Published in final edited form as:
Curr Biol. 2013 January 21; 23(2): 127–132. doi:10.1016/j.cub.2012.11.029.

Post-translational control of Cdc25 degradation terminates Drosophila's early cell cycle program

Stefano Di Talia[1], **Richard She**[1], **Shelby A. Blythe**[1], **Xuemin Lu**[1,2], **Qi Fan Zhang**[1], and **Eric F. Wieschaus**[1]

[1]Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA

SUMMARY

In most metazoans, early embryonic development is characterized by rapid mitotic divisions that are controlled by maternal mRNAs and proteins that accumulate during oogenesis [1]. These rapid divisions pause at the Mid-Blastula Transition (MBT), coinciding with a dramatic increase in gene transcription and the degradation of a subset of maternal mRNAs [2, 3]. In Drosophila, the cell cycle pause is controlled by inhibitory phosphorylation of Cdk1, which in turn is driven by down-regulation of the activating Cdc25 phosphatases [4, 5]. Here, we show that the two Drosophila Cdc25 homologues, String and Twine, differ in their dynamics and that, contrary to current models [4], their down-regulations are not controlled by mRNA degradation but through different post-translational mechanisms. The degradation rate of String protein gradually increases during the late syncytial cycles in a manner dependent on the nuclear-to-cytoplasmic ratio and on the DNA replication checkpoints. Twine, on the other hand, is targeted for degradation at the onset of the MBT through a switch-like mechanism controlled like String by the nuclear-to-cytoplasmic ratio, but not requiring the DNA replication checkpoints. We demonstrate that post-translational control of Twine degradation ensures that the proper number of mitoses precede the MBT.

RESULTS

The MBT marks the transition from the maternally controlled amplification of nuclear number to the zygotically directed program of differentiation and morphogenesis. Pre-MBT nuclear amplification is driven by an abbreviated cell cycle until the embryo reaches a defined ratio between DNA and cytoplasmic content (N/C ratio) [1, 6, 7]. At this critical N/C ratio, the cell cycle is rapidly reprogrammed through switch-like inhibition of Cdk1 [5]-driven by down-regulation of the two Cdc25 homologs present in Drosophila (String and Twine)-to ensure a pause.

To test if regulation of String and Twine protein rather than mRNA provides the switch controlling the rapid inactivation of Cdk1 at the MBT, we used live imaging of embryos maternally expressing His2Av-RFP to follow the behavior of nuclei, and String-GFP or

Corresponding Author: Eric F. Wieschaus (efw@princeton.edu).
[2]Present Address: Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, USA

Supplemental Information Supplemental Information includes Extended Experimental Procedures, 4 Figures and 4 Movies.

Twine-GFP expressed using the Gal4/UAS system and having the 3′UTR of SV40 (Figures 1A-B). Since 2-3 fold over-expression of Twine is reported to alter cell cycle behavior at the MBT [4], we selected UAS lines with expression levels below 50% of wild type (Figure S1A), thereby ensuring only a slight increase in total Twine levels. We confirmed the results obtained from live imaging experiments of the transgenic constructs by dual immunofluorescence quantification of String and Twine (Figure 1C).

Live imaging and immunofluorescence data show that String and Twine proteins preferentially localize to nuclei during interphase of cleavage cycles and during early cycle 14 (Figures 1A-B). Both proteins disperse in the cytoplasm during mitosis due to nuclear envelope breakdown (see Movies S1-S2). Interestingly, although both String and Twine are absent from nuclei by 20 minutes into cycle 14, the dynamics of their down-regulation are significantly different. Consistent with previous results [4, 5], down-regulation of String does not happen specifically at the MBT but precedes it, following a step-wise pattern of reduction during cycles 10-13 (Figure 1D). Twine, on the other hand, does not show significant down-regulation during cycles 10-13 (Figure 1E). High levels of Twine are still present in nuclei at the onset of cycle 14 (Figure 1E). Levels fall immediately thereafter and by 20 minutes into cycle 14 Twine is no longer detectable (Figure 1E). These results indicate that Twine, but not String, is down-regulated specifically at the MBT and could provide the required switch for the cell cycle pause.

In contrast to other systems [8, 9], quantification of the nuclear and cytoplasmic levels of both String and Twine indicates that they are not controlled by regulation of the nuclear-cytoplasmic transport or by the activity of the Anaphase Promoting Complex (see Supplemental Information and Figure S1).

