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Developmental gene amplification: insights into DNA replication and gene expression

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In the formation of a complex organism and the differentiation of specific cell types, there are often demands for high levels of particular gene products. These demands can be met by increasing transcription or translation, or by decreasing the rate of mRNA or protein turnover. Although these are the most common means to increase expression levels, there is another mechanism: gene amplification. Developmental gene amplification is a DNA replication-based process whereby specific genes are replicated above the copy number of surrounding sequences, resulting in an increase in the template available for transcription. Recent microarray studies in *Drosophila melanogaster* have identified two additional amplicons, suggesting that developmental gene amplification might be more widely used than was previously thought. Furthermore, work in both *Drosophila* and the related fly, *Sciara coprophila*, has yielded insights into the mechanisms, regulatory sequences and proteins controlling DNA replication during gene amplification, including a link between transcription factors and origin usage.

Introduction

Developmental gene amplification occurs in a variety of organisms at specific developmental stages (Table 1). Early studies involved genes encoding products needed in high amounts, such as rRNAs and structural proteins for

Table 1. Developmental amplicons

Organism	Cell type	Genes amplified	Mechanism of amplification	Fold amplification
Amphibians [2,3,15,73]	Oocyte	rRNA	rDNA excision from chromosomes, then rolling circle amplification	Up to several thousand-fold
Tetrahymena thermophila [1]	Macronucleus	rRNA	rDNA excision from original chromosome locus, then forms a palindromic rDNA chromosome (21 kb); Bidirectional replication gives an onionskin structure (see Figure 1)	Up to 10 000-fold over the 21 kb chromosome
Pterygotan insects (Coleoptera [4], Neuroptera [5], Orthoptera [6])	Oocyte (nurse cells in Coleoptera)	rRNA	rDNA excision, often followed by the formation of rDNA circles	Orthoptera: 14-fold; Coleoptera: sixfold
Bradysia hygida [74,75]	Salivary gland	Cocoon protein genes	Bidirectional replication from an endogenous chromosomal locus to form an onionskin structure (see Figure 1)	Locus (Puff) C4: 21-fold over 18 kb Puff B10: tenfold
Rhynchosciara americana [7,8,25]	Salivary gland	Communal cocoon protein genes	Bidirectional replication from an endogenous chromosomal locus to form an onionskin structure	Puff C3–22: 32-fold over 50 kb; Puff C8: 16-fold over 60 kb; Puff B2: fourfold
Sciara coprophila [10,18]	Salivary gland	Cocoon protein genes	Bidirectional replication from an endogenous chromosomal locus to form an onionskin structure	Puff II/9A: 18-fold over at least 35 kb; Puff II/2B: 17-fold
Drosophila melanogaster [12,13,20]	Ovarian follicle cells (form an epithelial layer around the egg)	Chorion genes, putative vitelline membrane enzymes, chitin-binding proteins and transporters	Bidirectional replication from an endogenous chromosomal locus to form an onionskin structure	DAFC-7F: 18–20-fold; DAFC-30B: fourfold; DAFC-30B: fourfold; DAFC-62D: 6.5-fold; DAFC-66D: 60–80-fold; All 75–100kb

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cocoons and eggshells. The rRNA genes of the protist *Tetrahymena thermophila* are amplified during the development of the transcriptionally active macronucleus [1], as are the rRNA genes in amphibian oocytes [2,3] and Pterygotan (winged) insect oocytes and nurse cells [4–6], to stockpile the machinery necessary for rapid embryonic development. Dipteran flies, including *Rhynchosciara americana* [7,8], *Bradysia hygida* [9] and *Sciara coprophila* [10], use gene amplification at multiple genomic loci in the larval salivary glands, presumably for the production of large quantities of structural proteins for cocoon construction. In another Dipteran fly, *Drosophila melanogaster*, amplification of two clusters of chorion (eggshell) protein genes in the ovarian follicle cells is seen [11,12] (Box 1).

Recently two additional amplicons were identified in *Drosophila* follicle cells by a comparative genomic hybridization (CGH) array approach. These amplicons contain genes encoding a variety of proteins, including transporters, proteases, chitin-binding proteins and two putative enzymes, encoded by *yellow-g* and *yellow-g2*, thought to be necessary for crosslinking proteins of the vitelline membrane or eggshell [13]. The observation that genes encoding enzymes are amplified in *Drosophila*, rather than just those encoding rRNA or structural proteins clearly required in abundant quantities, raises the possibility that gene amplification might be a more ubiquitous mechanism for coping with demands on gene expression throughout development than was previously appreciated. Recent studies in *Drosophila* and *Sciara* have provided insights into the mechanism of gene amplification, regulatory elements, and the roles of transcription factors and chromatin.

Mechanism of gene amplification

In addition to its intriguing developmental consequences, gene amplification serves as a powerful model for investigating the control of metazoan DNA replication. Gene amplification occurs in all of the organisms mentioned via a DNA replication-based mechanism. This contrasts with gene amplification observed in transformed mammalian cells, which probably arises from a recombination-based mechanism (for review see Ref. [14]).

During developmental gene amplification in amphibian and Pterygotan insect oocytes, in addition to *Tetrahymena*, the amplified DNA is first excised from the chromosome, then converted to an episome in amphibians [15] and Pterygotans [5,6,16] or a minichromosome in *Tetrahymena* (for review see Ref. [17]). We will focus on amplification mechanisms in the Dipteran flies, in which the amplicons are chromosomal and thus provide insights into replication origins and initiation control.

Gene amplification in Dipteran flies results from repeated firings of initiation zones and bidirectional fork movement to either side, producing a gradient of amplified DNA. The resulting 'onionskin' structure has been observed in electron microscopy studies and as replication intermediates by two- and three-dimensional gel electrophoresis experiments (Figure 1a) [18–23]. The salivary glands of the Sciarid flies and the *Drosophila* follicle cells are terminal tissues that are histolyzed shortly after amplification, so the amplified DNA does not become permanently rearranged or covalently integrated into the chromosomes, as might be the case if these cells underwent further divisions.

Gene amplification follows successive rounds of endocycling in *Sciara* and *Drosophila* (for review see Ref. [24]). Endocycles consist of alternating DNA synthesis (S phase) and gap phases without an intervening division of the chromosomes (mitosis). Whereas in gene amplification only specific portions of the genome are overreplicated, during endocycles the majority of the genome is replicated in each S phase. Endocycles produce polyploid or polytene cells, the distinction being that in the latter case the replicated copies of each chromosome are held in tight register.

In Dipteran flies, the gradient of copy number in the amplified regions is centered such that the genes in the center of the gradient (with the highest copy number) are the most abundantly transcribed, and those on the shoulders of the amplification gradient are only expressed at a low level or are not expressed at all in the amplifying tissues. This suggests that there could have been a selection for the origin of DNA replication to be adjacent to these highly expressed genes. Amplification levels vary among amplified regions, ranging from fourfold for the *Drosophila* amplicon in the follicle cells at cytological position 30B (DAFC-30B) to 60–80-fold for the chorion DAFC-66D amplicon [12,13,20]. The Sciarid fly amplification levels range from tenfold in *Bradysia* to between four- and 30-fold in *Rhynchosciara* [7,8,10,18,25]. Despite the differences in amplification levels in *Drosophila*, the extent of the gradient is similar for all amplicons, spanning 75–100 kb [12,13,20] (Figure 1b). If the selective force has been copy number increases of the central genes, those genes in the flanking regions of the gradient could be amplified passively and might not function in the amplifying tissues nor require amplification for expression. In addition to the increased copy number of amplified genes, amplification could augment transcription by interactions between replication and transcription proteins or by promoting an open chromatin conformation at the promoters of the amplified genes.

