Mast, a conserved microtubule-associated protein required for bipolar mitotic spindle organisation

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Running title: Multiple asters, a new microtubule-associated protein

Abstract

Through mutational analysis in *Drosophila,* we have identified the gene *multiple asters* (*mast*), that encodes a new 165 kDa protein. *mast* mutant neuroblasts are highly polyploid and show severe mitotic abnormalities including the formation of mono- and multi-polar spindles organised by an irregular number of MTOCs of abnormal size and shape. The *mast* gene product is evolutionary conserved since homologues were identified from yeast to man, revealing a novel protein family. Antibodies against Mast and analysis of tissue culture cells expressing an EGFP-Mast fusion protein show that during mitosis this protein localises to centrosomes, the mitotic spindle, centromeres and spindle midzone. Microtubule-binding assays indicate that Mast is a microtubule-associated protein displaying strong affinity for polymerised microtubules. The defects observed in the mutant alleles and the intracellular localisation of the protein suggest that Mast plays an essential role in centrosome separation and organisation of the bipolar mitotic spindle.

Keywords: centrosome/MAPs/microtubules/mitosis/Drosophila

Introduction

The mitotic spindle is a specialised structure required during mitosis for chromosome segregation, made up of microtubules (MTs) organised from microtubule organising centres (MTOCs) that in most animal cells correspond to centrosomes (reviewed in Zimmerman *et al*., 1999).

Assembly of the mitotic spindle starts during late G2, after centrosome replication. This transition is marked by a dramatic change in MT behaviour thought to be triggered by activation of MPF (Verde *et al*., 1992). At this stage, centrosomes begin to nucleate more and highly dynamic MTs forming well defined asters (Saxton *et al*., 1984) that are involved in centrosome separation and migration to opposite poles of the cell (Saunders *et al*., 1992). It is now thought that formation and maintenance of a bipolar spindle involves at least three families of molecular motors. These include the bipolar kinesins, C-terminal kinesins and cytoplasmic dynein (Sharp *et al.*, 1999; Sharp *et al*., 2000; Robinson *et al.,* 1999).

Recent studies on a new family of non-motor Microtubule Associated Proteins (MAPs), the dis1-TOG family, have suggested that these proteins may also play important roles in the organisation of the bipolar spindle. This family includes the human ch-TOG (Charrasse *et al*., 1998), *Xenopus* XMAP215 (Vasquez *et al*., 1994; Tournebize *et al*., 2000), *D. melanogaster* Msps (Cullen *et al*., 1999), *C. elegans* ZYG-9 (Kemphues *et al*., 1986, Matthews *et al*., 1998), *S. pombe* $p93^{dis1}$(Nabeshima *et al*., 1995), *S. cerevisiae* Stu2p (Wang and Huffaker, 1997) and *D. discoideum* DdCP224 (Gräf *et al*., 2000) proteins. These MAPs localise to either the centrosome/spindle pole body, spindle MTs, or both during mitosis or meiosis and have been implicated in the control of microtubule dynamics, stability of the mitotic apparatus, duplication of the centrosome and cytokinesis. Although all members of the dis1-TOG family have been shown to bind microtubules, the MT binding domain has only been determined for Stu2p, $p93^{dis1}$ and ch-TOG and this region falls outside the conserved

domains (Wang and Huffaker, 1997; Nakaseko *et al.*, 1996; Charrasse *et al*., 1998). The MT binding domain of ch-TOG is also found in other MAPs including tau2, MAP4 and MAP2b (Charrasse *et al*., 1998).

Here we report the identification and characterisation of a new *Drosophila* gene that we have named *multiple asters* (*mast*). Mutations in *mast* cause abnormal chromosome segregation associated with irregular centrosome separation and severely disrupted spindles. We show that the gene encodes a conserved 165 kDa MAP, defining a new conserved family of proteins that is related to the dis1-TOG family. We also show that the *mast* gene product localises to centrosomes, interphase and spindle MTs, centromeres and the spindle midzone. Our data suggests that Mast is required for centrosome segregation and organisation of the bipolar spindle.

Results

Identification and characterisation of the multiple asters (mast) mutations

The first *multiple asters* (*mast*^{P1}) mutant allele was identified by Omel'yanchuk and colleagues (Omel'yanchuk *et al*., 1997). Subsequently, we identified two other P-element induced alleles, *mast*^{P2} and *mast*^{P3}, from the Berkeley *Drosophila* Genome Project (BDGP) database. A fourth allele, *mast*^{P4}, an imprecise excision allele, was obtained after remobilization of the P-element in *mast*^{P1}.

mast^{P1} and *mast*^{P4} cause late larval/pupal lethality when homozygous or hemizygous over Df(3L)31A. *mast*^{P3} causes early embryonic lethality of homozygous individuals, suggesting the presence of a second mutation, since *mast*^{P3}/Df(3L)31A die during late larval/pupal stages. *mast*^{P2} allele is semi-lethal and viable adult homozygous, hemizygous or heterozygous over the other alleles are obtained. These adults are sterile, moreover testis and ovaries of *mast*^{P2} and *mast*^{P2}/Df(3L)31A adults are rudimentary.