To determine if degradation of maternal mRNA controls the protein decline we compared the protein dynamics with the mRNA dynamics measured by quantitative RT-PCR of precisely staged embryos. Consistent with previous results [4], we found that *string* mRNA decline follows protein decline with a significant lag (at least 15 minutes). At the beginning of cycle 14 *string* mRNA levels are still 60% of the cycle 12 levels, while the protein levels are almost undetectable (Figure 1D). Similarly, the decline in Twine protein precedes changes in mRNA levels; *twine* mRNA is constant from cycle 12 until about 15 minutes into cycle 14, and is rapidly degraded in the 20 minutes that follow (Figure 1E). The rapid down-regulation of Twine protein is observed significantly before the degradation of the mRNA and Twine is essentially absent when *twine* mRNA levels start declining (Figure 1E). Based on these observations, we conclude that mRNA levels do not control the dynamics of String and Twine prior to the MBT.

In haploid embryos, the N/C ratio associated with the MBT is achieved one cell cycle later than in diploids, and the cell cycle pause occurs in cycle 15 rather than 14 [6]. The 14th mitosis occurs about 15 minutes into cycle 14, at a time when both String and Twine would normally be almost absent (Figures 2A, 2C). We found that in haploid embryos, the down-regulation of both String and Twine is delayed by about one cell cycle (Figures 2A, 2C), but similar to diploids, the two proteins disappear significantly before the mRNA (Figures 2A-2D). For both proteins the data from diploids and haploids are similar upon rescaling them as a function of the N/C ratio rather than time (Figures 2B, 2D), indicating that the ratio regulates their levels.

Cell cycle control prior to the MBT is characterized by the lengthening of interphase due to a slow-down of S-phase, associated with an increased number of nuclei and nuclear content. We find that this slow-down is delayed in haploid embryos (Figure S2) and confirm that it requires DNA replication checkpoint activity [10, 11]. Live imaging of *grp lok* mutant

embryos that lack the main effector kinases (Chk1 and Chk2) of these checkpoints shows that lengthening of the late syncytial cycles is reduced and the gradual decline of String levels normally observed in the late cell cycles is absent (Figure 2E).

As *grp lok* embryos enter mitosis 13, chromosomes do not separate (Figure S2 and Movie S3) and nuclei enter an apparent interphase with the same nuclear density of the preceding cell cycle. Cell cycle driven periodic events (e.g. nuclear envelope breakdown and formation, chromosome condensation and decondensation) continue to occur in such embryos, although probably with defects (Figure S2 and Movie S3) and the embryo makes one or occasionally two extra attempts to execute mitosis before initiating cellularization. During this period, the increase in total His2Av-RFP signal per nucleus suggests that nuclei undergo extra rounds of DNA replication (Figure S2). String levels, which had failed to decline in the previous cycles, remain high during the defective mitosis 13 and 14 and are constant for about 15-20 minutes in cycle 15, at which point they disappear rapidly (Figure 2E).

Twine levels are unaffected during the cycles that precede the MBT in *grp lok* embryos (Figure 2F), although their down-regulation is delayed when measured relative to the premature entry into cycle 14 associated with the shortened cell cycles. Unlike String, Twine dynamics resembles those observed in wt. Protein levels only slightly decline until the last mitosis after which the protein disappears very rapidly. The kinetics of down-regulation of Twine at cycle 14 in wt and cycle 15 in *grp lok* are very similar (Figure 2F inset). We, therefore, propose that the abrupt down-regulation of Twine at the onset of the MBT does not require Chk1 and Chk2 activity and that the observed delay in Twine degradation in *grp lok* embryos is the indirect result of a delay in the onset of the MBT (see Discussion).

Based on the above observations, we propose that String down-regulation is the result of increased checkpoint activity, occurring gradually in an N/C ratio dependent manner and not controlled by a developmental switch. On the other hand, Twine is controlled by an MBT-specific developmental switch, which depends on the N/C ratio but does not require checkpoint activity.