The amplification profiles can be explained by regulation of the number of initiation events for each amplicon without control of replication termination. The replication forks in these polyploid tissues move slowly compared to S phase in diploid cells, and the time allotted for amplification in these terminally differentiated cells coupled with the rate of movement of the replication forks accounts for the width of the amplified domains [26,27]. Although it is not necessary to invoke replication termination sites for these amplicons, there do appear to be regions in which DNA sequence or chromatin structure impedes replication fork movement, because the amplification gradients in *Drosophila* and *Rhynchosciara* are not symmetrical [12,13,21,26]. Another interesting aspect of origin firing and replication fork movement at the *Drosophila* amplicons DAFC-7F, 30B and 66D is that all origin firings occur during a discrete period of development, and when initiation ceases only the existing replication forks undergo elongation [26]. DAFC-62D behaves in the same manner, except that it undergoes an additional round of origin firing at the end of differentiation that could provide more template for late gene expression [13].

Box 1. Where gene amplification occurs: the *Drosophila* follicle cells

The ovaries of *Drosophila* are made up of ovarioles (Figure I). Each ovariole is a string of several developing egg chambers of increasing age, with the youngest egg chambers and stem cells contained within the germarium (not shown in Figure I) at the anterior end of the ovariole, and the oldest egg chambers at the posterior, ready to be ovulated. Each egg chamber consists of three cell types, the 15 germline-derived nurse cells, a single oocyte and the somatically derived follicle cells that form an epithelial layer around the egg chamber. Based on various morphological markers, including the size of the egg chamber and positions of particular follicle cells, egg chamber development can be divided into various stages.

The follicle cells undergo several different types of cell cycles throughout egg chamber development. Initially, during stages 1–6, they use mitosis to increase their number to ≈1000 per egg chamber. By stage 7, mitosis has ended in the follicle cells and they begin endocycles. During the endocycles in stages 7–9, an S phase and a gap phase alternate without an intervening mitosis and cell division, and the DNA content of the cells increases to 16C. By stage 10a, the endocycles end, the follicle cells cease genomic replication and are poised to enter gene amplification.

In stage 10b, gene amplification begins at four loci throughout the genome, and can be visualized by immunofluorescence microscopy as four spots of BrdU incorporation. The four amplified loci are known as *Drosophila* amplicon in the follicle cells, or DAFC, and are also named for their cytological position on the *Drosophila* chromosomes. (DAFC-7F is on the X chromosome, DAFC-30B is on the second chromosome, and DAFC-62D and DAFC-66D are on the third chromosome.) High-resolution microscopy studies of replication factor localization and BrdU incorporation, in addition to real-time PCR quantitation of copy number along the amplified intervals, demonstrated that DAFC origins fire during stages 10b and 11 of egg chamber development (initiation). By the end of stage 11, the DAFC origins stop firing, and only the existing replication forks move out bidirectionally in an elongation-only phase during egg chamber stages 12 and 13. The exceptions to this are that DAFC-66D, the most highly amplified region, begins origin firing in stage 9, and DAFC-62D undergoes a final round of origin firing in stage 13, presumably to provide more template for transcription of genes needed late in oogenesis. Transcription of the chorion genes from DAFC-7F occurs in stages 11–12, before the burst of expression from the DAFC-66D locus in stage 13 [79]. Genes from the DAFC-30B and 62D amplicons begin transcription as early as late stage 10b [13].

By late stage 13, amplification has ended and the follicle cells continue to synthesize the eggshell, dorsal respiratory appendages and other crucial structures for the ensuing embryo. Once they have completed these tasks, the follicle cells are sloughed off. For a review see Ref. [80].

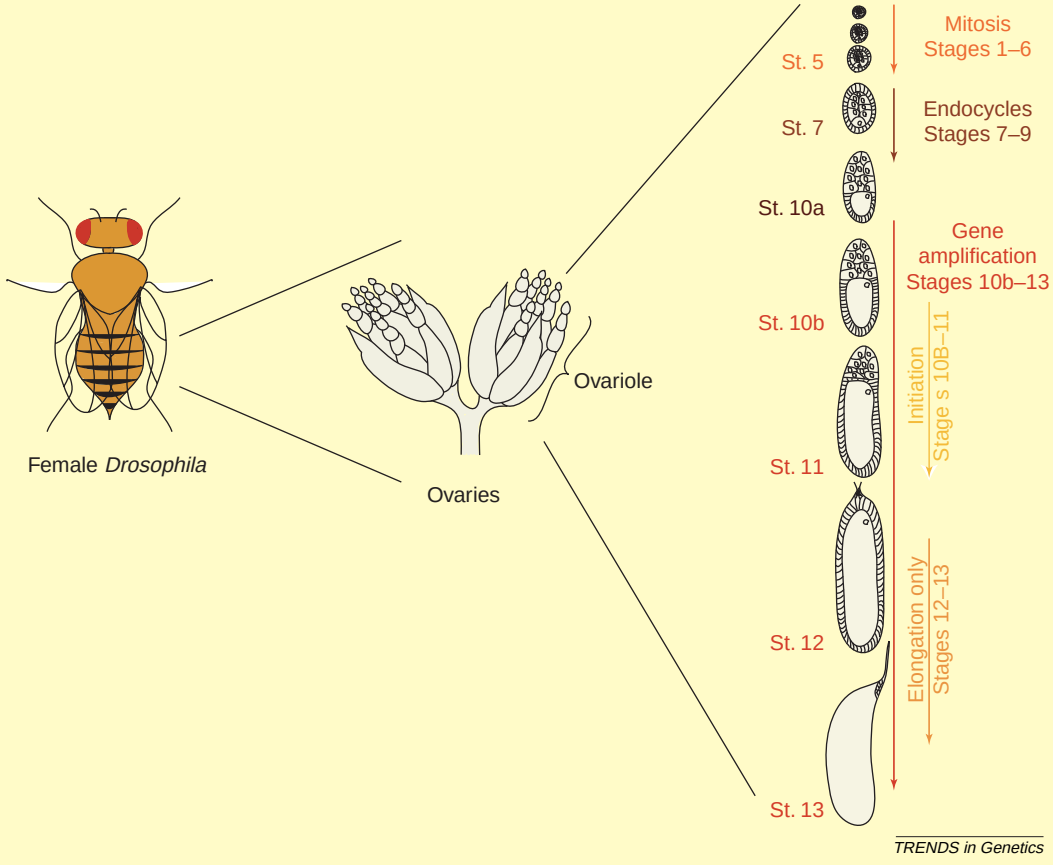

Figure I. The ovaries of female *Drosophila* consist of ovarioles, strings of egg chambers in various developmental stages. The youngest egg chambers are at the top, the oldest are towards the bottom. The cell cycle modifications undergone by follicle cells are marked, along with the developmental stages in which these occur.

Replication origins and enhancers in developmental gene amplification

Developmental gene amplification requires that a replication origin repeatedly fires in response to developmental signals. The control elements for this process have been defined most precisely for DAFC-66D, establishing a regulatory paradigm in which an enhancer-like regulatory element provides the developmental specificity for activation of an adjacent replication origin.

The regulator *ACE3* (Amplification Control Element for the third chromosome chorion cluster) (Box 2) and the origin *Oriβ* (Figure 2a) were delineated by a combination