Neuroblasts of homozygous or hemizygous larvae carrying *mast* mutant alleles show severe mitotic abnormalities (Fig. 1), including highly condensed chromosomes (Fig. 1B-F, J and K) that are frequently organised in circular arrangements (Fig. 1B and C), very few and irregular anaphases (Fig. 1H and I) and highly polyploid cells (Fig. 1C-F, J and K). Quantification of mitotic parameters shows that $mast^{P1}$ and $mast^{P2}$ do not cause a significant increase in the mitotic index. However, $mast^{P3}$/Df(3L)31A shows an elevated mitotic index and $mast^{P4}$ a severe mitotic arrest (Fig. 1L). Quantification of mitotic figures with respect to mitotic progression indicates that all mutant alleles cause a decrease in the number of cells in prophase, a significant increase of cells in prometaphase/metaphase and a decrease in the proportion of cells in anaphase or telophase (Fig. 1M). Quantification of the different types of mitotic abnormalities suggests that most alleles cause either a severe increase in the proportion of polyploid cells, metaphases with a circular chromosome configuration or abnormal anaphases (Fig. 1N). The effects on viability, mitotic phenotype and mitotic progression allowed us to order the alleles from least affected to very severe according to the following series: $mast^{P2} < mast^{P1} < mast^{P3} < mast^{P4}$. Taken together, these results indicate that mutations in *mast* cause severe abnormalities in chromosome segregation leading cells to arrest at prometaphase/metaphase. However, the arrest can be overcome and cells undergo multiple rounds of proliferation since most of them are polyploid.

Molecular cloning of the mast gene

In order to identify the mutated gene, we characterised the locus at the molecular level. We mapped by *in situ* hybridisation a single P-element insertion in the $mast^{P1}$ allele to the 78C1-C2 cytological region (data not shown) and cloned both sides of the insertion by plasmid rescue and inverse PCR. DNA sequence analysis with the BDGP databases indicated that it was a new gene and identified a number of ESTs that had already been partially

sequenced. We fully sequenced the largest cDNA (LD11488) of 5938 bp (these sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF250842). DNA sequence comparisons between the LD11488 cDNA and the corresponding genomic sequence, as well as, sequence from cDNAs isolated from adult heads and larva/early pupa, allowed us to determine the intron-exon organisation of the gene (Fig. 2A). The results indicate that there are at least two types of transcripts, a 5.2 kb transcript, present in cDNA libraries from adult heads and larva/early pupa, and a second transcript of 5.9 kb present in cDNA libraries prepared from embryos. Since *in situ* hybridisation to polytene chromosomes with the larger cDNA hybridises to a single site (data not shown), the simplest interpretation is that the two transcripts are produced by tissue-specific alternative splicing. The cDNA sequence contains a single ORF of 1491 amino acids, coding for a protein with a predicted molecular mass of 165.5 kDa and a pI of 9.17 (Fig. 2B).

We also characterised the molecular lesions in other *mast* alleles. The $mast^{P1}$ and $mast^{P3}$ alleles were obtained in different screens, however, they carry a P insertion at the same genomic position, 2317 bp upstream the predicted ATG. The P-element in $mast^{P2}$ is inserted 1679 bp from the ATG. The fourth allele, $mast^{P4}$, was obtained after remobilization of the P-element in $mast^{P1}$. Southern analysis of $mast^{P4}$ shows that it has lost part of the P element (including the ry^{+} gene). However, all genomic restriction fragments upstream from the insertion are unchanged, and it has a deletion of up to 1 kb of genomic sequence downstream from the insertion site (Fig. 2A). Furthermore, western analysis shows that a residual amount of full size protein is expressed in $mast^{P4}$ homozygous, confirming that the coding region of *mast* is not affected (see Fig. 2D). As a result of the remobilization of the insertion in $mast^{P1}$, we also obtained 39 independent lines that had lost the insertion, were viable and show normal mitotic parameters. These results, together with the genetic complementation data,

suggest that the late larval lethality and mitotic phenotypes are the result of mutations in the locus *mast*.