Since the rapid down-regulation of Twine at the MBT occurs prior to the loss of *twine* mRNA, it is possible that it reflects a switch-like decrease of the protein half-life. To test this hypothesis, we used an optical method employing photo-switchable proteins [12] to measure Twine half-life independent of its rates of synthesis. Using the Gal4/UAS system, we maternally expressed a fusion protein between Twine and Dronpa, a photo-switchable green fluorescent protein that can be converted to a non-fluorescent (dark) state by illumination with a 488 nm laser and can be switched back to the fluorescent state by illumination with a 405 nm laser [13]. Since native Dronpa is never produced in the dark state, protein translation does not contribute to the amount of protein that can be recovered from that state. After photo-switching, the amount of dark protein depends only on its degradation [12]. We estimated the protein degradation rate k_{deg} and half-life (half-life=$\ln(2)/k_{deg}$) by converting a measurable amount of protein to the dark state and analyzing the amount of that dark protein that remains after a given time interval and can be recovered to the bright state (Figures 3A-B, see Supplemental Information for details).

During cycles 11 to 13, we found that Twine is relatively stable with a half-life of about 20 minutes (Figure 3C). At the onset of cycle 14 the protein becomes highly unstable with a half-life of about 5 minutes (Figure 3C). The switch in protein stability is very abrupt and coincides with the beginning of cycle 14 (Figure S3 and Supplemental Information). These results indicate that the stability of Twine protein is developmentally controlled and rapidly decreases at the onset of the MBT.

Similar experiments in embryos expressing String-Dronpa indicate that String stability is regulated differently from Twine showing a gradual rather than switch-like decrease prior to the MBT (Figure 3A). String half-life is about 20 minutes at cycle 10 and steadily decreases as the length of the cell cycle increases (Figure 3D). By cycle 13 the half-life is about 5 minutes (Figure 3D).

To establish if the measured changes in half-lives (Figures 3C-D) are sufficient to account for the dynamics of String and Twine without invoking changes in mRNA levels or translation control, we computed the kinetics of String and Twine degradation predicted from the measured changes in stability under constant protein production rate (i.e. constant mRNA concentration and no translation control, Figure 3E). Numerical solutions of the mass-action kinetic equations show that the computed dynamics of Twine-Dronpa fluorescence can reproduce well the measured dynamics, and that a change in half-life from 20 to 5 minutes is sufficient to lower total proteins several fold at the MBT (Figure 3F). Similarly to Twine, numerical solutions of the kinetic equations describing String dynamics (Figure 3E) can reproduce the measured dynamics, suggesting that post-translational control contributes significantly to the regulation of String levels (Figure 3G). We conclude that prior to the MBT String and Twine are controlled through different post-translational mechanisms, and not through mRNA abundance or other translational controls.

Collectively, the previous experiments suggest that rapid turnover of Twine is required to avoid an extra mitosis prior to the MBT [14]. To test this hypothesis, we have screened various mutant forms of Twine tagged with GFP (data not shown). We have identified a mutant (lacking the region between amino acids 106 and 180), which does not undergo rapid degradation at the beginning of cycle 14, but retains similar localization and activity as wild type Twine (see Figure S3). Expression of this mutant form causes an extra division (28±2 minutes in cycle 14) in a high fraction of embryos (6/13 embryos, see Movie S4). Expression of Twine-GFP at similar levels does not result in an extra division (0/20 embryos). We, therefore, propose that control of Twine degradation ensures that the proper number of mitoses precede the MBT.

The pause in the cell cycle at the MBT is required for the morphogenetic process of cellularization, during which membranes enclose each nucleus into a cell. Following cellularization, the embryo gastrulates. During gastrulation, cells are programmed to divide in an intricate spatiotemporal pattern [15] through transcription of *string* [16, 17]. Altering the timing of these divisions can disrupt morphogenesis [18]. For transcription to control String accumulation and mitosis during gastrulation, it seems necessary that spatially uniform, maternal *string* and *twine* mRNA have been degraded ([4], Figure 1) and that the MBT pattern of protein degradation be reversed.