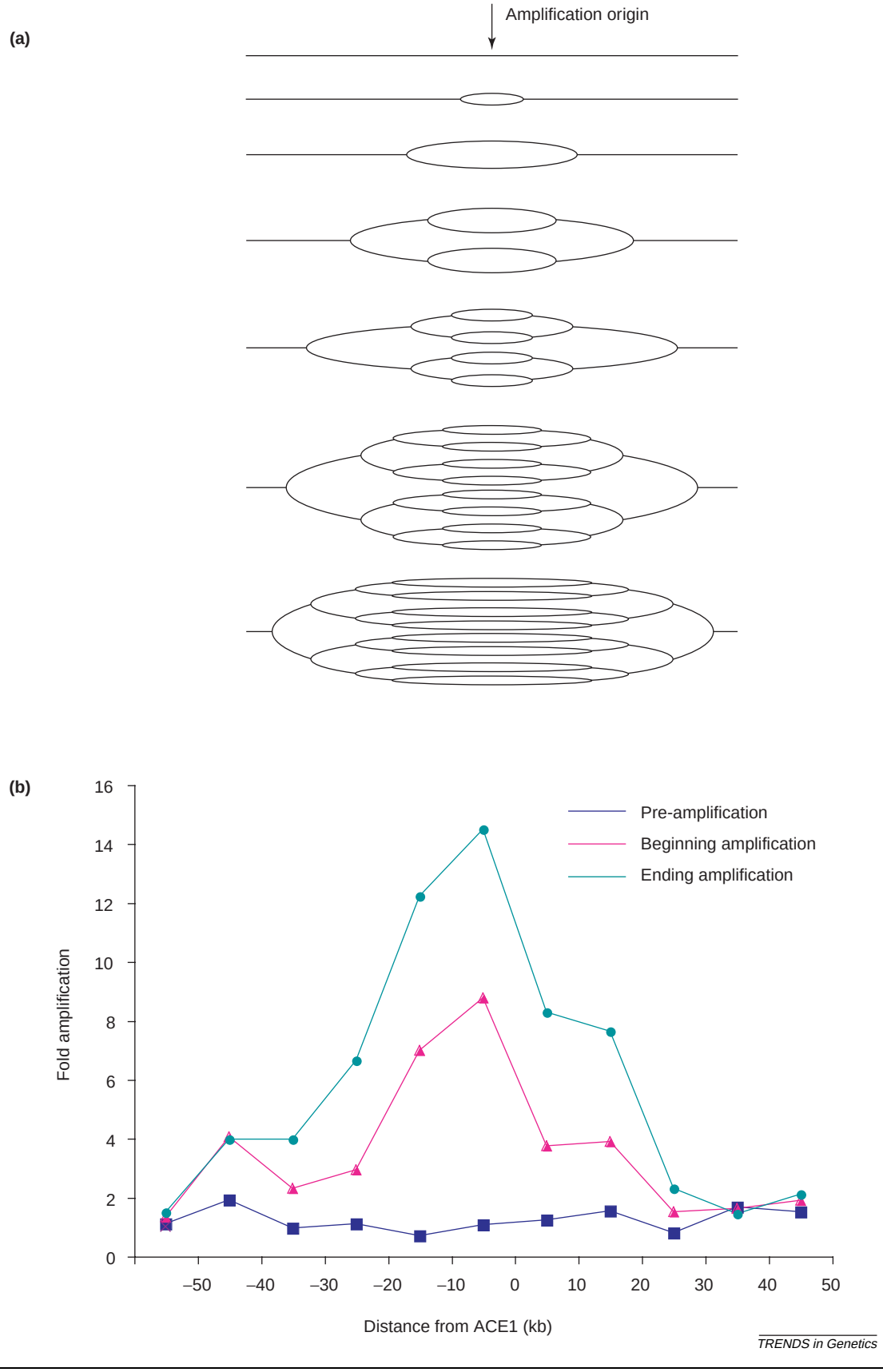

Figure 1. A schematic of the DNA copy number changes that occur during developmental gene amplification. **(a)** The onionskin structure of nested replication forks is generated from repeated firings of the central replication origin coupled with bidirectional replication fork movement. This structure has been observed by electron microscopy and two-dimensional gel analyses during gene amplification stages in the Dipteran flies and the protist *Tetrahymena*. **(b)** Quantitative real-time PCR measurement of copy number along the *Drosophila* DAFC-7F chorion amplicon during follicle cell differentiation [13]. In stages 1–8 of egg chamber development (blue), before gene amplification, the relative copy number along the amplicon is ≈1. As amplification begins, in stage 10b (pink), the centrally located origin region and chorion genes are copied first, whereas flanking sequences are copied in later stages by moving replication forks. By the end of gene amplification, at stage 13 (green), a gradient of copy number spanning 100 kb has been generated, with the peak of amplification in the center of the region.

