochromatin factor HP1 (Pak et al., 1997; Lidonnici et al., 2004). As such, ORC2 could act as a gatekeeper between euchromatin and heterochromatin or, alternatively, it could be implicated in the establishment or the maintenance of chromatin structure. After CycA overexpression, the observed lengthening of late S-phase is probably due to stable relocalisation of ORC2 to the heterochromatin that, concomitant with the high CycA activity, induces a complete replication of heterochromatin. In addition, under conditions of *CycA* loss of function where ORC2 does not relocalise to heterochromatin, we often observed that chromosomes were under-condensed. Similarly, irregularly condensed mitotic chromosomes were observed in *Drosophila* and in mammalian ORC2-depleted cells (Loupart et al., 2000; Prasanth et al., 2004). In any case, the fact that

heterochromatin can still be formed in the absence of ORC2 relocalisation suggests that ORC2 is necessary but not essential for heterochromatin maintenance. This dispensability of ORC2 has been also observed for endoreplication, as, in *orc2* mutants, endocycles occurred but cell ploidy was reduced (Park and Asano, 2008). These data are therefore in agreement with our results showing that CycA controls ORC2 localisation and modulates endocycles dynamics. Thus, lengthening of the late S-phase observed after CycA mis-regulations probably reflects a dual function for ORC2 in both DNA replication and chromatin structure.

Our data show that the timing of the early S phase was also affected under conditions of CycA mis-regulations. Early S-phase was lengthened under the CycA downregulation condition and conversely shortened after CycA overexpression. As our data showed that CycA begins to accumulate at the end of early S phase, this suggests that CycA controls the transition between early and late S phase. Under CycA downregulation conditions, we observed that ORC2 remained throughout the nucleus. This maintenance of ORC2 in the euchromatic regions may explain the delay in transitioning from early to late S phase as due to the stabilisation of a permissive state for euchromatin replication. Strengthening the case that CycA is important for this process, DSBs detected by γH2AV immunoreactivity are also induced under these conditions. These ectopic DSBs were not associated with ectopic heterochromatin foci (see Fig. 7B), arguing against them being caused by heterochromatin boundaries. This suggests that DSBs reflect partial replication due to the low level of Cdk activity. As such, our results imply that CycA is involved in the control of DNA replication per se. These results are in agreement with recent data of Ding and MacAlpin on DNA re-replication, suggesting that Cdk/CycA activity specifically inhibits pre-RC assembly in the euchromatin (Ding and MacAlpine, 2010).

Using DSBs to assay for replication defects, we have shown that ORC2 overexpression also induced similar replication impairment as CycA depletion. These results suggest that CycA-dependent ORC2 intra-nuclear re-localisation would be a way to deplete euchromatin of ORC2, concluding replication and generating a period during which euchromatic DNA is unable to relicense. This model is also consistent with observations made in other systems, for example, in CHO hamster cells where Cdk1/CycA was able to hyper-phosphorylate ORC1 and decrease its affinity for chromatin (Li et al., 2004). Moreover, it has also been shown that Cdk1/CyclinA can phosphorylate ORC2 in vitro (Remus et al., 2005). All these data support a model where CycA, in a dose-dependent manner, promotes ORC2 shuttling between euchromatin and heterochromatin as a mechanism to accomplish the early S phase (Fig. 7D).

Concluding remarks

Our data complete the current model of endocycle progression by incorporating the mitotic factor CycA. In this model, CycE activity oscillations, mediated in part by E2F1 activity, drive cyclic anti-parallel oscillations of APC/C-Fzr activity. Periods of low APC/C activity would permit accumulation of pre-RC complex proteins, such as ORC1 and Cdc6 (Narbonne-Reveau et al., 2008; Zielke et al., 2008). As such, these oscillating activities generate S- and G-phase time windows, which ensure that the genome is properly replicated once in every cycle. We suggest that CycA reinforces the exit from the replicative state, probably by controlling localisation and/or activity of pre-RC factors such as ORC2.

Finally, our work reveals a novel vision for the mechanism underlying endocycles. This mechanism appears not to be a subset of that controlling mitosis. Rather, we show here that, as the same factors participate in both processes, the mechanism involved in endocycles and mitotic cycles differs only by quantitative variations in protein levels and probably by the temporal expression of these factors.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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