To examine Cdc25 lifetimes under conditions that bypass endogenous mRNA regulation, we drove mRNA encoding the GFP and Dronpa forms of both String and Twine continuously during cellularization using both maternal and zygotic expression. Although mRNA levels remain high under these conditions (Figure S4), we found that the proteins are degraded with wild type kinetics at the MBT, but begins to re-accumulate about 50 minutes into cycle 14 (Figure 4A-B). Ultimately this uniform expression drives all ectodermal cells into mitosis, consistent with the observation that both proteins can drive an extra mitosis when reintroduced at cycle 14 [14, 19]. Using Twine-Dronpa, we estimated the half-life of Twine after it accumulates in interphase 14 (i.e. around the onset of gastrulation, coinciding with completion of cellularization and S-phase) and in the following interphase 15. We found that the protein no longer shows the instability characteristic of early cycle 14 and has a half-life (14±3 min, Figure 4C) more similar to that of the early cleavage cycles. String undergoes a similar change in stability, with a half-life now of 18±4 min (Figure 4D). These results

indicate that the high instability of String and Twine at the MBT are transient. Both proteins are again relatively stable following completion of cellularization and/or S-phase.

DISCUSSION

Inhibition of Cdk1 by phosphorylation results in the cell cycle pause at the MBT [5, 20, 21] and is driven by degradation of the activating phosphatases String and Twine. Here, we have shown that String and Twine are regulated differently during early embryonic development. We have also established that, contrary to earlier proposal suggesting that degradation of *string* and *twine* mRNA might control the cell cycle pause at the MBT [4], the levels of both proteins are mainly controlled through regulation of their stability.

String has a half-life of about 20 minutes at cycle 10, which decreases steadily with time so that at cycle 13 String half-life is of order of few minutes. String levels are controlled by the DNA replication checkpoints and by the N/C ratio, suggesting that such ratio is sensed during all phases of syncytial cycles and influences String degradation rate in a graded rather than a switch-like manner as observed for Twine (see below). Previous experiments had already indicated that the down-regulation of String begins prior to the MBT, is gradual and does not require zygotic gene expression [4]. Therefore, N/C ratio dependent control of String levels might not reflect an MBT–level control coordinating cell cycle and zygotic gene expression [22], but might arise from dose-dependent activation of Chk1 by the N/C ratio [23].

Differently from String, Twine is relatively stable (half-life ~20 minutes) until the beginning of cycle 14 when it is rapidly degraded. The pathways controlling Twine degradation respond to the N/C ratio. A similar switch in Twine stability is observed (although delayed) in the absence of DNA replication checkpoints. Given the insensitivity of Twine half-life to the activation of the checkpoints during cycles 11-13, we suggest that the delay may be caused by a lag in the onset of the MBT (zygotic transcription), and therefore not due to a direct effect of Chk1 and Chk2 on Twine stability. Furthermore, mutation of the three Chk1/Chk2 consensus sites (RxxS/T) in Twine has no effect on its dynamics (data not shown). We propose that a pathway targeting Twine for degradation by post-translational modification is activated at the MBT and terminates the early cell cycle program. Consistently, expression of a stabilized mutant Twine results in an extra mitosis prior to the MBT in a large fraction of embryos.

Degradation of mRNA is not an effective strategy to clear proteins and, unless the proteins were inherently unstable, might result in a relatively long lag between disappearance of the mRNA and the subsequent disappearance of the protein. Having all proteins that need to be degraded at the MBT be inherently unstable might be disadvantageous at other stages of development. We, therefore, speculate that one or few signaling pathways are activated at the MBT to degrade a specific subset of maternal proteins that would normally have longer lifetimes at other points in development. Elucidating the nature and regulation of such pathways will reveal central control mechanisms of embryonic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Drosophila Genomics Resource Center for plasmids and the Bloomington Drosophila Stock Center for fly stocks. We thank Oliver Grimm and Reba Samanta for help with experiments. We thank Jeff Farrell and Pat O'Farrell for discussion. This work was in part supported by the NIH (NICHD grant 5R37HD15587 to E.F.W., and

NRSA postdoctoral fellowship 1F32HD072653 to S.A.B.). S.D. was a Howard Hughes Medical Institute Fellow of the Life Sciences Research Foundation. E.F.W. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