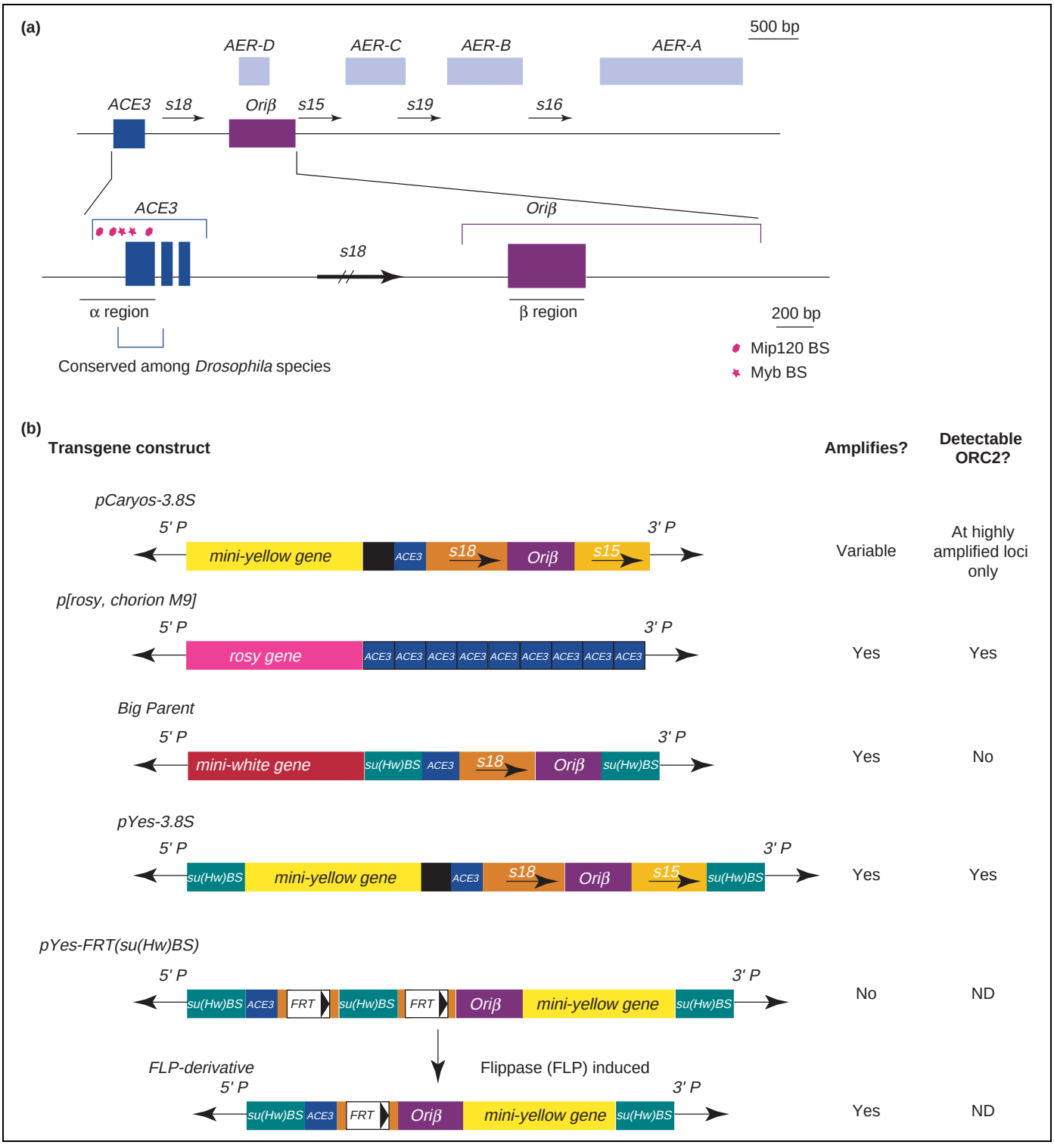

Figure 2. The replication control elements and chorion genes of DAFC-66D. **(a)** The chorion genes, *s18, s15, s19* and *s16*, are located within the region of peak copy number. The 440 bp enhancer-like region, *ACE3* (blue), has been dissected further in recent deletion studies using transgenes (zoom-in below), and a 320 bp region, the α region, appears to be conserved and necessary for amplification [54]. This portion of *ACE3* also contains binding sites for Myb and Mip120, which have previously been shown to be necessary for amplification [61]. The 884 bp *Oriβ* origin (purple) accounts for 70–80% of initiation events in the region. Recently a minimal region of *Oriβ*, the β region, has been shown to be crucial for amplification [54]. The replication stimulatory elements, *AER-A* to *-D* (lilac), are interspersed among the chorion genes [34]. **(b)** Transgenic constructs containing portions of the DAFC-66D amplicon have been introduced via P element transposons into exogenous portions of the genome to determine the sequence requirements for amplification. The *suppressor of hairy wing* binding sites (*su(Hw)BS*) (green) have been valuable in insulating such constructs from chromosomal position effects at the sites of insertion [33]. (Note that the *pCaryos-3.8S* construct and the *p[rosy, chorion M9]* constructs are not buffered by the *su(Hw)BS*.) The *p[rosy, chorion M9]* transgene can recruit visible ORC2 due to the number of *ACE3* copies it contains [51]. The *Big Parent* construct containing *ACE3, s18* and *Oriβ*, although buffered and capable of amplifying, is not able to recruit ORC2 protein in sufficient quantities to be visualized. Only with the addition of extensive sequences (*pYes-3.8S*) can ORC2 be visualized at the site of transgene insertion. Thus, it appears that there is a threshold amount of ORC that must be recruited to be visible, and *ACE3* and *Oriβ* serve as ORC nucleation sites [54]. The *pYes-FRT(su(Hw)BS)* construct, in which *ACE3* and *Oriβ* are separated by an insulator element that can be excised using the Flipase (FLP) recombinase (resulting in the *FLP-derivative* construct), was used to show that *ACE3* and *Oriβ* must have continuous chromatin to initiate amplification [31]. Note that the transgenes are not drawn to scale, and the black boxes within the *pCaryos-3.8S* and the *pYes-3.8S* constructs represent additional chorion sequences adjacent to *ACE3*. ND, not determined. Adapted with permission from Ref. [54].

Box 2. Definition of terms

ACE3: Amplification Control Element on the *Drosophila* third chromosome chorion amplicon.
Oriβ: Origin beta, the predominant amplification origin of the *Drosophila* third chromosome chorion amplicon.
AER-A to -D: amplification enhancing regions A to D for the *Drosophila* third chromosome chorion amplicon.
RIP mapping: replication initiation point mapping.
CGH array: comparative genomic hybridization array.
DUP (CDT1): double parked (Cdt1) protein, a component of the pre-replication complex.
MCM2–7: minichromosome maintenance proteins 2–7, components of the pre-replication complex.
PCNA: proliferating cell nuclear antigen, DNA polymerase delta processivity factor.
DAFC: *Drosophila* amplicon in follicle cells.
CDK: cyclin-dependent kinase.
DDK: dbf4-dependent kinase.
BrdU: 5′-bromo-2′-deoxyuridine, a nucleotide analog.
ChIP: chromatin immunoprecipitation.
Myb: myeloblastosis oncoprotein.
Mip: myb-interacting protein.
RBF: retinoblastoma protein homolog in *Drosophila*.
FLP: flipase recombinase, an enzyme that catalyzes site specific recombination.
FRT: FLP recombinase target, a DNA sequence recognized by the flipase recombinase.
ORC: origin recognition complex, a six-member protein complex that is part of the pre-replication complex.

of transgene experiments testing for amplification of DAFC-66D chorion amplicon sequences at ectopic genomic sites (Figure 2b) and two-dimensional gel analyses to map the sites of replication initiation. *ACE3*, located 5′ to the chorion genes, was shown to be crucial for amplification of chorion transgenic constructs, and a 440-bp fragment is sufficient to induce amplification at low levels with proper developmental specificity [28,29]. Notably, the *ACE3* sequences necessary for amplification were demonstrated to be distinct from promoter sequences controlling transcription of the adjacent *s18* gene [30]. Origin mapping by two-dimensional gel electrophoresis mapped the preferred site of origin activity to an interval 1.5 kb from *ACE3* known as *Oriβ* [19,20]. In contrast to *ACE3*, *Oriβ* was not sufficient to induce amplification of transgenes [31]. Although marked by a high A-T content and, for *ACE3*, conservation between *Drosophila* species [32], it remains to be determined whether these sequences will prove diagnostic for amplification control elements.

The relationship between *ACE3* and *Oriβ* has been deciphered in a series of experiments in which transgenes were buffered from surrounding chromatin (Figure 2b) [31,33]. The initial transgene constructs tested were vulnerable to position effects that inhibited amplification or provided potential ectopic replication origins (Figure 2b, *pCaryos-3.8S*). The chromatin insulator element (*su(Hw)BS*) was shown to protect chorion transgenes from position effects, resulting in consistent amplification at insertion sites [33]. In these buffered constructs, *ACE3* and *Oriβ* were sufficient for proper developmental amplification (Figure 2b, *Big Parent*), whereas *ACE3* alone was not sufficient [31]. This contrasts with unbuffered transgenes carrying only *ACE3*, suggesting that in the absence of insulator elements *ACE3* was capable of activating adjacent replication origins [29]. Chromatin insulator elements were used to examine the relationship between *ACE3* and *Oriβ* (Figure 2b, *pYes-FRT(su(Hw)BS)*, revealing a requirement for continuous chromatin between these elements. An insulator element placed between *ACE3* and *Oriβ* in transgenes nearly eliminated amplification, and subsequent removal of this insulator element by FLP and FRT-mediated recombination (Figure 2b, *FLP-derivative*) restored amplification [31]. In addition to *ACE3* and *Oriβ*, four other replication stimulatory elements, Amplification Enhancing Regions (*AER*)-*A* to *-D* (Figure 2a), have been mapped, of which *AER-D* resides within *Oriβ* [34].

During gene amplification in the *Sciara* salivary amplicon at Puff *II/9A*, two- and three-dimensional gel analyses indicate that initiation occurs in a 5.5-kb zone, and within this region a preferred 1 kb accounts for the majority of the origin firings [18,23] (Figure 3a). Furthermore, the precise nucleotide within the 1 kb region at which replication initiates has been isolated by the technique of replication initiation point (RIP) mapping [35]. The lack of a transformation system has impeded delineation of the sequences controlling amplification, although a replication-dependent DNaseI hypersensitive site at the left boundary of the initiation zone has been postulated to have a regulatory role [36]. In the related Sciarid fly, *Rhynchosciara*, two-dimensional gel analyses demonstrate that replication initiates in the salivary puff C3 from at most three sites in a zone of ≈6 kb and that this zone resides ≈2 kb upstream of the amplified gene *C3–22* [21].

Proteins regulating amplification

Genetic, biochemical, and cell biological approaches have demonstrated that the proteins involved in DNA replication during a normal cell cycle, including pre-replication complex (pre-RC) components, are also involved in gene amplification (Box 3). This further highlights the utility of this system to study replication control. An important insight emerging from the identification of regulatory proteins in amplification is the employment of transcription factors to control replication.

Replication proteins

Although mutation of essential DNA replication factors generally causes lethality, genetic screens in *Drosophila* have recovered weak mutations in several genes that affect gene amplification (Table 2). These hypomorphic alleles cause female sterility and thin eggshells due to reduced amplification and reduced production of eggshell components. Among this group are mutations in the *orc2*, *chiffon* (*dbf4*-like), *pcna* (*mus209*), *dup* (*cdt1*) and *mcm6* genes [37–42]. A weak allele of the essential gene *mus101* disrupts amplification, as does mutation of the *humpty dumpty* gene (previously known as mutant *fs(3)272–9*). Although *mus101* family members in other species have important roles in replication initiation, elongation, and in the replication checkpoint, the role of the *Drosophila* protein in amplification has not been defined [43–46]. The *humpty dumpty* gene product defines a new family of proteins, conserved among multicellular eukaryotes,

Box 3. Initiation of eukaryotic DNA replication

In eukaryotes, a complex of proteins assembles at replication origins to form the pre-replication complex (pre-RC), which is able to recruit the machinery for DNA synthesis (Figure I). The six-member origin recognition complex (ORC) was identified by its ability to bind the origins of *Saccharomyces cerevisiae* chromosomes [81], and it is conserved in metazoans. Once ORC is bound to origins of replication, it recruits CDC6 and DUP (CDT1), which in turn load the putative replication fork helicase complex, MCM2–7 [82–84]. Upon MCM2–7 loading, the origins are competent for replication initiation. Additional proteins are then recruited, including CDC45, which are required for origin firing and travel with the replication forks during elongation [85–88]. Proteins that function solely at the replication forks also are loaded, including the single-stranded DNA-binding protein RPA, the primase Polα, the clamp loader RFC complex, the polymerase processivity factor PCNA, DPB11, and the replicative polymerases Polδ and Pol ε (for a review see Ref. [60]). The activity of cyclin-dependent kinases (CDKs, including cyclin E and CDK2) and Dbf4-dependent kinase (DDK, composed of Cdc7 and Dbf4) regulates origin firing as well, with MCM2–7 and CDC45 as likely DDK targets [89,90]. Although this list of the factors involved in replication and their functions is not complete, the proteins listed are representative of the major factors functioning in gene amplification.

