RNA polymerase II kinetics in *polo* polyadenylation signal selection

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Regulated alternative polyadenylation is an important feature of gene expression, but how gene transcription rate affects this process remains to be investigated. *polo* is a cell-cycle gene that uses two poly(A) signals in the 3′ untranslated region (UTR) to produce alternative messenger RNAs that differ in their 3′UTR length. Using a mutant *Drosophila* strain that has a lower transcriptional elongation rate, we show that transcription kinetics can determine alternative poly(A) site selection. The physiological consequences of incorrect *polo* poly(A) site choice are of vital importance; transgenic flies lacking the distal poly(A) signal cannot produce the longer transcript and die at the pupa stage due to a failure in the proliferation of the precursor cells of the abdomen, the histoblasts. This is due to the low translation efficiency of the shorter transcript produced by proximal poly(A) site usage. Our results show that correct *polo* poly(A) site selection functions to provide the correct levels of protein expression necessary for histoblast proliferation, and that the kinetics of RNA polymerase II have an important role in the mechanism of alternative polyadenylation.

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Introduction

Transcription by RNA polymerase II (Pol II) is a complex and multistep process that consists of promoter binding, initiation, elongation and termination. For most eukaryotic genes, the nascent transcript is recognized by RNA processing enzymes, as it is being synthesized by Pol II. For instance, excision of non-coding introns to generate faithfully spliced messenger RNA (mRNA) occurs long before the polymerase has completed transcription of the whole gene (Moore and Proudfoot, 2009). Similarly, the nascent transcript is released from the transcribing polymerase by the action of a large processing protein complex (Shi *et al*, 2009) coupled to the addition of a poly(A) (pA) tail to the terminal 3′ end of the pre-mRNA and to transcriptional termination (for reviews, see Hirose and Manley (2000), Proudfoot (2004), Bentley (2005), Buratowski (2005) and Richard and Manley (2009)).

Although it was previously believed that the critical step for regulating transcription was at the stage of promoter binding and transcription initiation, recent advances have revealed that many genes are regulated at the phase that follows the promoter-paused Pol II entering into elongation (Core *et al*, 2008; Gilmour, 2009). At this stage, the speed at which Pol II transcribes a transcriptional unit may have an impact on the regulation of gene expression and may determine pre-mRNA processing (Caceres and Kornblihtt, 2002). In *Drosophila*, the *C4* mutation in the *RpII215* gene, which encodes the largest subunit of Pol II, changes amino acid 741 from Arg to His (Chen *et al*, 1993). *In vivo* this mutation induces Pol II α -amanitin resistance with a 50% slower transcription elongation rate than its wild-type counterpart (Coulter and Greenleaf, 1985; Chen *et al*, 1993) and reads less efficiently through DNA pause elements (Chen *et al*, 1996). In human cells, a mutation equivalent to *C4* has been shown to affect the alternative splicing pattern of the human fibronectin (FN) exon 33 (E33) EDI. Here, the ‘slow’ Pol II acts in concert with splicing factors to induce the inclusion of the alternative E33 (de la Mata *et al*, 2003, 2010). Additionally, in *Drosophila* *C4* embryos reslicing of the *Hox* gene *Ultrabithorax* is stimulated (de la Mata *et al*, 2003, 2010). Thus, a ‘kinetic coupling’ model in which the transcription elongation rate determines the outcome of the alternative splice site selection was put forward (Caceres and Kornblihtt, 2002).

The 3′ end processing complex is precisely regulated and specifically recognizes sequences in the nascent transcript that comprise the pA signal of the gene, which is typically AAUAAA, and a GU- or U-rich downstream RNA sequence element (for reviews, see Colgan and Manley (1997), Minvielle-Sebastia and Keller (1999), Wahle and Rueggsegger (1999), Zhao *et al* (1999), Edmonds (2002) and Shi *et al* (2009)). Additional sequences positioned either upstream (Carswell and Alwine, 1989; Gilmartin *et al*, 1992; Chen and Wilusz, 1998; Moreira *et al*, 1998; Hall-Pogar *et al*,

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2005; Hu *et al*, 2005; Venkataraman *et al*, 2005; Danckwardt *et al*, 2007) or downstream of the pA signal (Bagga *et al*, 1995; Arhin *et al*, 2002; Dalziel *et al*, 2007) may act as auxiliary elements by binding to components of the 3' end processing complex or to proteins that can influence the efficiency of this complex and consequently modulate the overall efficiency of mRNA 3' end formation.

Poly(A) sites are thus integral parts of the gene, signalling where in the precursor RNA cleavage and polyadenylation should occur. However, a large percentage of genes possess multiple pA sites that can be alternatively utilized. It is estimated that over half of human genes contain more than one pA signal and that alternative polyadenylation may be a major player in gene regulation (Lutz, 2008; Neilson and Sandberg, 2010; Lutz and Moreira, 2011). In some cases the choice of alternative pA sites is coupled with alternative splicing, which results in the production of different transcripts that are translated into different protein products with diverse cellular functions, for example, the membrane or secreted forms of immunoglobulin in B cells (Peterson and Perry, 1986; Lou *et al*, 1996; Takagaki *et al*, 1996; Edwalds-Gilbert *et al*, 1997; Peterson, 2007). In contrast, the presence of alternative pA sites in the 3'-most exon (tandem pA signals) directs the synthesis of mRNAs that share the same coding sequence but have different 3' untranslated regions (UTRs). This is usually coupled with the presence of regulatory elements in the 3'UTR that interact with RNA binding proteins or miRNAs, modulating mRNA stability, translation, mRNA transport and/or mRNA localization (Lewis *et al*, 1995; Huang and Carmichael, 1996; Jacobson and Peltz, 1996; Wickens *et al*, 1997; Preiss *et al*, 1998; Ghosh *et al*, 2008; Merritt *et al*, 2008; Newnham *et al*, 2010). Importantly, two recent genome-wide studies, in tumour cells and in activated versus resting T cells, have found that in general shorter 3'UTRs are indicative of enhanced cell proliferation (Sandberg *et al*, 2008; Mayr and Bartel, 2009). The shortening of the 3'UTRs resulting from alternative polyadenylation leads to the loss of regulatory elements in this region, such as miRNA target sites. Conversely, during mouse embryonic development, alternative polyadenylation was shown to be responsible for a progressive lengthening of mRNA 3'UTR (Ji and Tian, 2009). It was also recently shown that differences in the 3'UTR due in part to alternative polyadenylation, define molecular signatures that can distinguish tumour subtypes (Singh *et al*, 2009). In *Drosophila* the *enhancer of rudimentary* (*e(r)*) (Gawande *et al*, 2006) and the *suppressor of forked* (*su(f)*) are two examples of genes regulated by alternative polyadenylation (Audibert and Simonelig, 1998). *e(r)* produces two mRNAs due to two pA signals present in the 3'-most exon, but only the longer *e(r)* mRNA is specifically expressed in the female germline, which requires co-expression of the female-specific Sex-lethal (Wojcik *et al*, 1994; Gawande *et al*, 2006). *su(f)* contains three pA signals, the first one within intron 4, leading to the production of a truncated transcript, and the other two in the 3'UTR. It was suggested that the Su(f) protein regulates its own accumulation via a negative feedback loop that stimulates 3' end formation of the truncated *su(f)* RNA (Audibert and Simonelig, 1998).