1. O'Farrell PH, Stumpff J, Su TT. Embryonic cleavage cycles: how is a mouse like a fly? Curr Biol. 2004; 14:R35–45. [PubMed: 14711435]
2. Newport J, Kirschner M. A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. Cell. 1982; 30:687–696. [PubMed: 7139712]
3. Tadros W, Westwood JT, Lipshitz HD. The mother-to-child transition. Dev Cell. 2007; 12:847–849. [PubMed: 17543857]
4. Edgar BA, Datar SA. Zygotic degradation of two maternal Cdc25 mRNAs terminates Drosophila's early cell cycle program. Genes Dev. 1996; 10:1966–1977. [PubMed: 8756353]
5. Edgar BA, Sprenger F, Duronio RJ, Leopold P, O'Farrell PH. Distinct molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis. Genes Dev. 1994; 8:440–452. [PubMed: 7510257]
6. Edgar BA, Kiehle CP, Schubiger G. Cell cycle control by the nucleo-cytoplasmic ratio in early Drosophila development. Cell. 1986; 44:365–372. [PubMed: 3080248]
7. Newport J, Kirschner M. A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. Cell. 1982; 30:675–686. [PubMed: 6183003]
8. Busino L, Chiesa M, Draetta GF, Donzelli M. Cdc25A phosphatase: combinatorial phosphorylation, ubiquitylation and proteolysis. Oncogene. 2004; 23:2050–2056. [PubMed: 15021892]
9. Morgan, DO. The cell cycle : principles of control. New Science Press; London: 2007.
10. Sibon OC, Stevenson VA, Theurkauf WE. DNA-replication checkpoint control at the Drosophila midblastula transition. Nature. 1997; 388:93–97. [PubMed: 9214509]
11. Takada S, Kwak S, Koppetsch BS, Theurkauf WE. grp (chk1) replication-checkpoint mutations and DNA damage trigger a Chk2-dependent block at the Drosophila midblastula transition. Development. 2007; 134:1737–1744. [PubMed: 17409117]
12. Drocco JA, Grimm O, Tank DW, Wieschaus E. Measurement and perturbation of morphogen lifetime: effects on gradient shape. Biophys J. 2011; 101:1807–1815. [PubMed: 22004733]
13. Habuchi S, Ando R, Dedecker P, Verheijen W, Mizuno H, Miyawaki A, Hofkens J. Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. Proc Natl Acad Sci U S A. 2005; 102:9511–9516. [PubMed: 15972810]
14. Farrell JA, Shermoen AW, Yuan K, O'Farrell PH. Embryonic onset of late replication requires Cdc25 down-regulation. Genes Dev. 2012; 26:714–725. [PubMed: 22431511]
15. Foe VE. Mitotic domains reveal early commitment of cells in Drosophila embryos. Development. 1989; 107:1–22. [PubMed: 2516798]
16. Edgar BA, O'Farrell PH. Genetic control of cell division patterns in the Drosophila embryo. Cell. 1989; 57:177–187. [PubMed: 2702688]
17. Edgar BA, O'Farrell PH. The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. Cell. 1990; 62:469–480. [PubMed: 2199063]
18. Johnston LA. The trouble with tribbles. Curr Biol. 2000; 10:R502–504. [PubMed: 10898974]
19. Di Talia S, Wieschaus EF. Short-Term Integration of Cdc25 Dynamics Controls Mitotic Entry during Drosophila Gastrulation. Dev Cell. 2012; 22:763–774. [PubMed: 22483720]
20. Price D, Rabinovitch S, O'Farrell PH, Campbell SD. Drosophila wee1 has an essential role in the nuclear divisions of early embryogenesis. Genetics. 2000; 155:159–166. [PubMed: 10790391]
21. Stumpff J, Duncan T, Homola E, Campbell SD, Su TT. Drosophila Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis. Curr Biol. 2004; 14:2143–2148. [PubMed: 15589158]
22. Lu X, Li JM, Elemento O, Tavazoie S, Wieschaus EF. Coupling of zygotic transcription to mitotic control at the Drosophila mid-blastula transition. Development. 2009; 136:2101–2110. [PubMed: 19465600]
23. Conn CW, Lewellyn AL, Maller JL. The DNA damage checkpoint in embryonic cell cycles is dependent on the DNA-to-cytoplasmic ratio. Dev Cell. 2004; 7:275–281. [PubMed: 15296723]

24. Loppin B, Docquier M, Bonneton F, Couble P. The maternal effect mutation sesame affects the formation of the male pronucleus in Drosophila melanogaster. Dev Biol. 2000; 222:392–404. [PubMed: 10837127]

Highlights

- String and Twine are controlled by different post-translational mechanisms.
- DNA replication checkpoints control String, but not Twine, levels.
- Switch-like degradation of Twine at the MBT is required for the cell cycle pause.
- The instability of String and Twine is transient.