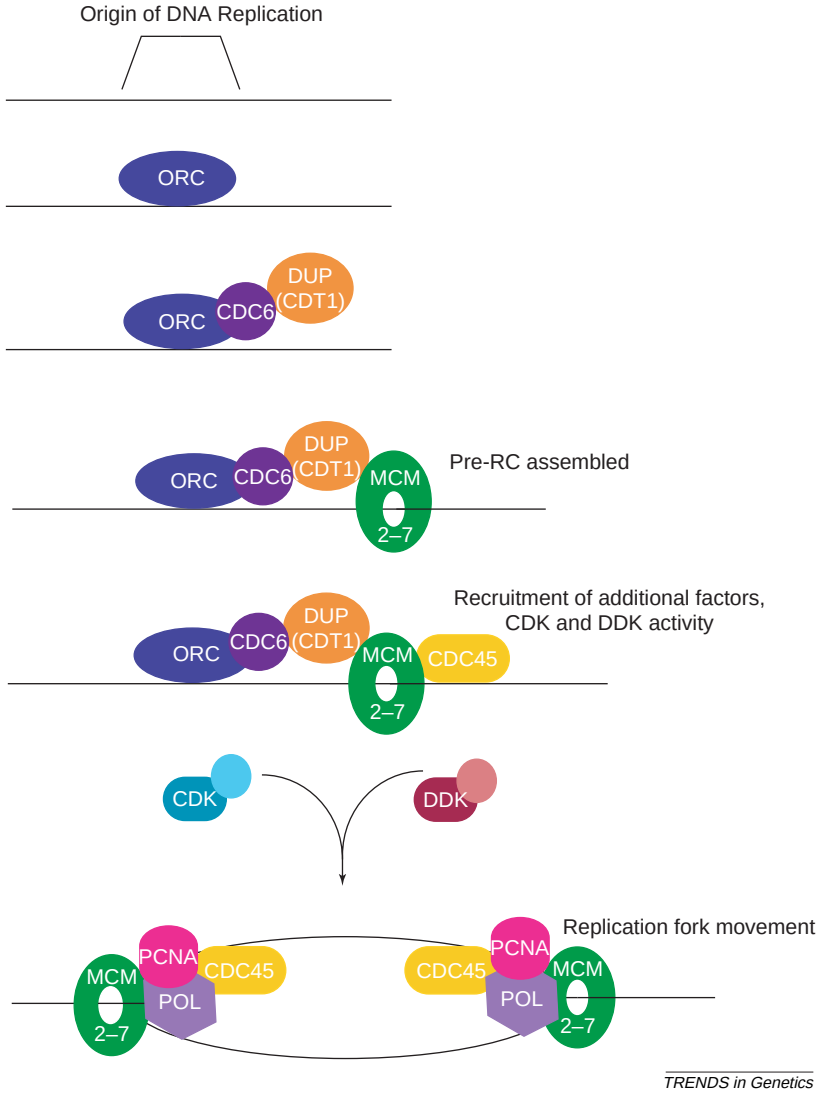

Figure I. The pre-replication complex is formed by the binding of ORC to origins, and the subsequent recruitment of DUP (Cdt1) and CDC6 to load MCM2–7 onto the chromosomes. Other replication factors, including CDC45, are recruited, and the CDK and DDK kinases both stimulate origin firing and inactivate some proteins so that origins only fire once per cell cycle. The replication fork machinery, including the polymerase complex and PCNA (among many other factors not shown here for simplicity), is recruited and replication forks begin their bidirectional progression.

necessary for genomic DNA replication and gene amplification, although a specific function in amplification has not been identified [37,47,48] (M. Botchan and B. Calvi, unpublished).

The amplification process in *Drosophila* is uniquely suited to the direct visualization of replication proteins, because in the follicle cells the majority of amplification occurs after genomic replication ends. Thus, bromodeoxyuridine (BrdU) incorporation during periods of amplification is observed solely at the four amplicons [48]. Similarly, immunolocalization of origin recognition complex (ORC) in the follicle cells dramatically changes from uniform localization throughout the nucleus during genomic replication to specific localization at the amplicons during stage 10, when amplification initiates [49,50].

