Supplemental Results

Expression of the SV40 early coding region in the early embryo

The 2.7kb SV40 early region encodes the LT, ST, and 17kT proteins through alternative splicing (Figure 1a) (Sullivan and Pipas, 2002).To express the early region of SV40 in early Drosophila embryos, we constructed transgenic flies that express this transcript under the control of the UAS-Gal4 system, a tool to control the spatial and temporal expression of ectopic proteins (Brand and Perrimon, 1993). Expression in this system can be modulated by temperature within the range permissive for Drosophila growth, 16-29°C, with highest levels achieved at 29°C. The SV40 early region transcript was expressed during oogenesis using a *nanos*-Gal4 driver (Van Doren *et al.*, 1998), in order to express early region proteins in the embryo maternally. To detect early viral proteins by Western blotting, we generated a polyclonal antibody in rabbits that recognizes ST, LT, and 17kT (Figure 1b and c, and unpublished data (S. Comerford and R. Hammer)). Surprisingly, ST but not LT or 17kT expression from the SV40 early region was detected in embryos with this antibody (Figure 1c) or with two commercial antibodies (pAB108 and pAB419, not shown). A similar construct, expressed in mouse liver, produced ST and LT (Comerford *et al.*, 2003) (Figure 1c). These results indicate that ST-encoding transcripts are the predominant mRNA species made in Drosophila embryos. This splicing bias was not simply due to genome position effect; since 3 independent lines showed exclusive expression of ST. ST expression in early embryos caused supernumerary centrosomes and lethality (Figures 1d and S1). In this report we focus on the effect of ST expression in early Drosophila development and the roles of PP2A subunits for its action.

Supplemental Discussion

ST-induced aneuploidy

A hallmark of most solid tumors is aneuploidy/chromosome instability (CIN), which is thought to be a causative factor in tumorigenesis (Rajagopalan and Lengauer, 2004). Expression of ST in embryos caused an increase in lagging chromosomes at anaphase and also the scattering and loss of chromosomes on the spindle apparatus. These phenotypes are consistent with perturbation of PP2A, since mutations in $PP2A^{B}$ (*tws*) cause chromosomes to lag at anaphase (Mayer-Jaekel et al., 1993), and RNAi of $PP2A^{B'-2}$ (*wdb*) causes chromosome scattering on the spindle (Chen *et al.*, 2007). Both of these chromosome segregation defects were observed in ST embryos and enhanced by PP2A subunit gene mutations. Moreover, the increase in cyclin E levels induced by ST may also contribute to the chromosome segregation defects we observed, since elevated cyclin E expression increases susceptibility for tumor formation, not only due to its ability to drive cell proliferation, but also to generate CIN (Loeb *et al.*, 2005; Spruck *et al.*, 1999). In addition, as discussed above, supernumerary centrosomes are also correlated to CIN and tumor progression (Brinkley, 2001; Fukasawa, 2007; Lingle *et al.*, 2002; Nigg, 2002; Pihan *et al.*, 2001). Thus, ST expression may contribute to CIN by causing chromosome segregation errors that are exacerbated by centrosome overduplication, either or both of which may be enhanced by increased cyclin E expression.

Supplemental Figure and Movie Legends

Figure S1. Embryonic lethality is correlated to ST-FLAG expression levels. Independent UASp-ST-FLAG transgenic lines showed variable lethality (**a**) that correlated directly with the levels of expression of ST-FLAG from each line (**b**). Embryonic lethality, measured as percent of embryos that fail to hatch, is similar between a line that expresses "native" ST from the SV40 early region construct, and a line that expresses higher levels of ST-FLAG. This indicates that the C-terminal 3xFLAG tag reduced the activity of ST, yet we achieved high enough expression to overcome this. Importantly, ST-FLAG expression results in the same phenotypes as the SV40 early region: centrosome amplification, aneuploidy, large microtubule asters, actin/cleavage furrow defects, and lethality (Figures 2a-d). The blot in (**b**) was probed with anti-ST antibody and anti-α-Tubulin as a loading control. All the lanes in (**b**) are from the same blot and same film exposure.

Whole embryo images of control and ST-FLAG embryos stained for α-Tubulin, CNN and DNA are shown in (**c**). Images of whole embryos at cycle 3, early syncytial blastoderm, and late syncytial blastoderm are shown with a higher magnification image shown below. Scale bars: 25 μm top rows, 10 μm bottom rows.

Figure S2. Cell cycle profiles of Kc cells following ST induction. Induction of vector or ST in stably-transfected Kc cell lines (as shown in Figure 2) showed no significant differences in the cell cycle stages at (**a, b**) 20 hours or (**c, d**) 42 hours. An example of a cell cycle profile for each sample is represented in (**a, c**) and the mean +/- SD of three profile measurements is represented in (**b, d**).

Figure S3. ST expression in Kc cells increases centrosome numbers without increasing chromosome numbers. Control and ST-expressing Kc cells, plus and minus induction with 1 mM $CuSO_4$ for 20 hrs, were stained with antibodies against CNN and Cid to label centrosomes and kinetochores, respectively. The results show that while centrosome numbers increase upon ST expression, the chromosome numbers do not increase. The scatter plot graphs in (**a**) show the centrosome and kinetochore counts for n = 50 cells. The horizontal bar in these graphs shows the number of kinetochores above and below 32 (the number expected for tetraploid Kc cells). The vertical line delineates 4+ centrosomes from <4 centrosomes. The numbers in each quadrant are the percent cells in each category. Note that approximately 30% of ST induced cells that are not polyploid have supernumerary centrosomes compared to 2-10% in the controls. The table in (**b**) shows the mean number of kinetochores for each sample shown in (**a**). Representative images of induced control and ST-expressing Kc cells stained for kinetochores and centrosomes are shown in (**c**). DNA is stained blue in the merged image.