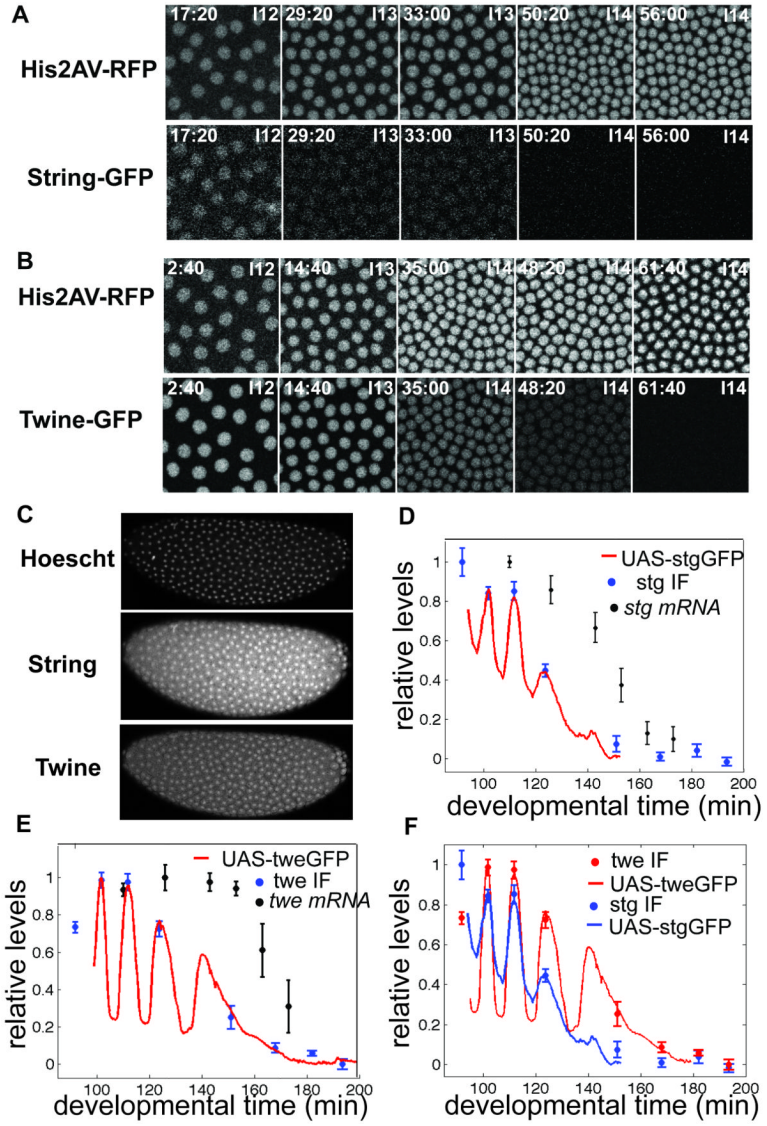

Figure 1. String and Twine undergo different down-regulations, which are not controlled by mRNA levels

Representative His2Av-RFP, String-GFP (**A**) and His2Av-RFP, Twine-GFP (**B**) images from time-lapse confocal microscopy. Time from the beginning of the movie as well as interphase number are reported in every panel. **C)** Images of an embryo used for immunofluorescence analysis. DNA was visualized by Hoechst staining, String using an anti-String rabbit antibody, Twine using an anti-Twine rat antibody. Comparison between the dynamics measured by live imaging, the dynamics inferred by immunofluorescence and the mRNA dynamics measured using q-PCR for String (**D**) and Twine (**E**). For both live imaging and immunofluorescence, we quantified the nuclear fluorescence intensity and normalized the data to the maximum value. **F)** Comparison between the protein dynamics of String and Twine. See also Figure S1 and Movies S1-S2.