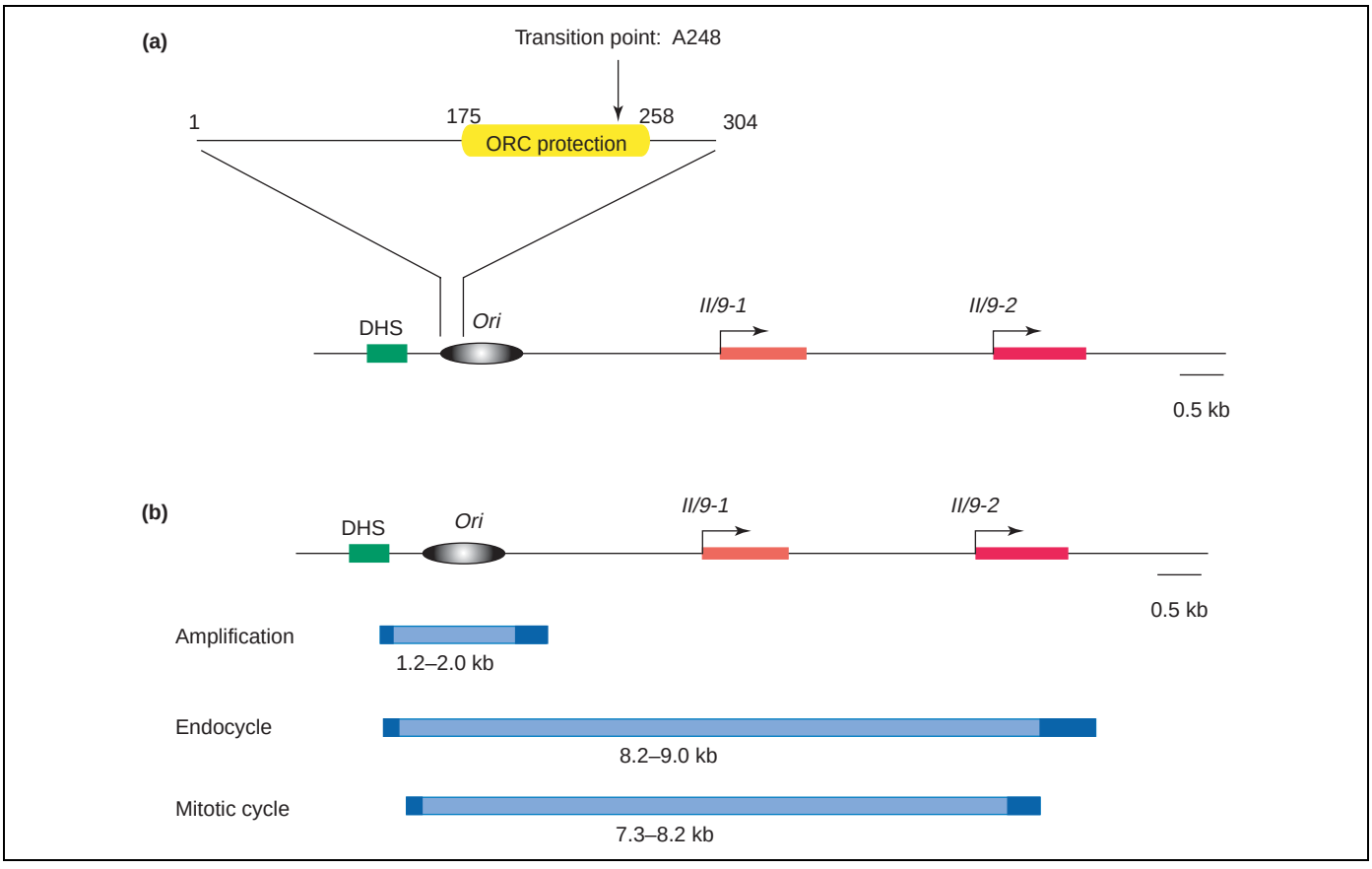

Figure 3. (a) A schematic of the *Sciara* Puff *II/9A* salivary gland amplicon. This region includes the 1 kb zone, mapped by two- and three-dimensional gel analyses, preferred for origin firing (*Ori*), the DNaseI Hypersensitive Site (DHS, green), hypothesized to have a regulatory role, and the amplified genes *II/9-1* and *II/9-2*. Within the origin zone, the precise nucleotide at which replication begins has been determined using RIP mapping. This site marks the transition from leading to lagging strand synthesis (Transition Point) and is nucleotide A at position 248 within the origin zone. Adjacent to this site, an ≈80 bp region to which recombinant *Drosophila* ORC protein binds *in vitro* has been determined. This same general region is bound *in vivo* by what is likely to be the *Sciara* ORC2 homolog [35]. **(b)** The zones of initiation in different developmental stages at the *II/9A* Puff have been determined, and the origin zone used during amplification falls within the zone used during endocycles and mitotic cycles. The left boundary of the initiation zones stays approximately the same, whereas the right boundary becomes restricted during amplification. Light blue indicates the conservative calculation for the size of the initiation zone, and dark blue indicates the liberal calculation for the zone based on nascent strand analysis data [65]. Adapted with permission from Ref. [65].

Chromatin immunoprecipitation (ChIP) and *in vitro* binding studies confirmed that ORC2 binds directly to *ACE3* and *Oriβ* during gene amplification [51,52]. However, recent studies demonstrate that the *Drosophila* ORC is incapable of binding sequence-specifically to *ACE3*, suggesting that binding specificity comes from additional proteins [53]. It has been suggested that *ACE3* and *Oriβ* are nucleating sites for ORC2 to spread along the chromatin, thus influencing the ability of the region to replicate [31,51,54]. By immunofluorescence, transgenes of *ACE3* multimers (Figure 2b, *p[rosy, chorion M9]*) recruit ORC2 *in vivo* [51], whereas buffered transgenes containing only *ACE3* and *Oriβ* are incapable of recruiting visible ORC2, despite being amplified in an ORC2-dependent manner [31] (Figure 2b, *Big Parent*). The addition of extensive sequences at the 5′ end of *ACE3* and the 3′ end of *Oriβ* to these buffered transgenes leads to the recruitment of ORC2 to visible foci (Figure 2b, *pYes-3.8S*), so perhaps a threshold amount of ORC2 must spread along the region before it can be detected [54].

Immunofluorescence microscopy also permits visualization of the distinct phases of replication initiation and elongation in *Drosophila* follicle cells (Figure 4) [26,55]. Those proteins involved solely in initiation, mainly ORC subunits, localize only during the initiation phase. ORC2 foci are present in stage 10 egg chambers but are no longer detectable by late stage 11 (Figure 4g for ORC2, green). Strikingly, BrdU incorporation is observed after the formation of ORC foci. Although BrdU initially overlaps the ORC spheres, it subsequently localizes on either side of ORC. As elongation continues, BrdU resolves to two bars (Figure 4a,c,h, for BrdU, green). Like BrdU, the replication elongation factors MCM2–7 and PCNA localize as foci and then resolve into the double bar structure, indicative of replication fork movement [26,55] (Figure 4d,f,i, for PCNA, green).

The analysis of replication proteins in follicle cells establishes their activity in amplification but also is significant in uncovering new activities of these proteins. Studies in yeast and *Xenopus* extracts highlighted a role for the pre-replication complex (pre-RC) protein DUP (Cdt1) solely in initiation [56–58]. During amplification, however, DUP (Cdt1) travels with the replication forks and thus might promote MCM2–7 localization at the forks during elongation [26] (Figure 4a,b,d,e,g,h,i, red). Furthermore, expression of a dominant-negative version of DUP (Cdt1) during the elongation-only phase of amplification potently inhibits BrdU incorporation, suggesting that DUP (Cdt1) is necessary for elongation [59]. The Dbf4 homolog in *Drosophila*, Dbf4-like, might have an

Table 2. Replication, transcription and chromatin remodeling factors implicated in *Drosophila* gene amplification

Protein	Amplification mutants	Cell biology	Molecular data
Replication factors			
ORC2 [26,37,49,51,52,64]	*orc2*^{fs293}	Localizes to amplicons stages 10a–11	Binds to *ACE3*, *Oriβ* and W–E2F1–Rbf–DP complexes
Double parked (Cdt1) [26,41,59,72]	*dup*^{PA77}	Localizes to amplicons stages 10b–13, goes from foci to double bar with BrdU	Co-immunoprecipitates with geminin; phosphorylated by Cyclin E and CDK2
MCM6 [26,42]	*mcm6*$^{fs(1)K1214}$	Localizes as DUP (Cdt1), using an antibody for MCM2–7	
PCNA [26,39]	*mus209*^{2735}, *mus209*^{B1}	Localizes as DUP (Cdt1)	
DBF4-like [38]	*chif*^{WD18}, *chif*^{WF24}, *chif*^{QW16}		Needed for ORC localization
CDC45 [76]		Localizes to amplicons in stages 10b, 11	
ORC5 [76]		Localizes to amplicons in stages 10b, 11	
ORC1 [50]	Overexpression	Localizes to amplicons in stages 10b–12	
MUS101 [43,77,78]	*mus101*^{K451}		BRCT repeats. Replication checkpoint?
Cyclin E [48]	Overexpression	Nuclear localization	
Dacapo [48]	Overexpression (inhibits amplification)		
HD (*humpty dumpty* gene product) [37,47,48] (M. Botchan and B. Calvi, unpublished)	*hd*$^{272-9}$	Required for genomic DNA replication and gene amplification	Defines new gene family with a single putative ortholog in each multicellular eukaryote
Transcription and chromatin remodeling factors			
RBF [64]	*rbf*^{14}, *rbf*^{120a}		Binds to *ACE3*, complexes with ORC2-E2F1-DP
E2F1 [49,64]	*e2f1*^{i1} reduced amplification, *e2f1*^{i2} overamplification		Binds to *ACE3*, complexes with ORC2–Rbf–DP
DP [49,64]	*dp*^{a1}		Binds to *ACE3*, complexes with ORC2–E2F1–Rbf
E2F2 [66,67]	*e2f2*$^{1-188}$, *Df(2L)e2f2*^{329}		Complexes with DP–Rbf; transcriptional role
Myb [61]	Follicle cell clones	Nuclear localization	Binds to *ACE3*, *Oriβ*
Mip130 [62]	Follicle cell clones, RNAi in Kc, S2 cells		Complexes with Myb, ORC1, 2 and 6; binds to *ACE3*, *Oriβ*
Mip120 [62]	RNAi in Kc, S2 cells		Complexes with Myb, ORC1, 2 and 6; binds to *ACE3*, *Oriβ*
Mip40 [62]	RNAi in Kc, S2 cells		Complexes with Myb, ORC1, 2 and 6; binds to *ACE3*, *Oriβ*
Caf1 p55 [62]	RNAi in Kc, S2 cells		Complexes with Myb, ORC1, 2 and 6; binds to *ACE3*, *Oriβ*
Rpd3 [68]	Follicle cell clones	Nuclear localization	Reduces origin firing when tethered to *ACE3*, *Oriβ*

additional role in amplification, by stabilizing the interaction of ORC with chromatin, as ORC2 does not localize to foci in the *chiffon* (*dbf4*-like) mutant [54]. This would be a novel role for the Dbf4-like protein, because in other organisms it acts at a step just before the initiation of DNA synthesis and not in the formation of the pre-RC (for review see Ref. [60]).