Figure S4. Mutations in the two $PP2A^{B'}$ subunits, *wrd* (B'-1) and *wdb* (B'-2), affect their expression. Schematic diagrams of the *wrd* (**a**) and *wdb* (**b**) genes and associated mutations are shown. While both subunit genes are highly conserved, we raised antibodies against the variant C-terminal regions. The $wrd^{KG01108}$ and $wdb^{EP(3)3559}$ mutants were obtained from the Bloomington Drosophila stock center. The 1.9 kb deletion in *wdb* ($wdb^{12-1)}$was generated by mobilization of EP(3)3559. (**c**) The two $PP2A^{B'}$ mutations used in this study, $wrd^{KG01108}$ and wdb^{12-1} have reduced levels of expressed B'-1 and B'-2 proteins, respectively. The $wrd^{KG01108}$ sample is from a homozygous mutant ovary compared to a wild type (Ore-R) ovary. The wdb^{12-1} mutant sample is from third instar larvae transheterozygous with a deficiency (Df(3R)R38.3) that

deletes *wdb*, compared to wild type larvae. (**d**) RNAi knockdown of both gene products in Drosophila S2 cells confirms the specificity of the antibodies for each $PP2A^{B'}$ gene product. The band labeled "NS" in (**c** and **d**) is a non-specific protein recognized by the anti-$PP2A^{B'-1}$ antiserum and serves as a loading control in (**d**).

Movie S1. Cleavage cycle in a control embryo. An embryo expressing eGFP-CNN and His2Av-mRFP to label centrosomes (green) and chromosomes (red), respectively was imaged at syncytial blastoderm cleavage stage through one complete cleavage division. Images were captured at 23˚C at 3-second intervals and animated at 15 frames per second.

Movie S2. Cleavage cycles in an ST embryo. An ST-FLAG embryo expressing eGFP-CNN and His2Av-mRFP to label centrosomes (green) and chromosomes (red), respectively was imaged at syncytial blastoderm cleavage stage through one cleavage cycle and part of the following cycle. Images were captured at 23˚C at 3-second intervals and animated at 15 frames per second.

Supplemental Materials and Methods

Fly Stocks and crosses

Transgenic flies were made by standard procedures. The PP2A mutants: *mts*$^{xe\text{-}2258}$ (PP2A^{C}), PP2A-29B^{EP2332} (PP2A^{A}), *tws*^{02414} (PP2A^{B}), *wrd*^{KG01108} (PP2A$^{B'\text{-}1}$), *wdb*$^{EP(3)3559}$ (PP2A$^{B'\text{-}2}$) and Df(3R)R38.3 were obtained from the Bloomington Stock Center. *wdb*$^{12\text{-}1}$ (PP2A$^{B'\text{-}2}$) is a 1.9 kb deletion generated by mobilization of a P element located in the 5' region of *wdb* (EP(3)3559) (Figure S2). Expression of transgenes from pUASp vectors in embryos was achieved by crossing transgenic lines to nos-GAL4VP16, which expresses GAL4VP16 in ovaries from the *nanos* promoter. Females from the cross were mated to wild-type males and incubated at either 25˚C or 29˚C (to vary the level of expression).

Plasmids

The SV40 early region (genomic sequence from positions 5763-2533) was cloned into the P element vector pUASp. ST cDNA and the ΔPP2A (also called "mut3") clone (gifts from Estelle Sontag) were amplified by PCR and cloned into pENTR/D-TOPO. Entry clones were then recombined into a vector containing a C-terminal 3X-FLAG tag (pPWF) using LR-recombination with the Gateway system (Invitrogen). ST cDNA in pPWF was named pST-FLAG. ST^{D44N} and ST^{C103S} were generated through site-directed mutagenesis with the ST Entry clone using QuikChange (Stratagene). The ST-expressing plasmid used for U2OS transfections was pCMV5/Smt (from E. Sontag). For expression of GST-ST fusion protein in *E. coli*, the ΔPP2A clone (which encodes amino acids 1-110) was cloned into the pGEX2* vector. The *wrd* (B56-1) and *wdb* (B56-2) sequences corresponding to amino acid regions 543-656 and 425-524,

respectively, were cloned into pRSETB plasmid for expression in *E. coli* as 6XHis-tagged fusion proteins for antibody production in rabbits.