Analysis of the protein sequence shows that Mast contains a 170 amino acid domain that shares limited homology with the proline-rich domain of MAP4 (Fig. 2B), that is thought to be involved in the high efficiency binding to MTs (Aizawa *et al*., 1991). It also contains two regions with significant homology to the HEAT repeat (Andrade and Bork, 1995), at positions 169-207 and 1414-1452 (Fig. 2B). This motif was identified in HUNTINGTIN, a protein associated to Huntington's disease and is also present in the 65 kDa regulatory subunit of PP2A (Hemmings *et al*., 1990) and in proteins of the dis1-TOG family (Tournebize *et al*., 2000). The Mast protein also contains two cyclin dependent kinase $p34^{cdc2}$consensus phosphorylation sites (Kennelly and Krebs, 1991) (Fig. 2B).

Expression of the mast gene in wild type and mutant tissues

In order to analyse the expression of the *mast* gene, we raised antibodies (Rb726) against a fragment of the Mast protein. The immunopurified antibody (IP726α) recognises the bacterially expressed protein and, in extracts from embryos, larvae and adult tissues, a large protein of 165 kDa (Fig. 2C). The protein is present during early embryogenesis, but it is highly expressed during late embryogenesis, in larval brains and ovaries, and significantly reduced in testis. In early embryos we also find a slightly larger protein that is specifically recognised by IP726α. We have also analysed the level of the Mast protein in homozygous mutant individuals and found that it is reduced in both $mast^{P1}$ and $mast^{P2}$ and barely detectable in $mast^{P4}$ when compared to the wild type control (Fig. 2D).

Evolutionary conservation of Mast

In order to determine whether the Mast protein is evolutionary conserved we performed searches against current databases. Mast shares significant identity with proteins encoded by two human cDNAs (KIAA0622 and KIAA0627; Ishikawa *et al*., 1998), three putative proteins in *C. elegans* (C07H6.3, R107.6 and ZC84.3; Wilson *et al*., 1994) and also limited identity with Stu1p from *S. cerevisiae* (Pasqualone *et al*., 1994) and its putative homologue in *S. pombe*, that we have called SpStu1p. Multiple alignment of the Mast sequence with those most closely related from other species (Fig. 3A) shows that all the proteins share identity throughout their sequence, however three regions (CR-1, CR-2 and CR-3) are more highly conserved (Fig. 3A and B). These results suggest that Mast and its homologues define a new evolutionary conserved protein family, that we have named Stu1-Mast.

Database searches also indicate that Mast shares identity to proteins from the dis1-TOG family, specially at the N-terminal half of the protein (amino acids 1-494), where they are 20-25% identical and 40-45% similar. Inside this region, there is a small domain of 18 amino acid residues that is highly conserved among these proteins (fig. 3B). Phylogenetic analysis including all sequences from the two groups suggests that they are evolutionarily close, but distinct, since they are positioned in different branches of the dendrogram (Fig. 3C).

Localisation of Mast during the cell cycle

To determine the intracellular localisation of Mast during different stages of the cell cycle we used IP726α for indirect immunofluorescence in S2 *Drosophila* tissue culture cells (Fig. 4). At interphase, Mast is focused on MTOCs and shows a punctuate pattern co-localising with α-tubulin. At prophase, Mast accumulates at the MTOCs, as shown by α-tubulin co-staining (Fig. 4). Double immunostaining with either anti-Centrosomin (CNN) or γ-tubulin antibodies also shows co-localisation with Mast (data not shown). In some cells, Mast also associates

with a rod-like structure present in interphase or mitotic cells (Fig. 4). This structure is stained by IP726α when cells are prepared by different fixation methods and in different cell types. At metaphase, Mast localises to centrosomes, the mitotic spindle and centromeres, maintaining this localisation during anaphase A. Later, at anaphase B, Mast appears concentrated at the spindle midzone, associated to polar MTs, and shows faint centrosomal localisation until late telophase. During very late telophase it localises at either side of the midbody and centrosomal localisation is barely detectable. After cytokinesis, Mast can be seen associated again with the rod-like structure (data not shown). This structure might correspond to the remains of the midbody.

