Supplement

Mechanism and Regulation of Twine (Cdc25) Protein Destruction in Embryonic Cell Cycle Remodeling

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Supplemental Data

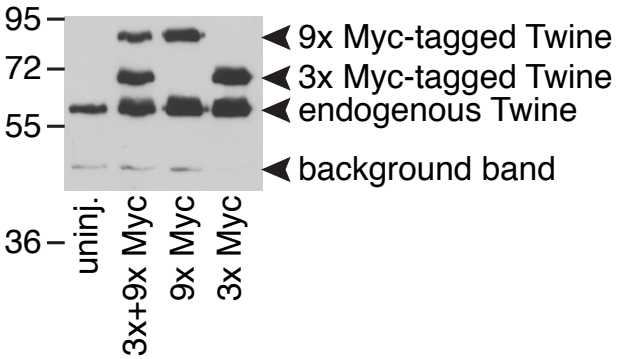

Figure S1, related to Figure 2. Characterizing the Twine antibody. Pools of embryos, either uninjected, or injected with mRNA encoding 3xMyc-tagged Twine or 9xMyc-tagged Twine were smashed and evaluated by Western blot. Arrows to the right of the blot label the suspected identity of the resultant bands, and the apparent weights from a pre-stained molecular weight marker are shown at left. There is only 1 documented or predicted isoform of Twine protein[34]. Twine antibody recognized two bands in extract from wild-type embryos: one with an apparent molecular weight of 47 kD and one with an apparent molecular weight of 59 kD, when compared to a pre-stained protein ladder (Fermentas PageRuler Plus). Injection of mRNA encoding 3xMyc- and 9xMyc-tagged Twine produced two additional bands, with apparent molecular weights of 68 and 84 kD. The 59 kD band's size is consistent with it being the untagged endogenous Twine, in comparison to the two bands produced by the mRNA encoding Myc-tagged Twine. We believe the 47 kD band is merely a background band that is sometimes, but not always, present in our experiments. Thus, we do not present it in the main figures.

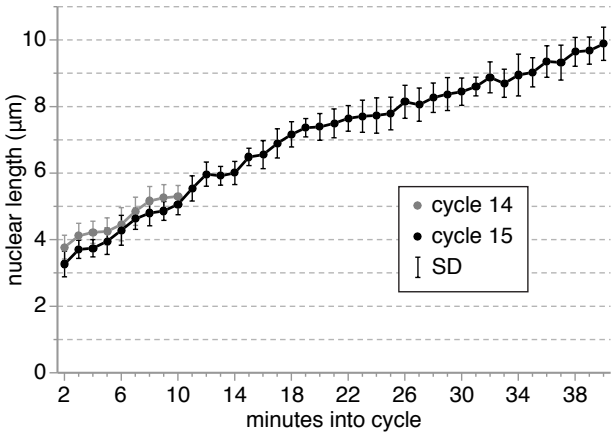

Figure S2, related to Figure 4C. Cellularization in *sesame* mutants. Nuclear length of *sesame* embryos injected with H2AvD-GFP mRNA during cycles 14 and 15, as cellularization attempts in cycle 14 and then succeeds in cycle 15. Five nuclei were measured at each timepoint from two embryos (for a total of 10 measured nuclei).

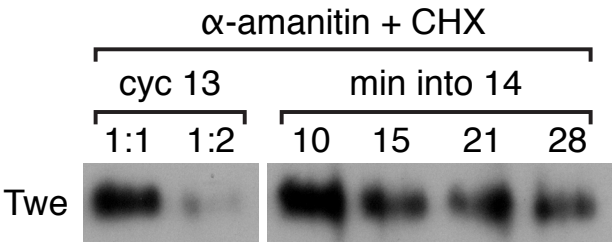

Figure S3, related to Figure 5D. Twine persistence after alpha-amanitin and cycloheximide. Embryos injected with alpha-amanitin and cycloheximide, either in cycle 13 (including a 1:2 dilution) and in cycle 14, timed visually on the scope.