Cell culture, transfections, and flow cytometry

Kc167 cells were cultured in CCM3 (Hyclone) + Penicillin/Streptomycin medium. Stable cell lines were generated by co-transfection with pMT-GAL4 (Klueg *et al.*, 2002) and either vector (pPWF) or pST-FLAG, and pCoPuro for drug selection using Effectene reagent (Qiagen). Cells were selected in the above medium + 2 μg/ml Puromycin (Sigma). Expression of ST-FLAG from these cells is induced by addition of $CuSO_4$, which activates expression of GAL4 transcription factor from the metallothionein promoter in pMT-GAL4. U2OS cells were cultured in DMEM + 10% FBS + Penn/Strep. Transient transfections into U2OS cells were performed using Lipofectamine 2000 (Invitrogen). pCMV5/Smt was co-transfected with pGFP-Histone (a marker for transfected cells), or pGFP-Histone was transfected alone as a control. Cells were processed for immunostaining and Western blotting 46 hrs post-transfection. Cell cycle analysis was accomplished through DNA staining with propidium iodide (PI) and flow cytometry as described in *Current Protocols in Cytometry* (2007). In brief, cells were washed in PBS and fixed in 70% ice-cold ethanol. Ethanol was removed and the cells resuspended to a final concentration ~10^6 ml^{-1} in PBS + PI/RNaseA/Triton X-100 (20 μg ml^{-1}, 200 μg ml^{-1} and 0.1% v/v respectively). Fluorescence was measured (Becton Dickinson, FACScan) for 15,000 cells per sample, and the data analyzed with FlowJo 8.7 software (Tree Star Inc, Ashland, OR) using a Watson Pragmatic fitting algorithm.

Immunofluorescent Staining

Embryos were collected on apple juice/agar plates for 3 hours. They were then fixed with methanol/ heptane. For actin staining and optimal astral microtubule fixation, embryos were fixed 2-3 min with 37% formaldehyde and the vitelline membrane removed by hand. Embryos were blocked in PBT (PBS + 0.1% Tween-20) + 5 mg/ml BSA for 1 hour at room temperature, incubated with primary antibodies in PBT + 5 mg/ml BSA overnight at room temperature, and incubated with secondary antibodies in PBT + 1 mg/ml BSA for two hours at room temperature. After each antibody application, embryos were rinsed twice in PBT and washed two times with PBT for twenty minutes. Embryos were left to settle onto a PBT/90% glycerol cushion overnight at 4°C prior to mounting. Embryos were imaged on a Leica SP2 confocal microscope using a 63X /NA1.4 oil immersion objective. Kc cells were fixed and stained as described (Kao and Megraw, 2004). Human cells were seeded onto four-well slides and cultured overnight. Cells were fixed 46 hours post-transfection in methanol. After fixation, cells were blocked with 1% goat serum + 5 mg/ml BSA + 0.1% saponin for thirty minutes. Cells were then stained as described for Kc cells. U2OS and Kc cells were imaged on a Zeiss axioskop using a 63X /NA1.4 oil immersion objective and a Coolsnap FX CCD camera with Metamorph software.

Embryo Hatching

Embryos were collected on apple juice/agar plates, and then lined up on fresh apple juice/agar plates. The plates were kept at room temperature for two days and were then counted under a dissecting scope to score hatching.

Immunoprecipitation

Embryos were collected for 3 hours on apple juice/agar plates. After chorion removal with 50% bleach, embryos were washed with extraction buffer (EB; 50 mM Tris pH 8.0, 150 mM NaCl). Embryos were then homogenized in approximately two volumes of EB + 1X protease inhibitor cocktail (Sigma). The extract was centrifuged for 15 minutes at 16,000xg at 4˚C and the supernatant was transferred to a new tube. Approximately one-tenth volume of 10% NP-40 was added to the extract and the extract was centrifuged for 10 minutes as above and the supernatant again transferred to a new tube. The extracts were then snap frozen in liquid nitrogen and stored at -80°C. Upon thawing, extracts were again centrifuged for 10 minutes as above. Colcemid (10 µM) was added to embryo extracts that were then incubated with anti-FLAG agarose (Sigma) pre-equilibrated with wash buffer (WB; 50 mM Tris pH 7.4, 150 mM NaCl) for two hours at 4°C. Following centrifugation, the pellet was washed five times over the course of 1 hr with WB + 0.5% Triton X-100 at 4°C, then twice more in WB. Protein was eluted with 20 µl 2X SDS-PAGE loading dye supplemented with fresh 2-mercaptoethanol (0.4 µM) for 5 minutes at 95°C. All centrifugations were 10,600xg for 5 seconds, unless otherwise indicated. The $PP2A^{C}$ IP was performed similarly as the FLAG IP, except 1µg of $PP2A^{C}$ monoclonal 1D6 was incubated with embryo extract + 10 µM Colcemid for 1 hour at 4°C and then the antibody + extract was added to 20µl pre-equilibrated Protein A sepharose (Amersham Biosciences). The Protein A sepharose was washed and eluted as above for FLAG IP.

Live-Imaging

Movies were captured as described in (Zhang and Megraw, 2007). The HandleExtraFileTypes.class plugin in Image J was used to convert the .avi files generated from

the Leica software into stacks, which were then converted to Quicktime movies at 15 frames per second with a Sorenson 3 Normal compression.