ChIP demonstrates that the *Sciara* II/9A origin is bound *in vivo* by a protein that cross-reacts with anti-XlORC2, and is likely to be the *Sciara* ORC2 homolog [35]. Recombinant ORC protein from *Drosophila* has been shown to bind to an 80-bp segment adjacent to the initiation site [35]. These important observations demonstrate that amplification in *Sciara* and *Drosophila* is probably controlled by the same machinery that controls DNA replication in a canonical cell cycle.

Transcription proteins

The *Drosophila* amplicons have yielded important insights into the action of transcription factors in regulating replication. The oncogene product Myb, and its binding partners Mip120, Mip130, Mip40 and the chromatin assembly factor complex 1 subunit Caf1 p55, were identified as a complex interacting with ORC and binding to *ACE3* and *Oriβ* [61]. Although Myb is essential for viability, its role in amplification was evaluated by making clones of mutant follicle cells. These studies showed Myb to be necessary for gene amplification, because mutant follicle cells failed to incorporate BrdU during stages when amplification should occur, although ORC2 and DUP (Cdt1) localized properly [61]. Binding sites for Myb and Mip120 have been identified within *ACE3*, and deletion of these sites from transgenes carrying *ACE3* and *Oriβ* resulted in almost no amplification from the transgenes [61]. These results indicate that the Myb and at least one of the Mip120 binding sites are necessary for amplification.

Knockout mutants of the *mip130* gene are sterile and lead to BrdU incorporation throughout the nucleus when amplification normally occurs [62]. These mutants also have decreased levels of Myb protein, as do *Drosophila* S2 or Kc cells in which Caf1 p55 or Mip120 levels have been decreased by RNA interference (RNAi), indicating that Myb must be in a complex to be stable. From these data, Mip130 and the other Mips form a complex that is involved in the repression of genomic replication, and Myb acts as a switch on this complex to stimulate replication. It is likely

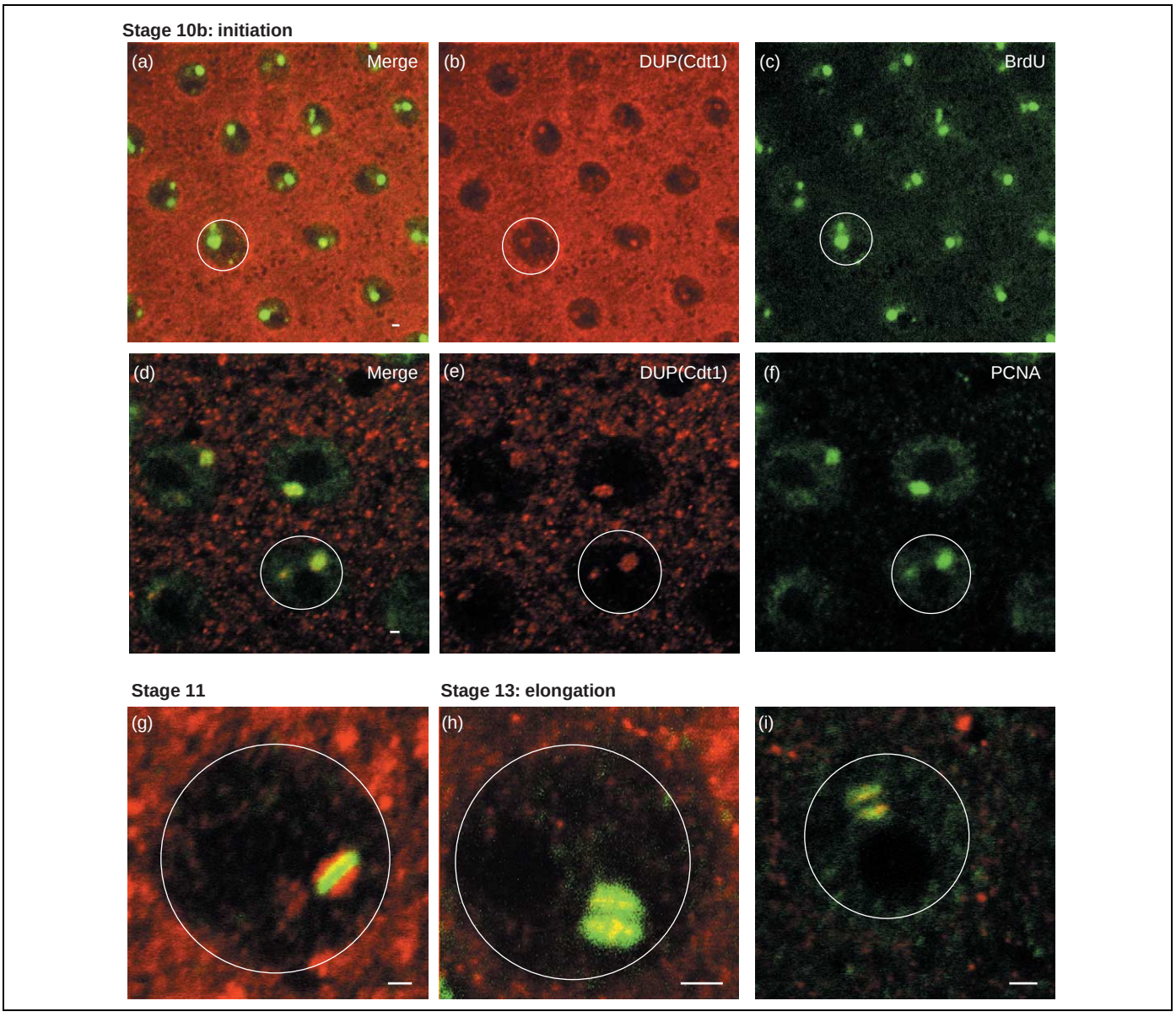

Figure 4. Visualization of gene amplification in *Drosophila* follicle cells by immunofluorescence microscopy. The *Drosophila* amplicons in the follicle cells surrounding the oocyte are uniquely suited to visualization, because amplification occurs after genomic DNA replication ceases. In panels (a) to (f), fields of follicle cells are shown. In panels (g) to (i), a single follicle cell nucleus is shown. White circles outline individual nuclei. **(a–c)** The DUP (Cdt1) protein (b, red) functions in replication initiation and localizes to the amplicons during stage 10b of egg chamber development, the initiation phase of amplification, and is coincident with sites of BrdU incorporation (c, green). **(d–f)** Much like DUP (Cdt1) (e, red), replication factors that function in elongation, such as PCNA (f, green) first localize as foci during initiation (stage 10b is shown here). **(g)** By stage 11, a time when initiation events are ending, the DUP (Cdt1) protein (red) moves away from the centrally located origin regions, to which the initiation protein, ORC2 (green), is bound. Shortly thereafter, initiation ends and ORC2 dissociates from the amplified regions, while elongation continues. **(h)** In stage 13, as the replication forks move further bidirectionally from the central origin regions, a double bar staining pattern is observed for BrdU (green), and for DUP (Cdt1) (red). **(i)** The elongation factor PCNA (green) also displays this pattern and colocalizes with DUP (Cdt1) (red) [26]. Scale bars represent 1 μm.

that Myb is always associated with the Mips, but remains poised to become activated, perhaps by phosphorylation [63] or another modification to change the activity of the complex. Thus, Myb could be specifically activated at amplification origins as part of a combinatorial signal to enable initiation at the appropriate time.

The retinoblastoma (Rb) tumor suppressor negatively regulates gene amplification via a direct effect at *ACE3*. In a weak *Rbf* mutant (one of two *Drosophila* Rb homologs), some follicle cells fail to exit the endocycle (the cell cycle preceding gene amplification), but in those cells that begin amplification, increased levels of BrdU incorporation are observed at the amplicons [64]. Similarly, a mutation in the transcription factor E2F1 that truncates the protein, making it unable to bind to Rbf, causes increased amplification [49]. A complex of Rbf–E2F1–DP–ORC was identified in follicle cell extracts, and E2F1 was shown by chromatin immunoprecipitation to colocalize with ORC at *ACE3* [64]. Thus, Rbf could limit origin firing via an association with ORC.