Polo was the first described member of an important family of cell-cycle kinases, the Polo-like kinases (Plks), and is involved in many critical steps in the cell cycle,

including mitotic entry, centrosome organization, spindle formation, chromosome segregation and cytokinesis (Sunkel and Glover, 1988; Llamazares *et al*, 1991; Moutinho-Santos *et al*, 1999; Glover, 2005). *polo* contains a proximal and a distal pA signal in the 3'UTR (referred herein as pA1 and pA2, respectively). Despite evidence that pA site selection in the 3' most exon of eukaryotic genes has a role in gene expression, the physiological impact of this mechanism on the development of an organism and the role of Pol II kinetics on alternative pA site choice is still poorly understood.

In this study, we show that in *RpII215* mutant flies, which display reduced Pol II elongation kinetics, Pol II occupancy along *polo* is altered and *polo* pA1 is used 3.5-fold more efficiently than in wild-type flies. An increase in proximal pA site usage was also observed for five other alternatively polyadenylated transcripts in *Drosophila*. Moreover, we show that in flies carrying a deletion of the *polo* pA2 signal, Polo is translated at low levels and as a result precursor cells of the abdominal epidermis do not proliferate during metamorphosis. This causes a defect in abdomen development and lethality. Consequently, we demonstrate that in transgenic flies *polo* pA2 signal is necessary for proper protein levels production, cell proliferation and viability in the living organism. Importantly, Pol II kinetics has a critical role in the selection of alternative pA sites in *Drosophila*.

Results

Pol II kinetics affects polo pA site usage

The *polo* gene, represented in Figure 1A, produces two mRNA species as previously described (Llamazares *et al*, 1991). The 3' end of these two mRNAs was mapped by sequencing cDNAs isolated from a Poly(A)⁺ 0–24 h embryonic mRNA library, and they were shown to differ in their 3'UTR due to alternative usage of two pA signals localized in this region (Llamazares *et al*, 1991). The pA1 signal is the fly variant ATTTAA present in 10–32% of the *Drosophila* genes analysed, while the pA2 signal is the variant AATATA present in 5–10% of the *Drosophila* genes (Figure 1A; Graber *et al*, 1999; Retelska *et al*, 2006).

To investigate the role of Pol II kinetics on pA site usage of *polo*, we used the *RpII215* *Drosophila* homozygous strain (referred herein as slow Pol II), which contains a C4 mutation in the largest subunit of Pol II reducing its transcription rate by 50% (Chen *et al*, 1996). We performed ChIP analysis on these flies, using an α -Rpb3 Pol II antibody to investigate Pol II distribution along the *polo* gene. Pol II occupancy was measured at nine positions within the *polo* transcription unit, the intergenic region and the promoter region of the next downstream gene, *snap* (Figure 1A), from chromatin sheared to an average size of 200–300 bp. Each position corresponds to a 125- to 150-bp amplicon separated by a minimum of 60 nucleotides (probes 7–8 and 8–9); the remaining probes are separated by >160 nucleotides. Quantification of the chromatin immunoprecipitation (ChIP) was done by real-time PCR using SybrGreen fluorescence (see Materials and methods). In wild-type flies, Pol II occupancy is reduced after pA1, suggesting that more pA1 than pA2 transcripts are being produced in adult flies (Figure 1A, left panel). This is in agreement with the quantification made in adult flies by real-time PCR (Figure 1E, *w¹¹¹⁸*) as well as with previously

Figure 1 RNA Pol II kinetics with a slower transcription elongation rate affects pA site selection. **(A)** Pol II ChIP across *polo* and *polo-snap* intergenic region. In the top panel, a schematic diagram is shown where *polo* and *snap* genes are depicted to scale (adapted from genome.ucsc.edu). ChIP was performed using α -Rpb3 antibody on wild-type (*w¹¹¹⁸*) and slow Pol II (*Rpl215* mutant, C4 mutation) adult flies chromatin. Numbers below each graph bar represent the position of real-time PCR primers. **(B)** *Rpl215* mutant generates similar levels of *polo* mRNA as compared with *w¹¹¹⁸*. Graph represents RT-qPCR quantification of *polo* mRNAs in adult flies, relative to *rp49* mRNA. **(C)** *Rpl215* mutant presents similar levels of Polo protein as *w¹¹¹⁸*. Western blot from total protein extracts of *w¹¹¹⁸* and *Rpl215* third instar larvae brains. MA294 Polo and DM1A α -tubulin antibodies were used. Quantification was made by densitometry (see Materials and methods) and Polo/ α -tubulin ratio was set at 1 for *w¹¹¹⁸*. **(D)** Representative gel of fractionated 3'RACE products of *polo* mRNA from *w¹¹¹⁸* and *Rpl215* adult flies is shown. Arrows indicate the bands corresponding to *polo* pA1 and pA2; -RT is a control reaction without reverse transcriptase. In both panels, phased-anchored oligodT was used for reverse transcription, as depicted in the diagram. **(E)** *Rpl215* shows an increase in the ratio of total/pA2 mRNAs. Diagram shows primer positions for qPCR analysis. Levels of total *polo* mRNAs and pA2 mRNAs were measured by real-time PCR and their ratio (total/pA2) in adult flies is shown. **(F)** *Rpl215* shows an increase in proximal site usage in several genes. RT-qPCR quantification was performed as in **(E)**. cDNA synthesis was performed with random primers (see Materials and methods). The ratio for *w¹¹¹⁸* was set at 1. For all the panels, error bars show s.e.m. from at least three independent experiments.

published results (Llamazares *et al*, 1991). The drop in Pol II occupancy that is seen after pA1 is indicative of *polo* termination, before it increases again over the closely spaced tandem gene *snap* (Figure 1A, left panel). However, in *Rpl215* flies

this pattern is altered, with lower but more even Pol II levels across the *polo-snap* locus (Figure 1A, right panel), which may be due to the mutation in the RNA polymerase II. In particular, no drop in signal was observed following pA1

(probe 7). The lower levels of Pol II over *polo-snap* do not reflect a decrease in the levels of *polo* transcripts (Figure 1B) or Polo protein (Figure 1C) produced. We then determined whether this altered Pol II distribution affects *polo* pA site selection, by 3'RACE and RT-qPCR in *RpII215* adult flies. 3'RACE analysis suggests that pA1 is used more efficiently than pA2 in *RpII215* flies (Figure 1D). In order to quantify this effect, we measured the ratio between the total levels of *polo* transcripts (*polo* pA1 + *polo* pA2) and *polo* pA2 mRNA levels by RT-qPCR. We observed that in *RpII215* adult flies, this ratio is increased 3.5-fold in comparison with the wild-type flies (Figure 1E), indicating that pA1 is used more efficiently than pA2 in the *RpII215* mutant. Our results clearly establish that *in vivo* the *Drosophila* slow Pol II affects *polo* pA site selection.