Supplemental References

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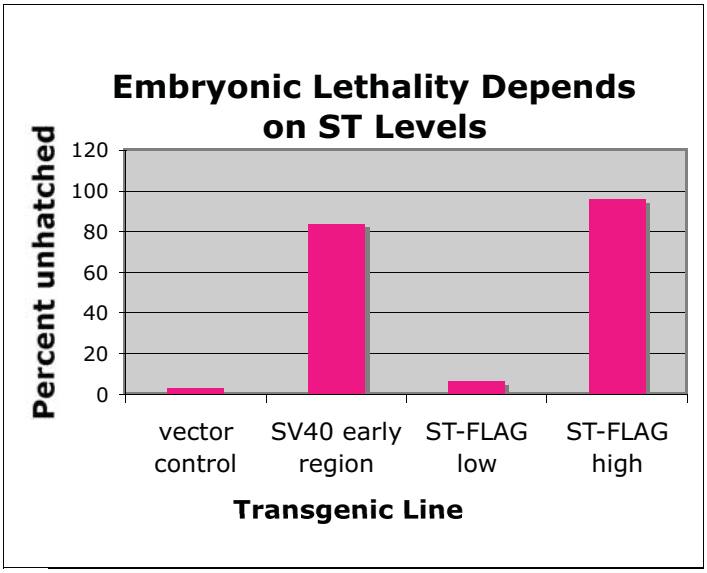

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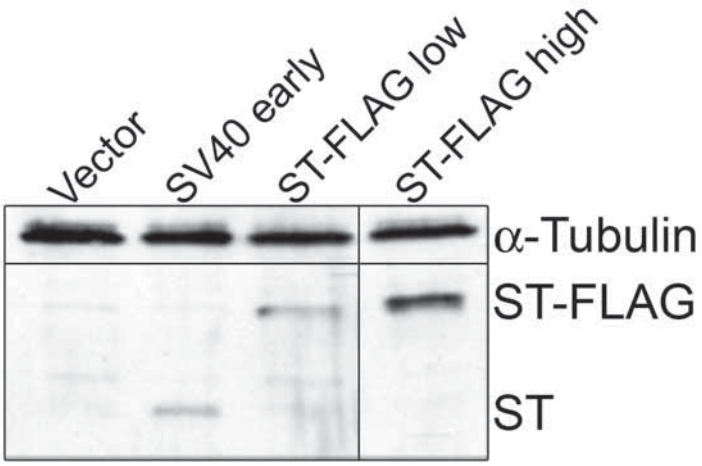