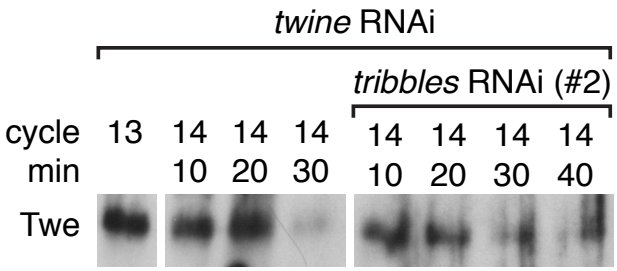

Figure S4, related to Figure 6F. Twine persistence after *tribbles* RNAi. Western blot, probed with anti-Twe, showing that in embryos treated with *twine* RNAi to prevent continued Twine translation, Twine persists longer after the MBT in embryos also treated with *tribbles* RNAi. (This uses a second, non-overlapping RNAi, compared to Figure 6F.) Lanes are labelled with cycle and time into cycle 14, determined visually on the scope.

Supplemental Experimental Procedures

Drosophila stocks. Most experiments were performed using embryos expressing Histone H2AvD-GFP [9]. Haploid embryos were obtained from *maternal haploid* mothers (y^1 w^a mh^1 / FM7a—Bloomington Stock 7130) [10] and *sesame* mothers (w ssm^{185b} / FM7c)[11]. Chk1-deficient embryos were obtained from *grapes* homozygous mothers (grp^{06034}, H2AvD-GFP / grp^{06034}, H2AvD-GFP—grp allele from Bloomington Stock 12219). Chk1, Chk2-deficient embryos were obtained from *grapes mnk* homozygous mothers (y^1 w^{1118} / w^{1118}; mnk^{P6} grp^{fs1} / mnk^{P6} grp^{fs1}) [22]. Embryos obtained from mothers homozygous for either *grapes* allele, or obtained from trans-heterozygous mothers (w^{1118} / +; mnk^{P6} grp^{fs1}/grp^{06034}) had similar phenotypes and did not hatch.

Preparation of Twine antibody. The Twine CDS was amplified by PCR from whole embryonic cDNA (0–3 hr) with Twe-pET28 primers (agctcat[catATG]GCGAGCAAG-CGGCTAATG and agctaata[gcggccgc]CTCGGCGTA-GAGCAGTCGTGAAC) and cloned into pET28c vector using the NdeI and NotI restriction sites, then the construct was transformed into BL21(DE3) cells. Two liters of culture were grown in 2xYT and induced with 1mM IPTG for 3 hours at 30°C to produce 6xHis-Twine, which was then purified using nickel agarose beads according to the manufacturer's instructions (Qiagen). Rabbits were then immunized using the purified protein and immune sera were obtained by Pacific Immunology. A purification column was packed with 6xHis-Twine protein coupled to cyanogen bromide-activated Sepharose beads (Invitrogen). The antibody was then purified by passing 7-fold diluted sera over the column, washing with PBS + 500mM NaCl, and eluting with 500uL of 100mM glycine pH 2.5 into 100uL of 1M Tris pH 7.0, which was then dialyzed into PBS.

Western blotting. Single embryos were prepared in two separate fashions, depending on whether they were staged by nuclear length (Figs. 2A and 5B) or time after mitosis 13 (Figs. 3A–E, 4A–B, 4D, 5A, 5C–D, 6D, 6F, S3, and S4). For those staged by nuclear length, collections of embryos were fixed in 1:1 methanol:heptane then rehydrated, and the DNA was stained using Pico Green (Molecular Probes). They were examined on a confocal microscope, and selected embryos were transferred to 2X SDS sample loading buffer (0.1 M Tris at pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, bromophenol blue) and boiled for 8 min. Embryos chosen by time after mitosis 13 were affixed to a coverslip and observed on an inverted confocal microscope. Selected embryos were gently released from the coverslip by applying gentle pressure under the embryo, perpendicular to the anterior–posterior axis, using the longest bristles of a trimmed paintbrush. Complete release of the embryo was confirmed by microscope. They were then fixed in small aliquots of 1:1 heptane:methanol, kept on ice, transferred to 2X SDS buffer, vortexed 2x8sec with glass beads, then boiled 8 min. For all samples, volumes were normalized and 1/2 of each sample was loaded on a 10% acrylamide gel, electrophoresed, and transferred to Immobilon-P membrane (Millipore). The blot was blocked for 2 hours with 3% milk, incubated with 1:1000 Twine antibody overnight in 3% milk at 4°C, incubated with 1:10000 Donkey anti-rabbit-HRP (Jackson Labs) for 1 hour at RT, washed 4 times with PBST, once with PBS, and then visualized with Super Signal West Femto ECL (Pierce) and Blue X-ray film (Phenix).