To extend this observation to other alternatively polyadenylated genes in *Drosophila*, we tested seven other previously described (GenBank, NCBI) or *in silico* predicted (Joel Graber, personal communication) genes, with a similar genomic structure to *polo*, that is containing two pA signals in the 3'UTR: *abd-B* (Abdominal-B, a member of the bithorax complex in *Drosophila*; Sanchez-Herrero *et al*, 1985), *lace* (a homologue of the LCB2 subunit of serine palmitoyltransferase; Adachi-Yamada *et al*, 1999), *stlk* (Ste20-like kinase), *CG6024* (Artero *et al*, 2003), *cycD* (Cyclin D; Finley *et al*, 1996), *drep-3* (DNA fragmentation factor-related protein 3; Inohara and Nunez, 1999) and *stg* (String, a Cdc5-like phosphatase; Edgar and O'Farrell, 1989). Using RT-qPCR with specific probes for these genes, we measured the ratio between total RNA levels and longer transcript levels (produced by distal pA signal selection) in *RpII215* and *w¹¹¹⁸* flies. Five out of the seven genes analysed showed an increase in total/distal RNA levels in *RpII215* flies in comparison with the wild type similar to that found for *polo* (Figure 1F). *CG6024* shows a three-fold increase and *Abd-b*, *stlk* and *lace* show a 1.5- to 2-fold increase. *Drep-3* and *stg* show a slight increase in this ratio. These results clearly show that Pol II elongation kinetics affects alternative pA site selection in other genes and suggests that it may be a general effect in *Drosophila*.

***polo* pA2 is required for *Drosophila* viability and abdominal development**

Having established that slow Pol II kinetics correlate with increased pA1 site usage, we then addressed the question of the function of the *polo* distal pA2 site *in vivo*. We therefore

determined the consequence of mutation/deletion of each of the two tandem pA sites located in the *polo* 3'UTR (Figure 2C). Flies that express *polo* tagged with *gfp* at the N-terminus containing either one of the two pA signals were generated, using a previously described transgene *gfp-polo* (Moutinho-Santos *et al*, 1999). Point mutations were introduced in the pA1 signal (ATTAAA to GTTAAC), thus generating the transgene Δ pA1 that allows the expression of the longer *polo* transcript (Figure 2A). A second transgene, Δ pA2, was generated by deletion of pA2 signal and the entire downstream region (Figure 2A). The point mutations in pA1 and the deletion of pA2, inactivating pA1 and pA2, respectively, were therefore sufficient to allow the selective expression of only the longer or shorter *polo* mRNA in each transgenic line as shown by northern blot analysis in Figure 2B (open and black arrows, respectively). In this northern blot, *polo* pA1 and *polo* pA2 mRNAs are detected at similar levels in brains of third instar larvae in a wild-type background (Figure 2B) in contrast to what was observed in adult flies. This difference may reflect the nature of the developmental stage used and is in agreement with previous results (Llamazares *et al*, 1991). All transgenic lines express GFP-Polo protein throughout fly tissues (Supplementary Figure S1).

To study the role of *polo* pA signals *in vivo*, the viability of homozygous lines for the transgenes was analysed in a *polo⁹* mutant background, which has been described as the strongest hypomorphic allele identified for *polo* (Donaldson *et al*, 2001). This mutant was generated by insertional mutagenesis of a P-element into the 5'UTR of *polo*, 176 nucleotides upstream of the ATG codon, where it interferes with *polo* transcription (Donaldson *et al*, 2001). *polo⁹* homozygous third instar larval brains have barely detectable levels of Polo protein that are not sufficient for the larvae to develop beyond this developmental stage, and consequently die (Donaldson *et al*, 2001). We first determined if the *polo* mRNA and protein levels contributed by the transgenes were sufficient to rescue the third instar larval lethal phenotype displayed by *polo⁹* individuals in the transgenic flies *gfp-polo;polo⁹*, Δ pA1;*polo⁹* and Δ pA2;*polo⁹*. As can be seen, the *gfp-polo* and Δ pA1 transgenes rescue *polo⁹* phenotype and the flies reach adulthood (Table I). In contrast, nearly all Δ pA2;*polo⁹* individuals died during pupariation (Table I). These results clearly show that the longer *polo* mRNA produced using pA2 is required for normal *Drosophila* development beyond the late third instar larval stage, more precisely,

Figure 2 Deletion of *polo* distal pA2 signal causes abnormal formation of the fly abdomen. (A) Schematic representation of the *gfp-polo*, Δ pA1 and Δ pA2 transgenes driven by the endogenous *polo* promoter. The two pA signals ATTAAA and ATATAA are indicated. In the Δ pA1 transgene, the proximal pA signal was mutated to GTTAAC. (B) Δ pA1 and Δ pA2 transgenes utilize pA2 and pA1 signals, respectively, as indicated by the arrows. Northern blot of *w¹¹¹⁸*, *gfp-polo*, Δ pA2 (lines 2.3 and 5) and Δ pA1 (lines 12.1 and 9.1) from third instar larvae brains total RNA. The asterisks indicate the two *polo* mRNAs present in *w¹¹¹⁸*, the black arrows the shorter transcripts present in Δ pA2 lines and the open arrows the longer transcripts present in Δ pA1 lines. All fly lines analysed contain the endogenous *polo* mRNAs that serve as internal controls (denoted by *). (C) *polo* 3'UTR sequence. Bold sequence indicates proximal (pA1) and distal (pA2) pA signals. The arrows indicate the cleavage site of the transcripts produced by usage of pA1 and pA2. (D) *polo* pA2 is required for abdominal development. Dorsal view of female abdomens from *w¹¹¹⁸* control (panel 1); the seven tergites of the adult abdomen are indicated as A1–A7. Panels 2–4: *gfp-polo*, Δ pA1 (line 12.1) and Δ pA2 (line 2.3) individuals in a *polo⁹/TM6B* background. Panels 5–7: *gfp-polo*, Δ pA1 (line 12.1) and Δ pA2 (line 2.3) individuals in a *polo⁹* background. (E) *polo* mRNA quantification by RT-qPCR in the transgenic fly lines in a *polo⁹* background. Total RNA was extracted from two adult flies of each genotype (for Δ pA2;*polo⁹*, two escapers were used) in a *polo⁹* background, and from *w¹¹¹⁸* flies. After cDNA synthesis with random hexamers, RNA levels were quantified using *gfp*-specific primers for amplification of the transgene-derived transcripts (light grey bars) or primers for exons 4 and 5 for amplification of the total *polo* RNAs present (dark grey bars). In all genotypes, *rp49* was used for normalization. The difference between total *polo* (dark grey bars) and *gfp-polo* (light grey bars) corresponds to *polo⁹* contribution. Error bars show s.e.m. from at least three independent experiments.