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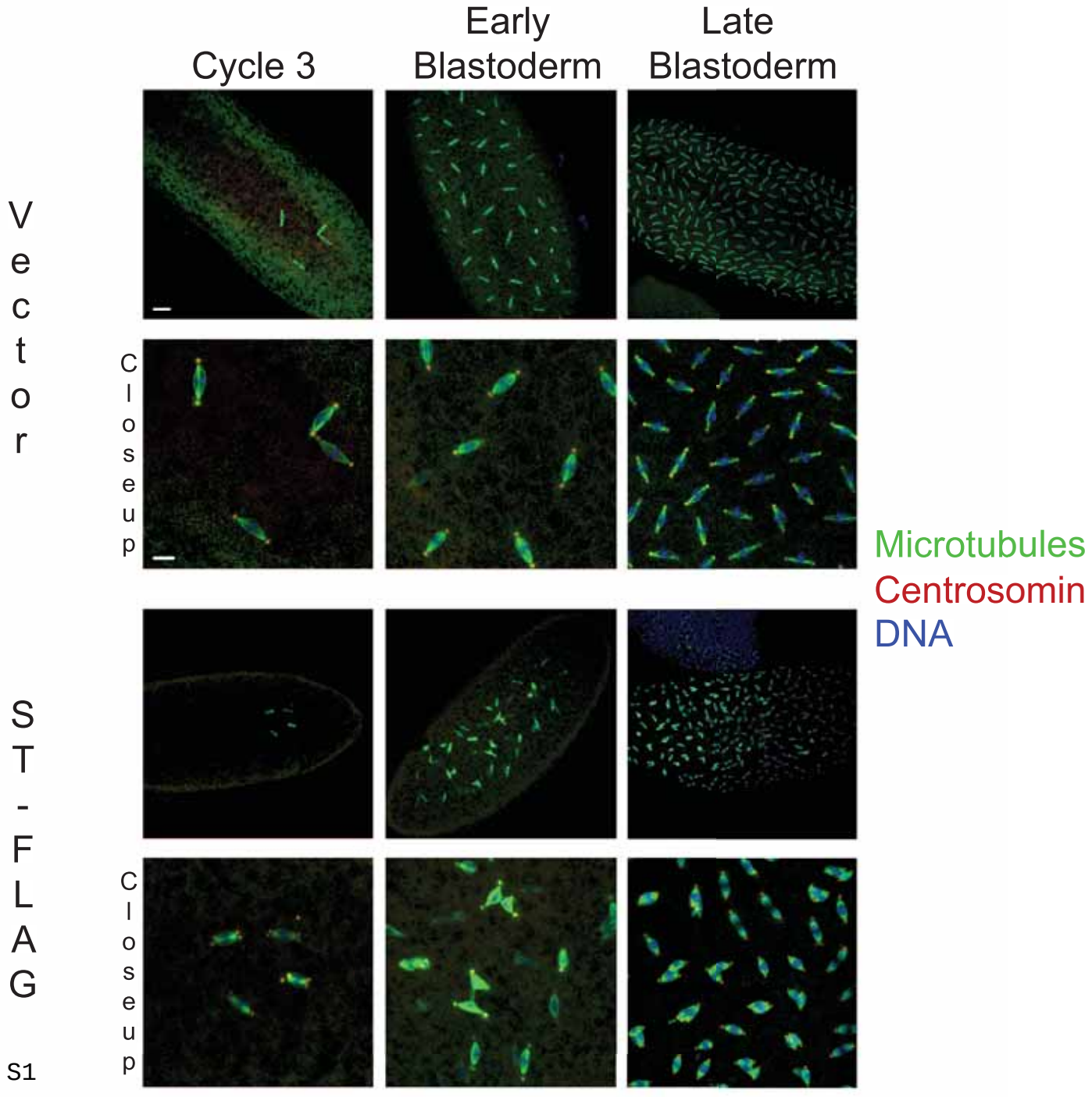
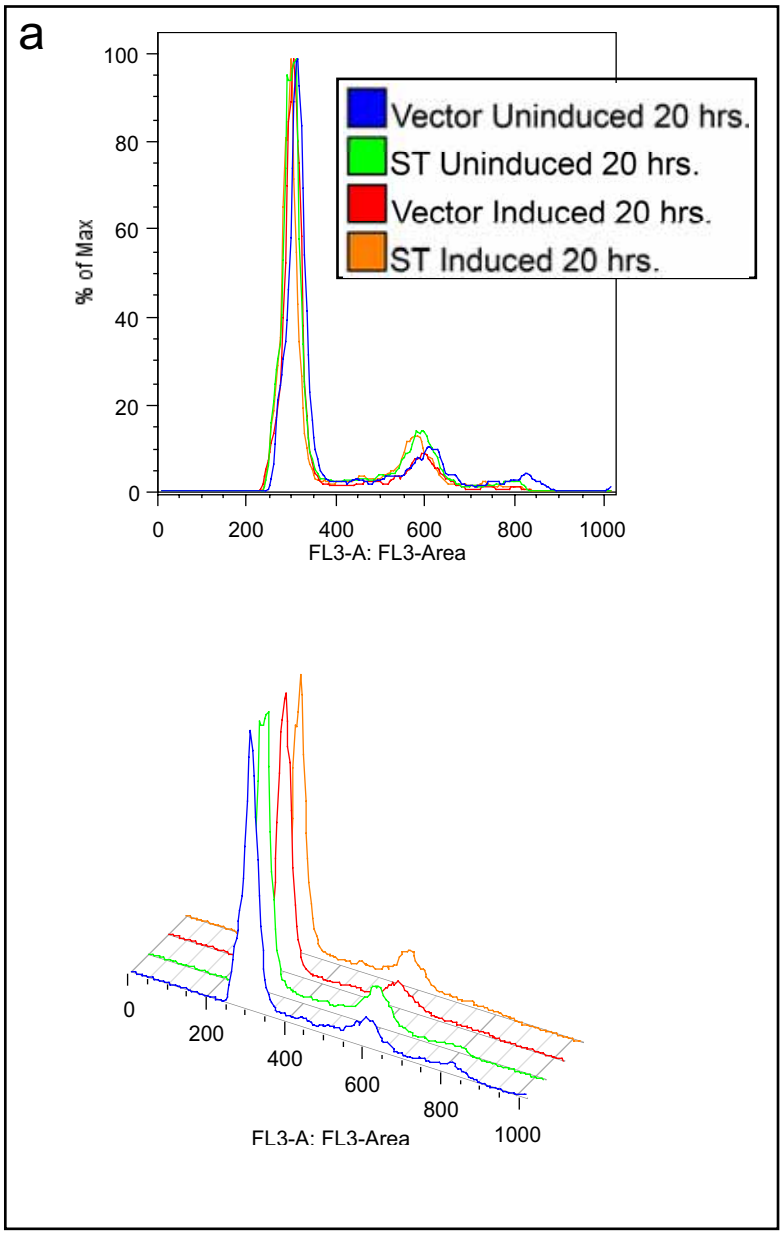