There are hints that transcription factors could influence *Sciara* amplification [65]. Although transcription of the *II/9-1* gene does not begin until amplification ends, the promoter of *II/9-1* is occupied by RNA Pol II during amplification stages, but not during mitotic cycles or endocycles. The presence of RNA Pol II is thought to limit

the righthand boundary of the initiation zone during amplification (Figure 3b). This effect could be due to RNA Pol II occupying positions used by ORC in mitosis and endocycle replication, thus forcing ORC to different positions and causing a change in the preferred sites of initiation during amplification [65].

Regulation of reinitiation at amplification origins

The two key puzzles in the regulation of amplification are how replication is restricted to specific genomic sites and how the amplification origins, unlike genomic replication origins, are capable of repeated firing. Recent work has identified some of the players affecting origin specificity and reinitiation, thus providing clues to regulatory mechanisms.

A simple mechanism for specification of amplification origins would be a specificity factor, induced at the correct stage that facilitates formation of the pre-RC and initiation at amplification origins. In *Drosophila*, no amplification-specific factor has been recovered from genetic screens for amplification mutants. Rather, the locus specificity of amplification is lost in mutants generally affecting replication, transcription or chromatin, suggesting that a combinatorial signal provides specificity. In *Rbf*, *E2F2*, and *DP* mutants, ectopic DNA replication occurs during stages when amplification should occur, and ORC is observed at ectopic sites or throughout the nucleus [49,64,66,67]. These mutants also display transcriptional derepression of crucial replication factor genes [67]. The resultant increase in replication protein levels could cause ectopic DNA replication. In other studies, overexpression of the ORC1 replication protein led to continued genomic replication when amplification should occur, consistent with levels of replication factors being crucial for preventing genomic replication during amplification [50]. This explains the inhibition of genomic replication but fails to account for the restriction of replication to the four DAFC sites. The Myb complex might help to direct replication at the amplicons, because mutation of *mip130* causes genomic rather than restricted replication, and Myb is necessary for amplification [61,62].

In addition to regulation by transcription factors, acetylation of histones at the amplicons has a role in restricting replication to these sites. Histones H3 and H4 at and around *ACE3* and *Oriβ* are hyperacetylated during gene amplification. Histone acetylation at the DAFCs is coincident with ORC2 in both its temporal and spatial localization at the origins ([68] and T. Hartl *et al.*, unpublished). Furthermore, the acetylation does not colocalize with BrdU or DUP (Cdt1) at the replication forks, indicating that the acetylation pattern observed is not due to the assembly of new chromatin after the replication forks have passed ([68] and T. Hartl *et al.*, unpublished). Several specific histone H4 residues were shown to be acetylated at chorion origins, including lysine 8 [68], a mark previously associated with transcriptional activation, in addition to lysines 5 and 12, modifications associated with DNA replication (T. Hartl *et al.*, unpublished). The sufficiency of each of these histone modifications alone or in combination with other modifications to affect gene amplification currently is unclear. However, limiting acetylation to the DAFCs is necessary for restricting amplification to these sites, because mutation of a histone deacetylase gene, *rpd3*, results in increased and genome-wide acetylation, genomic replication and promiscuous ORC localization [68]. The histone acetyltransferase responsible for acetylation at DAFCs, and the mechanism targeting it to these sites, remain to be identified.

A relevant question regarding the control of amplification specificity is whether amplification origins also serve as replication origins during a normal cell cycle. The answer could differ between the *Drosophila* and *Sciara* amplicons. In *Drosophila*, *ACE3* and *Oriβ* do not act as origins in Kc tissue culture cells [69]; however, this is the only cell line or tissue that has been examined thus far. In *Sciara*, the zone of initiation in Puff *II/9A* has been determined in mitotic and endocycling developmental stages, in comparison with amplification stage tissue, using nascent strand analysis and quantitative PCR [65] (Figure 3b). The initiation zone in all three developmental stages possesses the same left boundary. However, the right boundary defines a smaller interval for initiation during amplification, such that replication initiates ≈2.3 kb further from the *II/9-1* gene than in the pre-amplification stages. This is the first demonstration that an origin used during gene amplification resides within the same region used for initiation during a mitotic cell cycle, yet it indicates that the boundaries of initiation set up during embryogenesis can change throughout development.

Perhaps the most confounding question about gene amplification is how the cell suspends the rule of replicating DNA once and only once per cell cycle to enable refiring of amplification origins. The answer could lie in the events preceding gene amplification. In *Tetrahymena* and Dipteran flies, the cells in which amplification occurs undergo endocycles, in which synthesis and gap phases alternate with no intervening mitosis, before gene amplification. *Tetrahymena* macronuclei reach a ploidy of ≈45C, *Sciara* salivary gland cells reach 8192C, and *Drosophila* follicle cells achieve 16C before amplification begins. In the endocycles, mitotic cyclins that normally block reinitiation are shut off and cyclic expression of cyclin E serves as the oscillator to trigger replication and then permit resetting of replication origins [70,71]. Levels of cyclin E might also affect the transition from the endocycle to amplification in *Drosophila* follicle cells. When endocycles cease in egg chamber stages 9 and 10a, cyclin E levels are high and might serve to inhibit the formation of prereplication complexes at genomic origins, inhibiting further endocycles [48]. In this model, cyclin E also promotes origin firing at the amplified loci, possibly by acting positively on an as yet uncharacterized amplification factor. Recent work also shows that follicle cells enter gene amplification in a late S–G2-like state, based on having high levels of phosphohistone H1 staining, a marker of G2, cyclin E and CDK2 activity in these cells (T. Hartl *et al.*, unpublished). Perhaps a forced initiation of replication from a G2 state overcomes normal blocks to reinitiation of replication.

The DUP (Cdt1) replication protein is also likely to have a role in enabling amplicons to undergo multiple rounds of initiation. This protein is a crucial regulator

Box 4. Outstanding questions

- How is replication permitted at these specific amplicons?
- How are other replication origins repressed during gene amplification?
- How are origins able to escape normal controls on re-replication and repeatedly fire?
- Is there an amplification-specific protein that provides the proper developmental and origin specificity?
- What are the common sequence characteristics of the DAFCs, and are these elements interchangeable between DAFCs?
- What are the important regulatory elements present in the *Sciara* Puff *II/9A*?
- What are the roles of Dbf4-like DDK and DUP (Cdt1) in amplification? Have they adopted new functions that are crucial for the regulation of gene amplification?
- Is amplification used in other developmental contexts and in other organisms?

of initiation, activating origins by recruiting MCM2–7 (for review see Ref. [60]). The role of DUP (Cdt1) in activating origins is regulated by its association with an inhibitor protein, Geminin, and by degradation after replication initiates [60]. Alterations to DUP (Cdt1) regulation could account for reinitiation at the DAFCs. Evidence in support of this has been reported from analysis of *geminin* mutants [72], and from DUP (Cdt1) expression studies. Misexpression of full-length DUP (Cdt1) causes genomic rereplication, whereas, strikingly, expression of a DUP (Cdt1) C-terminal fragment lacking degradation signals leads to overamplification [59].

Chromatin accessibility and histone acetylation could have crucial roles in the ability of amplicons to fire repeatedly, as evidenced by experiments in which a histone deacetylase or chromatin repressor was tethered adjacent to *ACE3* and shown to reduce amplification [68]. By contrast, tethering of a histone acetyltransferase increased amplification levels [68]. This is consistent with the observation that mutation of *Rbf*, encoding a histone deacetylase recruiter, leads to increased DNA replication [64].

Concluding remarks

Analyses of developmental amplicons have identified an enhancer-like replication element that interacts with replication origins to trigger loading of replication proteins and multiple rounds of reinitiation. In addition to replication proteins common to genomic replication, transcription factors and histone-modifying enzymes affect the activity of amplification origins. The CGH array approach led to the isolation of two new amplified regions in *Drosophila* follicle cells whose properties suggest that amplification could be a widespread strategy for augmenting expression of developmentally important genes. The field is poised to expand our knowledge of developmental amplicons, the functions of amplified genes, and the regulation of gene amplification throughout various developmental contexts in the life cycle of a complex organism (Box 4).

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