during metamorphosis. Strikingly, analysis of the few $\Delta pA2; polo^9$ adult escapers indicated that these individuals have strong defects in abdominal morphology, with the

complete absence or incorrect formation of tergites (Figure 2D, panel 7) while all other adult structures appear normal. Panels 2–4 show abdomens prepared from transgenic

Table 1 Viability—deletion of *polo* distal pA2 causes high lethality levels

Strain	% Flies homozygous for <i>polo</i> ⁹ carrying each of the transgenes
<i>gfp-polo; polo</i> ⁹	91
$\Delta pA1; polo9 (line 9.1)$	100
$\Delta pA1; polo9 (line 12.1)$	63
$\Delta pA2; polo9 (line 2.3)$	0.1 ^a
$\Delta pA2; polo9 (line 5)$	0.6 ^a

Quantification of the viability of the transgenic lines $\Delta pA1; polo$ ⁹, lines 9.1 and 12.1 and $\Delta pA2; polo$ ⁹, lines 2.3 and 5 (presented as the percentage of flies that reached adulthood). For each strain, crosses were performed to rescue the *polo*⁹/*polo*⁹ allelic combination.

^aMost individuals die as pharate pupae.

flies, in a *polo*⁹/*TM6B* background, containing the transgenes plus one copy of *polo* (*TM6B*). Panels 5–7 show transgenic flies in a *polo*⁹ homozygous background. Transgenic *gfp-polo; polo*⁹ adult flies have normal tergites as expected, while $\Delta pA1; polo$ ⁹ present a mild abdominal phenotype (Figure 2D, panels 5 and 6). The possibility that the phenotype may arise as a result of the insertion of transgenes into the same genomic region in both transgenic lines was excluded (Supplementary Figure S2). In spite of the almost undetectable endogenous *Polo* in *polo*⁹ (Donaldson *et al*, 2001), we quantified the remaining *polo* mRNA contribution by this allele, as well as the levels produced by each transgene, in all the transgenic *polo*⁹ homozygous lines, by RT-qPCR (*gfp-polo; polo*⁹, $\Delta pA1; polo$ ⁹ and $\Delta pA2; polo$ ⁹; Figure 2E). Using specific primers for the *gfp* transgene (light grey bars) or primers that amplify both endogenous *polo* and *gfp-polo* mRNAs (dark grey bars), it is clear that the transgenes are principally responsible for *polo* mRNA levels (Figure 2E). The small difference between the bars corresponds to the endogenous *polo* contribution. Moreover, $\Delta pA2; polo$ ⁹ flies show higher mRNA levels than $\Delta pA1; polo$ ⁹ flies. This clearly indicates that the phenotype presented by $\Delta pA2; polo$ ⁹ individuals (Figure 2D, panel 7) is due to the lack of *polo* pA2 transcript and that selection of the pA2 signal is essential for normal development of the abdomen and viability of the fly.

pA2 is required for abdominal histoblast proliferation at metamorphosis onset and for correct *polo* protein expression

The high levels of lethality for $\Delta pA2; polo$ ⁹ observed during the pupa stage suggested that *polo* pA2 was essential at this stage of development. Additionally, the phenotype presented by $\Delta pA2; polo$ ⁹ is in agreement with that found in mutants for *arrowhead* (*awh*) and *escargot* (*esg*) genes, two other genes previously reported as necessary for the formation of the adult abdomen (Hayashi *et al*, 1993; Curtiss and Heilig, 1995). In *esg* mutants, the histoblasts—small diploid cells that arise during the last stages of embryogenesis (Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980)—undergo DNA endoreplication resulting in polyploid cells which fail to proliferate, leading to adult flies with an abdominal phenotype very similar to $\Delta pA2; polo$ ⁹ flies (Hayashi *et al*, 1993). Upon entering metamorphosis, the histoblasts undergo very rapid proliferation and from 15 h after pupa formation they expand, replacing all larval poly-

tene epidermis cells (Ninov *et al*, 2007), starting to form a complete adult epidermis layer. Therefore, we analysed the histoblasts in pupae collected 26–27 h after pupa formation (Figure 3A). At this stage, histoblasts are already proliferating, replacing larval epidermis cells and forming over 50% of the adult abdomen epidermis (Roseland and Schneiderman, 1979). As expected, in *gfp-polo; polo*⁹ and $\Delta pA1; polo$ ⁹ pupae normal histoblast proliferation can be clearly seen: histoblasts are the small cells (delimited by the dotted line in Figure 3A). Larval epidermis cells (delimited by solid line in Figure 3A) are larger and easily distinguished from histoblasts. However, in $\Delta pA2; polo$ ⁹ pupa epidermis only the large polytene larval cells are observed and histoblasts could not be detected in pupae at this stage (Figure 3A). Moreover, this phenotype is only observed in a *polo*⁹ homozygous background (Supplementary Figure S3). In conclusion, our results show that in the absence of the *polo* pA2 transcripts, histoblasts are not detected during metamorphosis.

The absence or incorrect formation of the tergites in $\Delta pA2; polo$ ⁹ flies could result either from a failure in the specification/formation of abdominal histoblasts before the entrance to the pupal stage, or from the inability of these cells to proliferate during this developmental stage. To distinguish between these two possibilities, $\Delta pA2; polo$ ⁹ third instar larvae were dissected and immunostained with anti-Headcase (Hdc) antibody, used as a marker for abdominal histoblasts, showing that histoblasts are clearly present in *gfp-polo; polo*⁹, $\Delta pA1; polo$ ⁹ and $\Delta pA2; polo$ ⁹ larvae (Supplementary Figure S4A). Furthermore, all histoblast cells are strongly stained for Cyclin B, as expected from G₂-arrested cells (Supplementary Figure S4A). Moreover, quantification of the number of histoblasts in each nest shows that these larvae have similar number of cells in each histoblast nest for the different abdominal segments (Supplementary Figure S4B). Taken together, these results indicate that $\Delta pA2$ abdominal histoblasts are correctly formed during embryogenesis and are able to maintain the G₂ arrest until the larvae stage. Consequently, the longer *polo* transcript derived from pA2 usage is essential for the proliferation of these cells at the onset of metamorphosis.