Figure S1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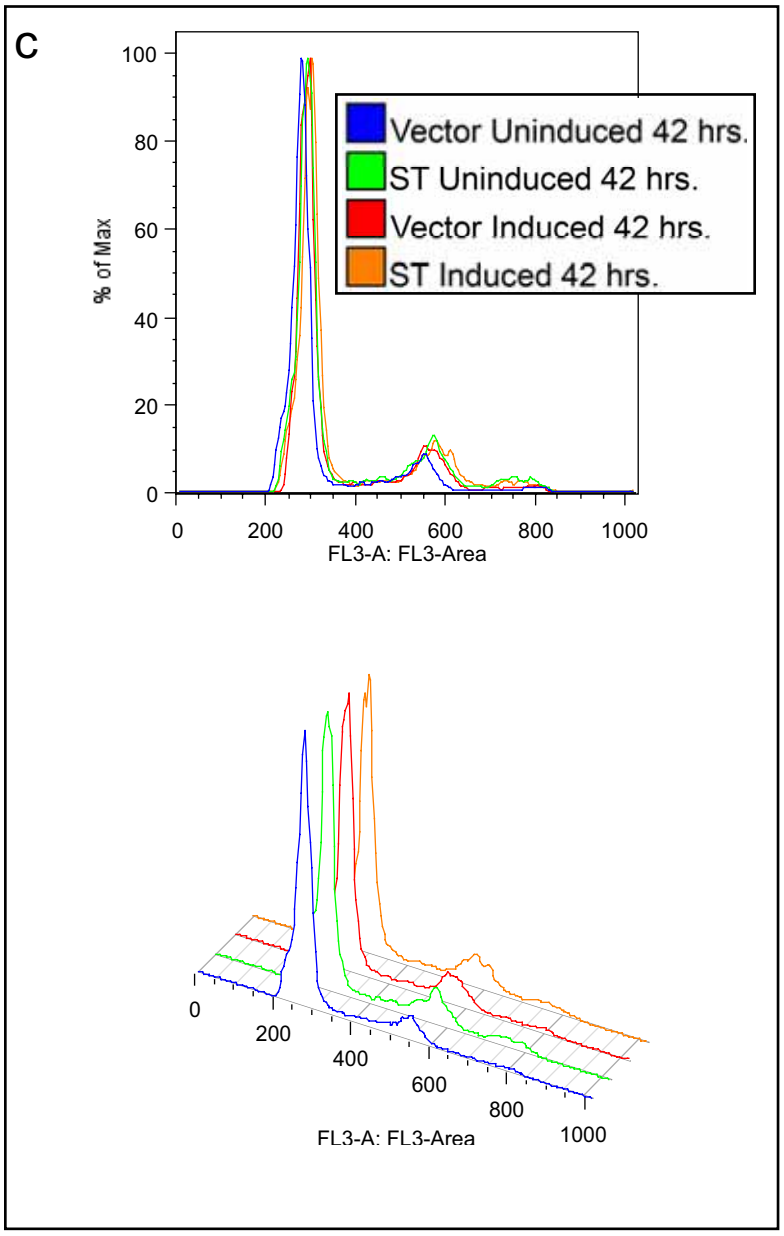
ST Uninduced 20 hrs.
Vector Induced 20 hrs.
ST Induced 20 hrs.
% of Max
FL3-A: FL3-Area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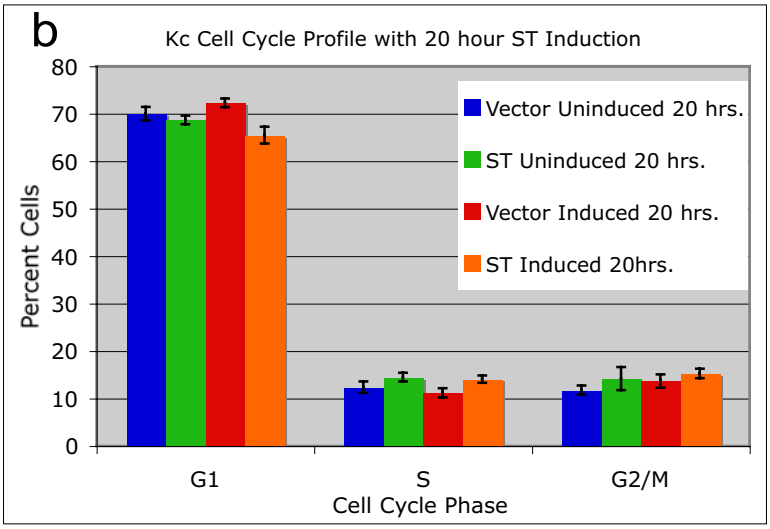
ST Induced 42 hrs.
% of Max
FL3-A: FL3-Area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Kc Cell Cycle Profile with 20 hour ST Induction