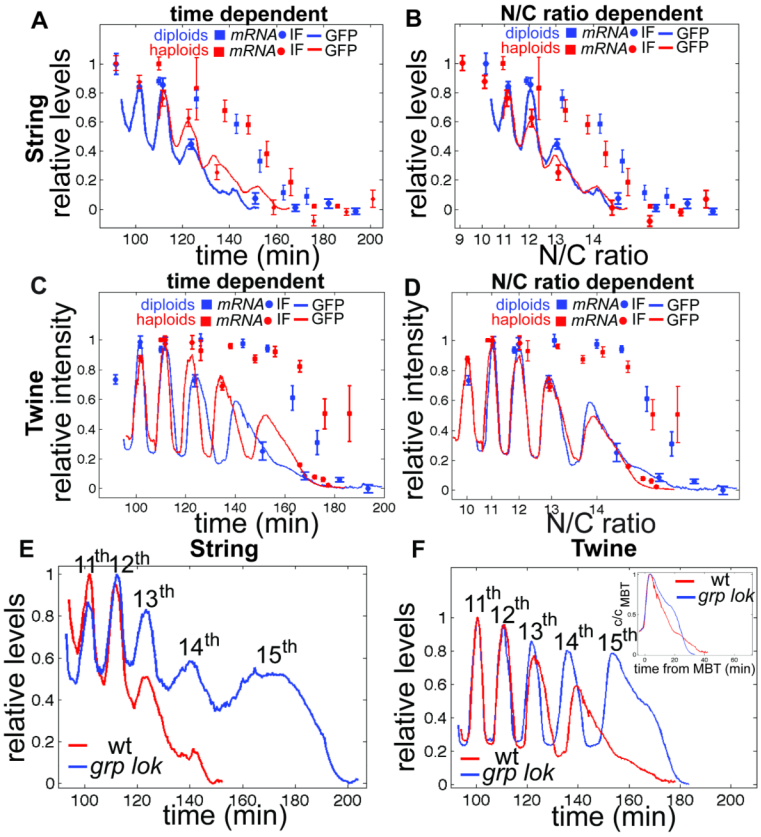

Figure 2. String and Twine levels are controlled by the N/C ratio but respond differently to the DNA replication checkpoints

String-GFP fluorescence intensity, endogenous String intensity measured with immunofluorescence and mRNA levels measured by q-PCR for diploid and haploid embryos (embryos laid by mothers mutant for the gene *sesame* (*ssm*) [24]) as a function of time (**A**) and the N/C ratio (**B**). Twine-GFP fluorescence intensity, endogenous Twine intensity measured with immunofluorescence and mRNA levels measured by q-PCR for diploid and haploid embryos as a function of time (**C**) and the N/C ratio (**D**). Comparison of the String-GFP (**E**) and Twine-GFP (**F**) dynamics in wt and *grp lok* embryos. The inset in (**F**) shows the dynamics of Twine-GFP in the last maternal cycle in wt (cycle 14) and *grp lok* (cycle 15). Fluorescence intensities were normalized to c_{MBT} defined as the maximum concentration in the last maternal cycle. See also Figure S2 and Movie S3.