Having determined that deletion of *polo* pA2 was detrimental to the fly, we went on to investigate the expression of GFP-Polo obtained from the different transgenes in late pupal development, using pupae in a heterozygous background (*polo*⁹/*TM6B*), to allow proliferation of the histoblasts. We observed a significant decrease of the GFP-Polo signal in histoblasts of $\Delta pA2$ pupae (Figure 3B; histoblast cells are delimited by a dotted yellow line). Quantification of the fluorescent levels throughout the whole pupa epidermis, using the same individuals, showed that while *gfp-polo* and the $\Delta pA1$ transgenes express similar levels of GFP-Polo a 30% decrease in fluorescence is observed for the $\Delta pA2$ transgene (Figure 3C). To elucidate if this is due to a decrease in the mRNA produced, we quantified the mRNA by RT-qPCR. As can be seen in Figure 3D, there is no significant difference between the mRNA levels produced by each transgene; thus, the decrease in protein levels observed in $\Delta pA2$ flies is unlikely to be due to a simple reduction in the mRNA levels. These results thus suggest that the appropriate levels of Polo protein in abdominal histoblasts may only be achieved when the distal pA2 site is used.

Figure 3 $\Delta pA2$ transgene causes a failure in abdominal histoblasts proliferation and produces lower Polo protein levels in histoblasts. (A) Pupa epidermis from *gfp-polo*, $\Delta pA1$ or $\Delta pA2$ pupae transgenes in a *polo⁹* homozygous background was dissected at 26–27 h after pupa formation and stained with DAPI. *gfp-polo;polo⁹* pupa epidermis exhibited large polyploid nuclei (circled), the larva epidermis cells (LECs). LECs were surrounded by smaller cells (highlighted by dotted lines), which are the proliferating abdominal histoblasts. $\Delta pA1;polo⁹$ pupa epidermis show a pattern very similar to that observed in *gfp-polo;polo⁹* tissues. In $\Delta pA2;polo⁹$, only LECs are observed, indicating that at this stage, pupa epidermis is mainly composed of larval cells. Scale bar is 50 μm . (B) GFP-Polo expression (green) in abdominal histoblasts in pupae epidermis (26–27 h after pupa formation). Pupae from *gfp-polo*, $\Delta pA1$ (line 12.1) and $\Delta pA2$ (line 2.3) transgenic flies in a *polo⁹/TM6B* background were collected and the epidermis was dissected. The DNA (red) was stained with DAPI. The yellow dotted surrounding area encompasses some of the histoblast cells present in each image. Scale bar is 10 μm . (C) The levels of GFP-Polo were determined in the abdomen histoblasts, by quantification of the GFP fluorescence levels in *gfp-polo*, $\Delta pA1$ (line 12.1) and $\Delta pA2$ (line 2.3) pupae in a *polo⁹/TM6B* background, 26–27 h after pupa formation. (D) The levels of *gfp-polo* transcripts were determined by RT-qPCR in *gfp-polo*, $\Delta pA1$ (line 12.1) and $\Delta pA2$ (line 2.3) whole pupae in a *polo⁹/TM6B* background, 26–27 h after pupa formation. The levels of *gfp-polo* RNAs were normalized to *rp49* RNA. Error bars represent s.e.m. from three independent experiments.

To identify the molecular mechanism responsible for the decrease in Polo protein when pA2 is deleted, we used a *Drosophila* cell line to assay for the stability of the

endogenous *polo* mRNAs. Upon treatment with actinomycin D and measurement of RNA decay by RT-qPCR, we observe that both *polo* RNAs and *polo* pA2 RNA levels are maintained

Figure 4 *polo* pA2 RNA is stable and the corresponding 3'UTR confers higher transability to a reporter gene in *Drosophila* cells. (A) Endogenous *polo* pA2 RNA is stable. The decay of the endogenous *polo* RNAs and *Vha68-1* RNA was monitored at the indicated time points after addition of actinomycin D (5 µg/ml). The levels of *polo* RNAs were normalized to *rp49* RNA in three independent experiments and are plotted against time. RNA half-lives ($t_{1/2}$), calculated from the decay curves, are longer than 6 h, except for *Vha68-1* that is ~30 min. (B) Representation of the firefly luciferase reporter constructs with the shorter *polo* 3'UTR (pA1) or the longer *polo* 3'UTR (pA2) (pA1 signal mutation: ATTAAA→GTTAAC). The region for specific qPCR primers is underlined. (C) pA1 produces three-fold less protein than pA2. Kc cells were transfected with the pA1 and pA2 reporters and 3 days later firefly luciferase activity and the corresponding mRNA levels were measured. Y axis represents luciferase values normalized to the mRNA levels. Error bars represent s.e.m. from at least three independent experiments.

for at least 6 h (Figure 4A), suggesting that both transcripts are equally stable. As a control for the actinomycin D treatment, we used the *Vha68-1* (*vacuolar H⁺ ATPase subunit 68-1*) RNA which displays a half-life of ~30 min, as previously shown (Behm-Ansmant *et al*, 2006). We then tested if the different 3'UTRs present in each transcript were responsible for the different levels of protein produced. The 3'UTR ending at pA1 was fused downstream of a *Luciferase* reporter gene to produce the pA1 plasmid (Figure 4B). In a construct containing the whole 3'UTR fused to *luciferase*, the pA1 signal was mutated (ATTAAA→GTTAAC) to produce plasmid pA2 (Figure 4B); the resultant plasmids, pA1 and pA2, were transfected into Kc cells. The mRNA 3'ends formed from each construct were confirmed by 3'RACE and to distinguish translational output from mRNA turnover, luciferase translational activity was normalized to *luciferase*-reporter mRNA levels to obtain the

Figure 5 Overexpression of *polo* leads to an increase in pA1 utilization and a reduction in GFP-Polo protein levels. (A) *MZ1061-Gal4;UAS-polo:polo⁹* overexpresses Polo. L3 brains were analysed by western blot and a 2.3-fold increase in Polo was observed in comparison with the control—*w¹¹¹⁸*. Quantification was made by densitometry (see Materials and methods) and Polo/α-tubulin ratio was set at 1. Polo was detected with MA294 and α-tubulin with DM1A antibodies. (B) Overexpression of Polo causes a switch in the pAs ratio. Total RNA was extracted from L3 larvae brains of the *MZ1061-Gal4;gfp-polo:polo⁹/UAS-polo:polo⁹* progeny (labelled as overexpression in the figure). The strain *MZ1061-Gal4;gfp-polo:polo⁹* was used as control. Total/pA2 mRNA levels were quantified by RT-qPCR with specific primers for both transcripts in the coding sequence and primers between the two pAs. A 2.5-fold change was detected in comparison with the control. (C) GFP-Polo protein expression was analysed by western blot, using the same conditions as in (A). When *polo* is overexpressed, a four-fold decrease in GFP-Polo is observed.

translation efficiency (defined in Supplementary Figure S5). Significantly, we found that pA1 causes a three-fold decrease in translation as compared with pA2 (Figure 4C). These results clearly indicate that the longer *polo* mRNA is more efficiently translated than the shorter transcript. This suggests that the correct levels of Polo are defined by pA2 signal selection, in agreement with the *in vivo* results obtained with the transgenic fly lines.