Immunofluorescence. Embryos were fixed in 3.7% formaldehyde for 20 minutes and then devitellinized by shaking in heptane:methanol (unless embryos had been injected, in which case they were devitellinized by hand with a tungsten needle). An exception was embryos used to visualize spindle enrichment which were fixed for 5 minutes in 1:1 heptane:methanol. Fixed embryos were cleared with xylene (5 min 1:1 xylene:methanol, 1 hr xylene, 5 min 1:1 xylene:methanol, 2x5 min methanol) then rehydrated slowly (5 min each 1:3, 1:1, 3:1 PTX:methanol, then 3x5 min PTX). They were blocked using 15% normal goat serum (Jackson Labs) for 2 hours and incubated with 1:500 anti-Twine in 15% normal goat serum overnight at 4°C. In Fig. 1C, tubulin was stained with a mixture of 1:50 each AA12.1, AA4.3, and E7 mouse monoclonals (DSHB). They were then stained for 15 minutes with 1:500 Pico Green (Invitrogen, Molecular Probes), washed 4x15min with PTX, then reblocked with 15% normal goat serum for 30 minutes. 1:1000 goat anti-Rabbit-Alexa-546 (Invitrogen, Molecular Probes) was added to this and incubated at RT for 2 hours. Embryos were then washed 5x20min in PTX, and mounted in Fluoromount-G. Slides were imaged on a spinning-disk confocal microscope as previously described [16] and visualized using Volocity 6 (Perkin Elmer).

Embryo Injection. Embryos were injected as previously described [8]. dsRNA were injected at concentrations of

approximately 1-2mg/ml in PBS. Cycloheximide (Sigma-Aldrich) was injected at 1mg/ml in 1% DMSO. Alpha-amanitin (Calbiochem) was injected at 1mg/ml. mRNAs were injected at 800ug/µl, unless otherwise noted. Mitotic cyclin dsRNA was produced as previously described[16]. *tribbles, tribbles #2*, *string*, and *twine* dsRNA were produced in the same manner, but using *tribbles* dsRNA primers ([gggcgggt]ATCAGCGCACAGCCTAGTCA and [gggcgggt]ATGGCCATAGATGGTGCTCC), *tribbles #2* dsRNA primers ([gggcgggt]CTTCCACATGTACCTGCCAGT and [gggcgggt]TGGAAGGCAGTGAGTTCTTGT), *string* dsRNA primers ([gggcgggt]GCCGAAAATTCTGCCAGCTATGGGA and [gggcgggt]TAGGCTTTGCTGAAGTCGCCGATT), or *twine* dsRNA primers ([gggcgggt]GCAACGACAGAAGTCAGCATACCCA and [gggcgggt]TGCTCTGTGTCCACACAACGTGGAA). mRNA was produced as previously described [8]. Template for *twine* mRNA was produced as previously described[8]. *tribbles* mRNA was produced from construct JFP2, which was made by amplifying the *tribbles* CDS with the Tribbles-SF449 primers (gtcaca[ccatgg]ATAACAGTAGCGGTCAAAACAGC and cgc[gaattc]CTTGGTGAGATCAAATTCCAATG) and cloning it into SF449 [35] by the NcoI and EcoRI sites.

Antibody Verification. pBS-XBG-Twe was constructed by amplifying the Twine CDS with the primers ataagaat[gcggccgc]GCGAGCAAGCGGCTAAT and gatcgg[actagt]CTCGGCGTAGAGCAGTC and cloning it into the pBS-XBG backbone[8] via the NotI and SpeI sites. pBS-XBG-Twe-3xMyc was constructed by amplifying the 3xMyc tag with the primers gatcgg[actagt]AGAGGTGAACAAAAGTTGATTTCT and aatcgg[ggtacc]CTAGACTCTAGATGATCCGTTCAA and cloning it into pBS-XBG-Twe by the SpeI and Acc65I sites. pBS-XBG-Twe-9xMyc was constructed by amplifying the 9xMyc tag with the primers gatcgg[actagt]TCTGCTGCTAGTGGTGAAC and aatcgg[ggtacc]TTAGCTAGTGGATCCGTTCAA and cloning it into pBS-XBG-Twe by the SpeI and Acc65I sites. The mRNAs were produced as described above.

Supplemental References

34. Flybase FlyBase Polypeptide Report: Dmel\twe-PA. flybase.org. Available at: http://flybase.org/reports/FBpp0080412.html [Accessed June 7, 2012].
35. Foley, E., O'Farrell, P. H., and Sprenger, F. (1999). Rux is a cyclin-dependent kinase inhibitor (CKI) specific for mitotic cyclin–Cdk complexes. Current Biology *9*, 1392–1402.