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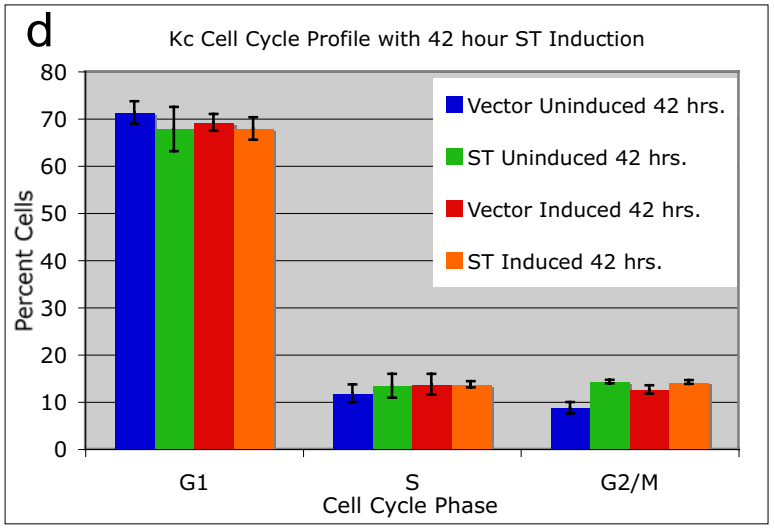
ST Uninduced 20 hrs.
Vector Induced 20 hrs.
ST Induced 20hrs.
Percent Cells
G1
S
G2/M
Cell Cycle Phase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Kc Cell Cycle Profile with 42 hour ST Induction
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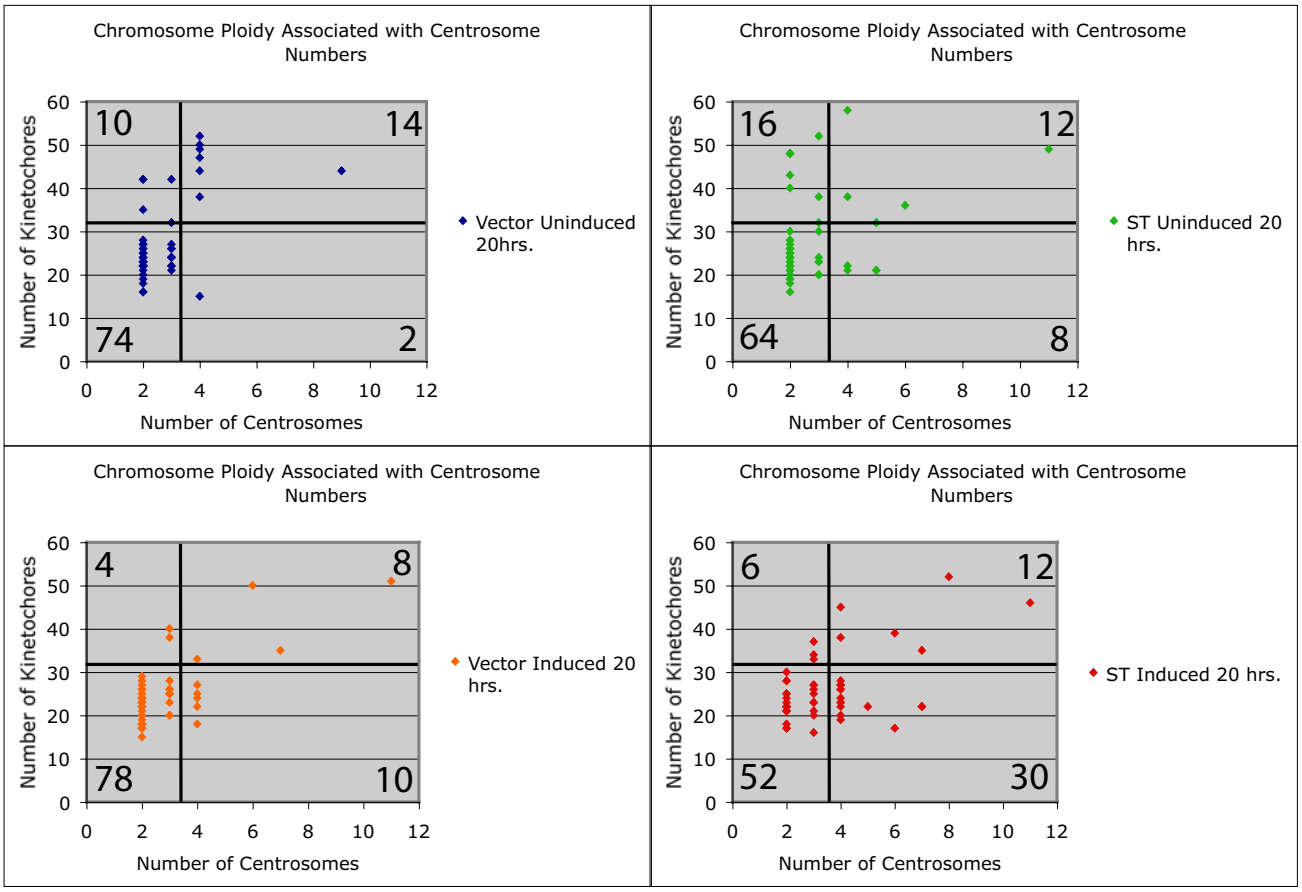
ST Induced 42 hrs.
Percent Cells
G1
S
G2/M
Cell Cycle Phase