Finally, we asked if the levels of utilization of pA1 and pA2 could be regulated by *in vivo* overexpression of Polo using the previously established GAL4/UAS system (Brand and Perrimon, 1993). Using this system, it was previously shown that Polo is overexpressed by 2.5-fold which does not affect its physiological function in mitosis (Martins *et al*, 2009). Thus, using a neuroblast driver (*MZ1061-Gal4*) we tested the correlation between the expression of each transcript and protein levels in a tissue-specific manner. For this, *MZ1061-Gal4; gfp-polo:polo⁹/TSTL* females were crossed with *MZ1061-Gal4;UAS-polo:polo⁹/TSTL* males and the *polo⁹*

homozygous brain progeny was analysed; in these flies, *polo* cDNA with the full 3'UTR is transcribed from an upstream inducible element (Mirouse *et al*, 2006). As shown in Figure 5A, Polo is overexpressed 2.3-fold relative to the wild-type control, levels that are in agreement with previous studies (Martins *et al*, 2009). Using RT-qPCR, we then measured the ratio of pA1 to pA2 mRNAs in the *polo*⁹ homozygous F1 generation taking strain *MZ1061;gfp-polo:polo*⁹/*TSTL* as a control. Significantly, overexpression of Polo results in a 2.5-fold increase in the total/pA2 mRNA ratio (Figure 5B). We further determined the amount of GFP-Polo protein levels by western blot analysis and observed a four-fold decrease in GFP-Polo in the flies where *polo* was overexpressed (Figure 5C). Taken together, these results show that overexpression of *polo* induces a switch from pA2 to pA1 mRNA and a consequent decrease in the protein levels. These results suggest the existence of a potential auto-regulatory feedback loop mechanism.

Recent studies have shown that in cancer cells alternative polyadenylation in general selects shorter 3'UTRs and consequently there is an increase of protein production (Mayr and Bartel, 2009). This was shown to be partly due to the presence of miRNA target sites in the extended 3'UTRs. We have searched in the available databases and prediction programs for miRNAs that could potentially target *polo* transcripts and found dme-mir-8 and dme-mir-1016 (Stark *et al*, 2007). The predicted miRNAs were quantified and tested in Kc cells. However, we could rule out an effect of dme-mir-8 and dme-mir-1016 overexpression on *polo* 3'UTR (Supplementary Figure S6).

Discussion

Expression levels of the essential cell-cycle regulatory gene *polo* are critical to *Drosophila* development. We show in these studies that Polo protein levels are regulated by the relative usage of two alternative pA sites (pA1 and pA2) present in the *polo* gene terminal exon. Unexpectedly, we show that pA2 is essential for fly viability whereas the pA1 site is non-essential. The molecular explanation for this specificity is that *polo* mRNA utilizing pA2 is translated more efficiently than *polo* pA1 mRNA thereby generating sufficient protein for cell proliferation. Thus, we show in these studies that deletion of pA2 displays a lethal phenotype (Table I; Figure 2D). Our studies further suggest that levels of Polo protein are auto-regulated by the selective usage of these two pA sites. Thus, *polo* overexpression results in a switch in pA site selection from pA2 to pA1 with consequent downregulation of Polo expression (Figure 5).

Since many genes in flies and other eukaryotes possess alternative pA sites in terminal exons, we investigated the possibility that general transcription kinetics might also have a role in alternative polyadenylation. To do this, we employed the well-characterized *C4* fly mutant, which has a 50% decrease in Pol II elongation rate (Greenleaf *et al*, 1979). Interestingly, we show that this results in increased utilization of the *polo* pA1 site (Figure 1D and E). In particular, in *C4* flies, *polo* pA1 is used 3.5-fold more efficiently than in the wild type. We further demonstrate that the effect of Pol II kinetics in alternative pA site selection is likely to be general as analysis of five additional *Drosophila* genes possessing alternative terminal exon pA sites showed a similar switch to

upstream pA site usage in the *C4* mutant. This clearly indicates that Pol II kinetics has an important role in alternative polyadenylation site selection. Significantly, human Pol II carrying the equivalent mutation to the *Drosophila* *C4*, when transfected into human cells, affects alternative splicing of a FN minigene. Inclusion of the alternative EDI exon in this system increases ~4-fold (de la Mata *et al*, 2003), a similar level to that obtained for alternative *polo* pA signal selection (Figure 1E). *C4* flies also show a difference in Pol II occupancy across the *polo* gene (Figure 1A). Thus, in wild-type flies Pol II levels are reduced downstream of pA1, while in *C4* flies this pattern is disrupted. Presumably, the slow Pol II enables the pA1 signal on the nascent transcript to be exposed to the polyadenylation machinery for a longer time before Pol II transcribes pA2. Therefore, pA1 will be processed before pA2 is transcribed pointing to a mechanism that relies on the rule of 'first come, first served' (Figure 6A). When the Pol II elongation rate is higher, as in wild-type flies, it transcribes through pA1 and pA2 more efficiently, so that the polyadenylation machinery processes both pA signals on the nascent transcript (Figure 6A). This resembles the mechanism described above for EDI alternative splicing where slow Pol II preferentially includes the alternative EDI exon which is normally excluded, because it allows the machinery time to assemble on the spliceosome (de la Mata *et al*, 2003, 2010). Our results now indicate that both alternative polyadenylation and alternative splicing depend on Pol II kinetics. In view of recent findings that highlight the importance of alternative pA signal selection (Sandberg *et al*, 2008; Ji and Tian, 2009; Mayr and Bartel, 2009; Singh *et al*, 2009), our results now suggest a general molecular mechanism for this process.