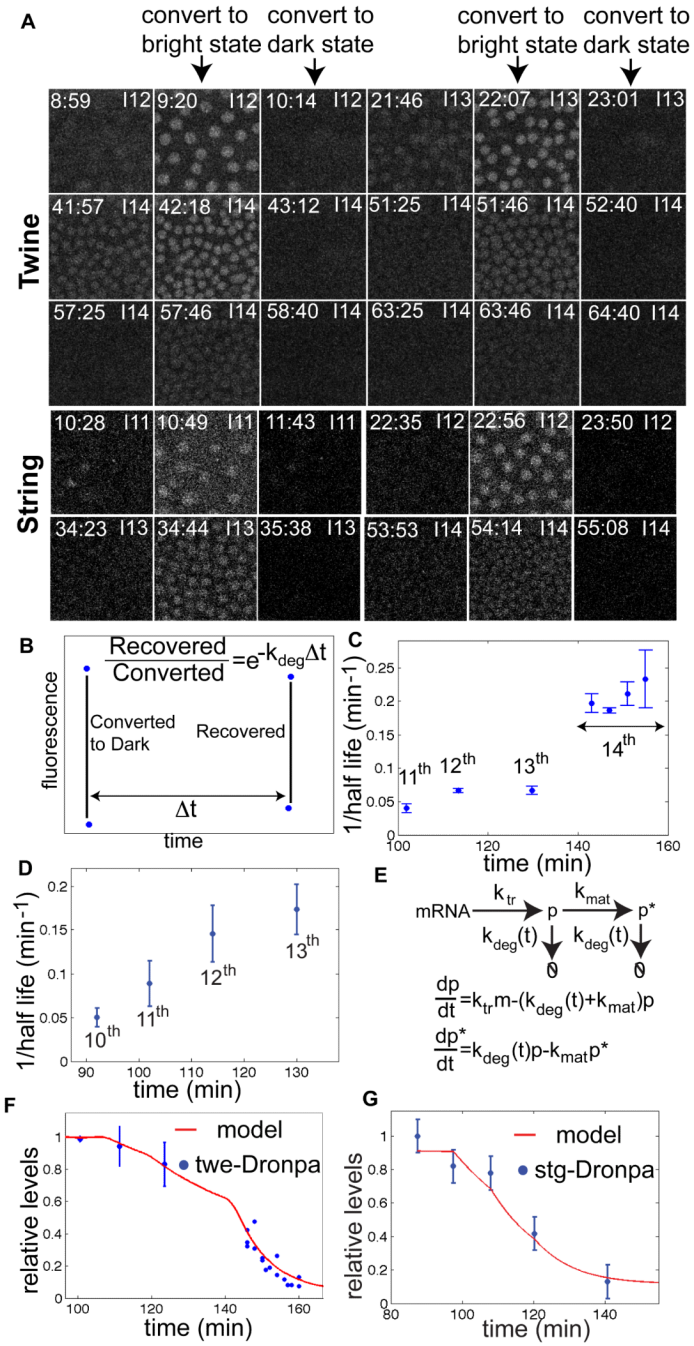

Figure 3. A post-translational switch controls Twine degradation at the onset of the MBT

A) Representative Twine-Dronpa and String-Dronpa images for the measurement of protein stability. Time from the beginning of the movie as well as interphase number are reported in every panel. **B)** Conceptual scheme for the measurement of protein degradation rate. Degradation rate of Twine (**C**) and String (**D**) as a function of inferred developmental time. **E)** The mass action reactions and equations describing Twine and String dynamics. String-Dronpa and Twine-Dronpa can exist in two states (an immature state p and a mature fluorescent state p*) with first order transition from the immature to the mature state (with rate k_{mat}). m indicates the amount of mRNA, k_{tr} the protein translation rate and $k_{deg}(t)$ the

time dependent degradation rate measured experimentally. Comparison between the measured fluorescence intensity and the numerical solution of the mass action kinetic equations for Twine (**F**) and String (**G**). See also Figure S3.

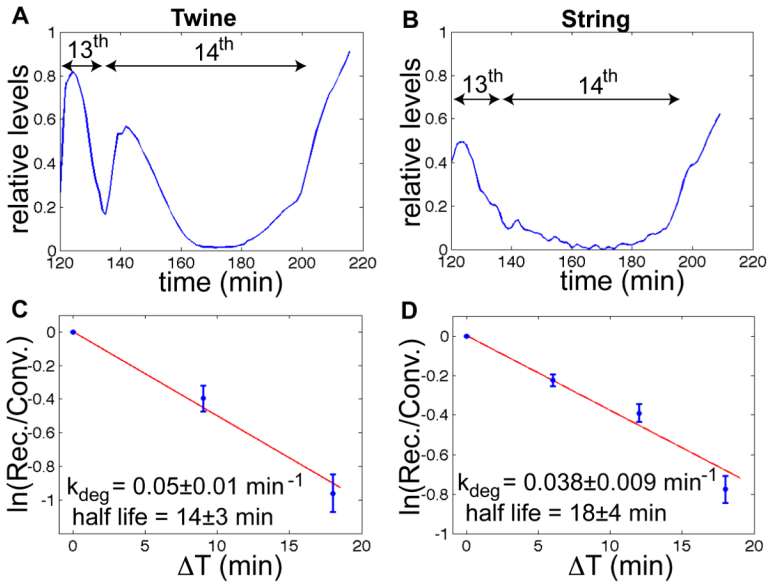

Figure 4. The signals targeting Twine and String for degradation are transient

A) Quantification of Twine dynamics in embryos expressing Twine-GFP maternally and zygotically. **B)** Quantification of Twine dynamics in embryos expressing String-GFP maternally and zygotically. Estimate of the degradation rate (and half-life) of Twine (**C**) and String (**D**) following their reaccumulation in interphase 14 and interphase 15. See also Figure S4 and Movie S4.