Figure S2

a



b

Sample:	Vector Uninduced 20 hrs.	ST Uninduced 20 hrs.	Vector Induced 20 hrs.	ST Induced 20 hrs.
Mean Number of Kinetochores	27.8	28.48	24.78	26.02

c

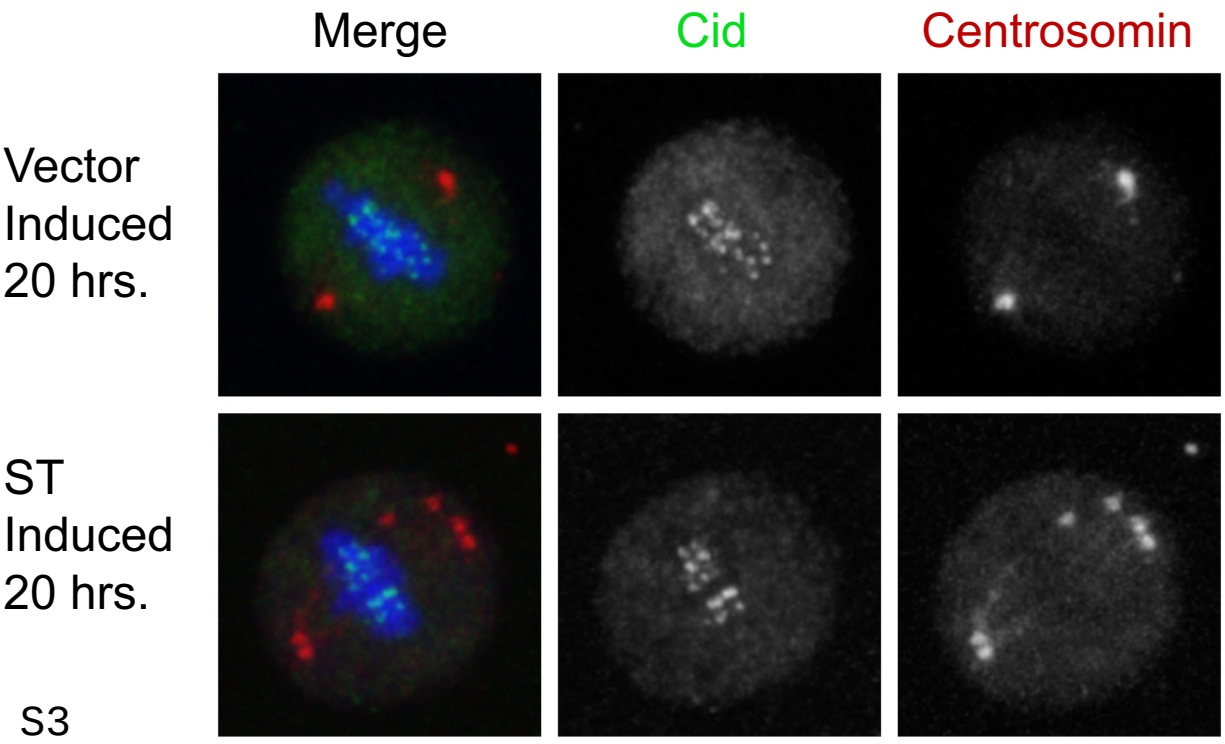
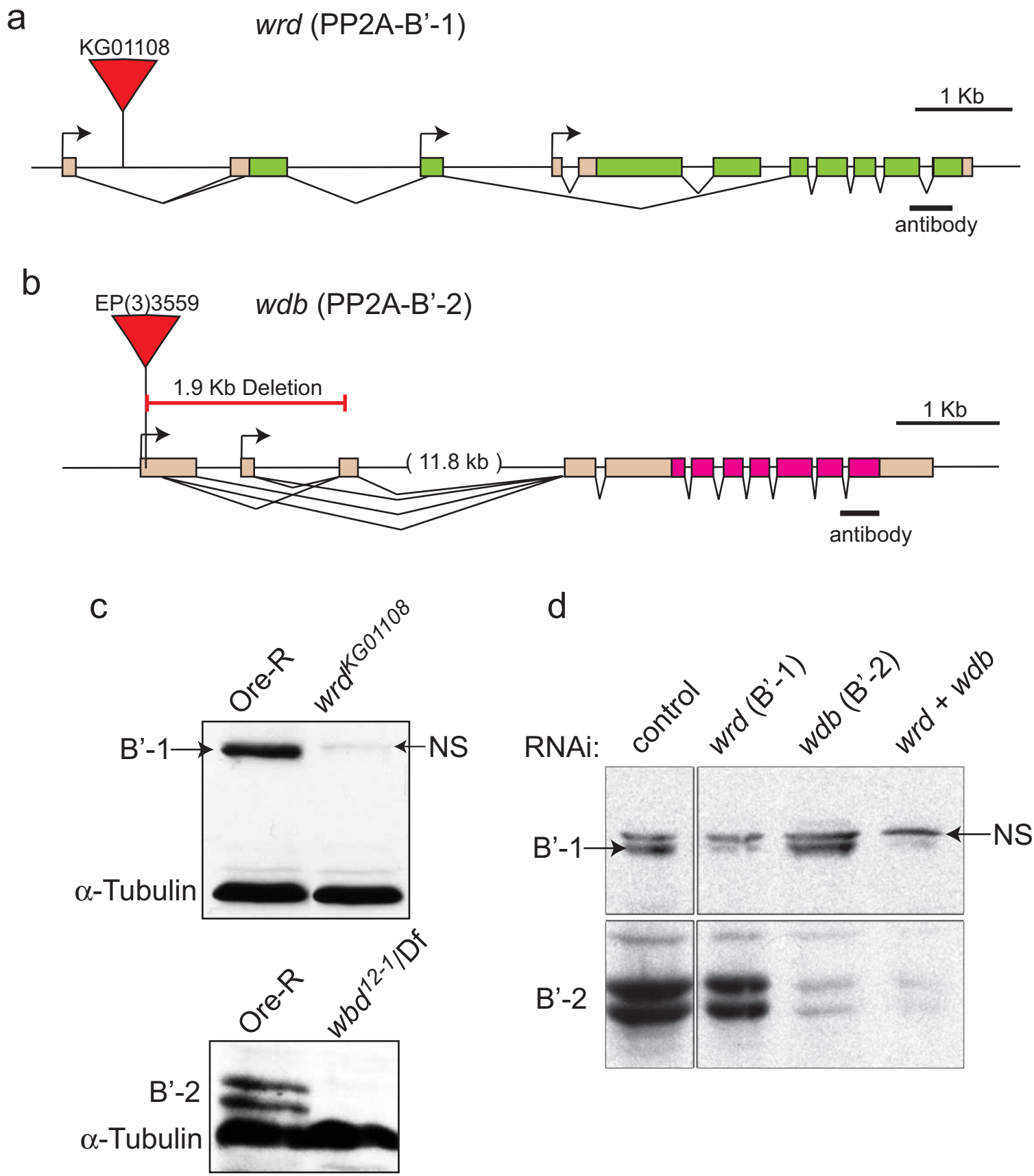


Figure S3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(11.8 kb)
antibody
c
Ore-R
wrdKG01108
B'-1
NS
α-Tubulin
Ore-R
wbd12-1/Df
B'-2
α-Tubulin
d
RNAi:
control
wrd (B'-1)
wdb (B'-2)
wrd + wdb
B'-1
NS
B'-2

Figure S4