The physiological consequences of correct *polo* pA signal choice *in vivo* as shown in this study are profound: pA2 is essential for abdominal histoblast proliferation, development of the adult epidermis and viability of the transgenic flies. The lethality and strong abdominal phenotype observed in *gfp-poloΔpA2;polo*⁹ flies are due to the fact that these flies lack *polo* pA2 mRNA and consequently cannot express sufficient levels of Polo protein (from *polo* pA1 mRNA) for flies to survive the pupa stage. This phenotype is in agreement with earlier studies showing that *polo*¹/*polo*² individuals express low levels of Polo protein (Herrmann *et al*, 1998) and present an abnormal development of the abdomen (Sunkel and Glover, 1988). We anticipated that the *C4* mutant would display a similar phenotype to *gfp-polo ΔpA2* flies since it downregulates pA2 usage. However, we observe a developmental defect similar to the so-called 'Ubx effect' (Greenleaf *et al*, 1980; Martin *et al*, 1988). We note that slow Pol II elongation will impact on many genes so that any phenotype observed is likely to derive from complex genetic effects as previously reported (Greenleaf *et al*, 1980). We also note that *polo* pA2 mRNA is still produced at significant levels in the *C4* mutant (Figure 1D and E), which may produce sufficient protein for development of the abdomen, as opposed to the *ΔpA2* transgenic flies that lack *polo* pA2 mRNA (Figure 2B) and show a decrease in Polo protein production in the histoblasts (Figure 3B and C).

Two genes in humans (*hap* and *Bzw1*) where alternative distal pA site usage results in increased levels of protein production have been previously described (Qu *et al*, 2002;

Figure 6 Proposed working model for the role of Pol II kinetics on pA site selection. **(A)** When Pol II elongation rate is low, Pol II spends more time transcribing *polo* allowing the pA factors (represented by a triangle and an hexagon) to assemble on the pA1 signal presented by the nascent pre-mRNA, leading to an increase in pA1 utilization, and therefore producing four-fold more *polo* pA1 mRNA. In conditions where Pol II processivity is high, Pol II transcribes more rapidly through pA1 and pA2. Under these conditions, both pA1 and pA2 signals are present on the nascent transcript and exposed to the polyadenylation machinery at the same time. Consequently, both *polo* pA1 and pA2 mRNAs are formed. **(B)** The longer *polo* mRNA, produced by pA2 signal selection and containing a longer 3'UTR, is more efficiently translated into protein than the shorter pA1 mRNA. Consequently, selection of pA2 is important for the viability of the fly. Levels of Polo protein are controlled by a negative feedback loop mechanism *via* pA site selection; in the presence of high levels of Polo, pA1 signal is predominantly selected, causing a decrease in the levels of Polo protein produced.

Yu *et al*, 2006). In T cells and cancer cell lines, proximal pA site selection in the 3'UTR results in a relief from microRNA repression with the same final effect of an increase in protein production (Sandberg *et al*, 2008). However, a considerable proportion of genes do not follow this pattern, pointing to the existence of other regulatory elements in the different 3'UTRs (Mayr and Bartel, 2009). Presumably, *polo* is one such gene as we show that its expression is not regulated by dme-mir-8 and dme-mir-1016 overexpression (Supplementary Figure S6). Instead regulated *polo* expression relies on the fact that the choice of pA1 by the transcriptional/processing machinery leads to a decrease in the translation of Polo (Figure 4). This indicates that Polo protein expression is modulated by pA signal selection and by translational control through the 3'UTR, suggesting the presence of regulatory elements in the different 3'UTRs (P Pinto and A Moreira, unpublished

results). As Polo is a master regulator of the cell cycle, we predict that the consequences of this type of control will be critical to the cell.

Histoblasts have a very high proliferation rate and go through very rapid cell cycles at the onset of metamorphosis (Madhavan and Schneiderman, 1977; Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980; Ninov *et al*, 2007, 2009), therefore would be predicted to be especially sensitive to Polo levels. The lack of *polo* pA2 mRNA in $\Delta pA2$ flies leads to a reduction in Polo protein levels with the consequent block of histoblast proliferation. This will in turn result in a subsequent failure to correctly develop the adult epidermis. Consistent with our studies, loss of Polo was previously shown to cause G₂ arrest (Rehwinkel *et al*, 2005).

Interestingly, we also show that, in flies where Polo is overexpressed, the shorter mRNA produced by pA1 usage is more abundant than the longer mRNA produced by pA2 selection (Figure 5A and B). This suggests that Polo controls its own expression levels by an auto-regulatory loop, where higher levels of Polo lead to preferential recognition of pA1. As this mRNA is not efficiently translated (Figure 4C), this will lead to a decrease in the protein levels produced (Figure 6B). It was previously shown that upregulation of *su(f)* leads to an auto-regulatory feedback loop through the recognition of a weak intronic pA site (Audibert and Simonelig, 1998). This results in the production of a truncated polypeptide leading to a shut down in gene expression. In the case of *polo*, a more subtle process is evident, as pA1 selection generates a functional transcript, even though it is not efficiently translated into protein.

Taken together, our results suggest that Pol II kinetics have an important role in pA site selection. Moreover, our results reinforce the view that tight regulation at the level of pA signal selection is necessary for the cell. The importance of precisely choosing the correct pA signal to control cell viability during development is underlined by our studies.

Materials and methods

Plasmid constructs

$\Delta pA1$ transgene: pXB7 $\Delta pA1$ was constructed by site directed mutagenesis (adapted from Makarova *et al* (2000)) using pXB7 (Moutinho-Santos *et al*, 1999) as a template. pXB7 $\Delta pA1$ was digested with *Bst*API and *Eco*RI and the 1576-bp fragment was subcloned into pCR-*gfp-pol*o to generate the pCR-*gfp-pol*o $\Delta pA1$ plasmid. pCR-*gfp-pol*o $\Delta pA1$ was digested with *Cci*NI and *Xho*I and the 7717-bp DNA fragment was subcloned into pW8 to generate pW8-*gfp-pol*o $\Delta pA1$ (named $\Delta pA1$). **$\Delta pA2$ transgene:** constructed by PCR amplification with the pshA1 genomic and not pA1 oligonucleotides. The PCR product was subcloned into pW8-*gfp-pol*o. For luciferase assays, *polo* 3'UTR ($\Delta pA1$ and $\Delta pA2$) was PCR amplified from the above constructs, subcloned into pCR $\text{\textcircled{R}}$ II-TOPO (Invitrogen) and subsequently subcloned into the *Eco*RI restriction site immediately downstream of the firefly coding region in the pAc5.1C-F-Luc plasmid. miRNA coding regions were PCR amplified from genomic DNA (~400 bp surrounding the mature miRNA) and subcloned into pCR $\text{\textcircled{R}}$ II-TOPO (Invitrogen). miRNA reporters were subcloned into *Kpn*I and *Xho*I restriction sites of pAc5.1A vector. All constructs used in this study were fully sequenced.

Total RNA isolation and northern blot analysis

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. Northern blot was performed as described

(Sambrook and Russell, 2001). Quantifications were made by densitometry using a GS-800 densitometer (Bio-Rad).

Drosophila stocks

u¹¹⁸ and *Rp11215*, carrying the RNA Pol II 215 C4 allele, were obtained from the Bloomington Stock Centre. *UAS-polo* and *polo⁹/TM6C* flies were kindly given by Jean-Louis Couderc (INSERM, France) and David Glover (University of Cambridge, UK), respectively. For the overexpression studies, *MZ1061;gfp-polo:polo⁹/TSTL* females were crossed with males *MZ1061;UAS-polo:polo⁹/TSTL* and the *polo⁹* homozygous progeny was analysed (Mirouse *et al*, 2006). The strain *MZ1061;gfp-polo:polo⁹/TSTL* was used as a control. All stocks were grown at 25°C using standard culture conditions and media.

Viability quantification

For the *gfp-polo; polo⁹/TM6B*, Δ *pA1; polo⁹/TM6B* and Δ *pA2; polo⁹/TM6B* crosses were performed to rescue the *polo⁹/polo⁹* allelic combination. From these crosses, flies homozygous for *polo⁹* were quantified over the total number of flies.

Adult abdomen preparation

Flies were cut between the thorax and the abdomen and all appendages were removed. Abdomens were incubated in a lactic acid:ddH₂O (3:1) solution overnight at 60°C, mounted in a fresh solution and incubated overnight at 60°C. The images were acquired with a Leica DM LB microscope (Leica Microsystems) with a 3 CCD color camera vision module (DONPISHA) and treated with the Adobe Photoshop 7.0 Software.

Pupa epidermis preparation

The dissection of 26–27 h pupae was performed according to Gompel and Carroll (2003). The epidermis was fixed in 1.85% (v/v) formaldehyde in fixation buffer (0.1 M PIPES pH 6.9, 1 mM EGTA pH 6.9, 1% (v/v) Triton X-100 and 1 mM MgCl₂) for 10 min at RT. After fixation, the epidermis was washed twice for 5 min at RT in PBT (1 × PBS, 0.03% (v/v) Triton X-100) and mounted in 6 µl of VectaShield with DAPI (Vector Laboratories). The images were acquired with the AxioImager Z1 microscope (Carl Zeiss), with a b/w CCD camera Axiocam MR ver. 3.0 (Carl Zeiss) and treated using the Adobe Photoshop 7.0 Software. The number of histoblasts present in each nest represents the average number of cells in two pupae of each genotype.

Immunostaining of third instar larvae abdominal histoblasts

The protocol used was adapted from Weaver and White (1995) and Sullivan *et al* (2000). Third instar larvae were incubated in 1 × PBS at 55°C for 10 s, cut along the dorsal–ventral axis and all internal tissues were carefully removed. The cuticles were then fixed in 100 µl of 3.7% (v/v) formaldehyde in PBT (1 × PBS, 0.1% (v/v) Triton X-100) for 20 min at RT and washed three times in 100 µl of PBT. Blocking was performed for 1 h, at RT, in 200 µl of PBT with 10% (v/v) FBS. The cuticle was incubated ON at 4°C with 200 µl of α -Hdc antibody (1:5), kindly provided by Robert White (Weaver and White, 1995), and α -CycB antibody (Jacobs *et al*, 1998) (1:3000) in PBT with 10% (v/v) FBS. The following day, the cuticles were washed three times for 10 min in 200 µl of PBT and mounted in 20 µl of VectaShield with DAPI (Vector Laboratories). The images were acquired with an inverted Laser Scanning Confocal Microscope Leica SP2 AOBs SE (Leica Microsystems) and deconvolved and projected onto a single plan using the Huygens Deconvolution Software (SVI). The images were treated using the Adobe Photoshop 7.0 Software.

Quantification of GFP levels in 26–27 h after pupa formation pupae epidermis

The images were acquired using a Laser Scanning Confocal Microscope Leica SP2 AOBs SE (Leica Microsystems) and GFP fluorescence measured using the Leica Confocal Software, version 2.61 Build 1538 LCS Lite (Leica Microsystems). For each genotype, two pupae were analysed. For each sample, one plane was chosen and several GFP measurements were made throughout the epidermis. The GFP levels were quantified using the following formula: mean × area/number of cells, where the mean is the average level of GFP fluorescence measured for each sample.

cDNA synthesis and RT–qPCR

cDNA synthesis was performed using either oligodT or random hexamers with Superscript III following the manufacturer's instructions (Invitrogen). cDNA was then quantified in an iQ5 Bio-Rad real time PCR machine with iQTM Sybr® Green Supermix. *rp49* was used for normalization in all assays.

Western blot

Western blotting was performed as previously described (Moutinho-Santos *et al*, 1999). For Polo, MA294 antibody was diluted 1:80 and α -tubulin was detected using mAB DM1A (Sigma) diluted 1:8000. Quantifications were made by densitometry using a GS-800 densitometer (Bio-Rad).

mRNA stability assays

Kc cells were treated with actinomycin D (5 µg/ml), total RNA was isolated at designated time points and cDNA synthesis was performed using random hexamer primers with SuperScript III (Invitrogen) following the manufacturer's guidelines, followed by qPCR. mRNA half-life was calculated from the decay curves.

3' UTR and miRNA functional assays

Kc cells were transfected in 6-well plates using Effectene transfection reagent (Qiagen) with 100 ng of the Firefly luciferase reporter plasmid (F-Luc) containing the SV40 late polyadenylation signal (control) or the whole *polo* 3'UTR fused downstream to the Firefly luciferase reporter, 400 ng of the Renilla luciferase transfection control plasmid (R-Luc) and 500 ng of the plasmid (pAc5.1/V5-HisA, Invitrogen) expressing the miRNA primary transcripts. Three days after transfection, Firefly and Renilla activities were measured using a Dual-Luciferase reporter assay system (Promega). Total RNA was also isolated and northern blot (miRNA assay) or RT–qPCR (3'UTR assay) on oligodT synthesized cDNA was performed with Quantace Sensimix NoRef in a Rotor-Gene 6000 (Corbett LifeScience) using Firefly- and Renilla-specific primers as described above.

Chromatin immunoprecipitation

ChIP was performed as previously described (Schwartz *et al*, 2003). Each value for ChIP experiments was derived from IPs of at least three independent adult fly samples. Extracts were sonicated at 4°C on the medium-sonication setting of Bioruptor® (Diagenode) with 20 s bursts/30 s cool off, to achieve average fragment sizes of 200–300 bp. For the immunoprecipitations, 4 µl of rabbit anti-Rpb3 was used (Adelman *et al*, 2006). Primer sequences are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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performed the experiments. AM, CES and NJP analysed the data. AM and PABP wrote the paper with substantial contribution from NJP.

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Conflict of interest

The authors declare that they have no conflict of interest.

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