To confirm the specificity of the immunofluorescence labelling, the coding region of *mast* was cloned both in *Drosophila* and mammalian transfection vectors to express an EGFP-Mast fusion protein in S2 and HeLa cells. As control, we expressed EGFP alone and verified that it has a homogeneous distribution in S2 or HeLa cells (data not shown). However, the EGFP-Mast fusion protein follows a pattern of localisation in both cell types similar to the one described in the previous section (Fig. 5). In interphase, EGFP-Mast signal is strongly associated to MTOCs and to a fibrillar network that resembles MT bundles. This extensive fibrillar network is not observed in transfected cells treated with colchicine (data not shown). At prophase, the protein localises to the cytoplasm and shows accumulation to the centrosome. Later, during prometaphase/metaphase, spindle association is clearly evident, as well as, localisation to the centrosomes and centromeres. During early anaphase, EGFP-Mast appears more diffused, although, at later stages, centrosomes, spindle MTs and the spindle midzone show accumulation of the protein. In telophase, both S2 and HeLa cells show EGFP signal at the midbody and centrosomes. These results indicate that Mast associates with MTs and centrosomes during most of the cell cycle but undergoes accumulation to additional structures during mitosis.

Microtubule binding assays

Indirect and direct localisation shows that Mast associates to MTs throughout the cell cycle. To determine whether Mast is a MAP, we performed subcellular fractionation and followed the protein using IP726α. The results show that during sequential purification of MTs from embryo extracts Mast remains tightly bound to the insoluble fraction of polymerised MTs and can be partially released from the polymer after incubation in high salt (Fig. 6). These *in vitro* results support the *in vivo* intracellular localisation data suggesting that Mast associates with MTs.

Localisation of Mast in cells arrested with colchicine

Since Mast is a MAP, we wanted to determine whether its localisation to the various compartments of the mitotic apparatus depends upon active MT polymerisation. Accordingly, S2 tissue culture cells were incubated in the presence of colchicine for various periods of time, fixed and immunostained with IP726α and with an α-tubulin (Fig. 7A-B) or γ-tubulin (Fig. 7D) antibody. The results show that after 8 or 16 hours incubation no significant spindle MTs are present in these cells (Fig. 7B-D). In all cells analysed, Mast shows co-localisation with γ-tubulin (Fig. 7D) suggesting that microtubules are not required to maintain its localisation at the centrosome. Furthermore, in the absence of MTs, Mast shows strong accumulation at the primary constriction of highly condensed chromosomes (Fig. 7B-D). In order to determine whether this localisation corresponds to the centromere, isolated mitotic chromosomes from S2 cells were stained for both Mast and the mitotic kinase Polo that has been previously shown to accumulate at this site (Logarinho and Sunkel, 1998). The results obtained show that Mast and Polo co-localise at the centromere of isolated chromosomes (Fig. 7E).

Organisation of the mitotic apparatus in mast mutant neuroblasts

To characterise the organisation of the mitotic apparatus in *mast* mutant cells, neuroblasts from mutant larvae were immunostained to visualise the spindle (α-tubulin) and centrosomes (CNN, γ-tubulin or CP190). Since the results with all three centrosomal markers are very similar, only CNN staining is shown (Fig. 8). The wild type control cell at metaphase shows a typical neuroblast bipolar spindle (Fig. 8A). CNN stains the centrosomes in a ring-like pattern that is clearly seen in the amplified image shown on the left panel. All other images are from *mast* mutant cells that display various degrees of disorganisation of the mitotic apparatus. Extensive analysis of mutant neuroblasts stained with α-tubulin to reveal mitotic MTs suggests that the overall morphology of MTs is not the same as the wild type controls. A comparison between control (Fig. 8A) and mutant (Fig. 8B) cells at metaphase highlights the differences. While in the wild type cell MTs are generally straight and form tight bundles, in mutant cells MTs are generally irregular in shape and do not appear always straight. Occasionally, mutant cells are able to organise bipolar spindles, however, both poles appear associated with large asters (Fig. 8B). CNN staining shows that spindle poles contain an irregular number of ring-like structures. A higher magnification of the MTOCs indicates that in this cell, one of the poles contains at least 9 centrosomes while the other pole contains several poorly defined centrosomes. Many cells were found to contain only a single MTOC with a large associated aster and multiple ring-like structures that are stained by CNN (Fig. 8C and D). Some cells contain a single aster and show no organised ring-like CNN positive structures but a rather diffused staining in the form of a ball that localises to the middle of the condensed chromosome mass (Fig. 8E). We also observe many cells in which chromosomes are organised into a sphere with MTs emanating from a number of irregular size foci that are

all CNN positive and occasionally display pairs of ring-like structures reminiscent of two adjacent normal centrosomes (Fig. 8F).

Finally, in various combinations of mutant alleles we observed highly polyploid cells that contain both CNN positive centrosome aggregates inside the chromosome mass, as well as, other CNN positive aggregates in the surrounding cytoplasm (Fig. 8G). These cytoplasmic aggregates are also capable of nucleating MT asters. These results suggest that *mast* mutant cells are capable of replicating their centrosomes but either can not segregate them properly or they segregate but later collapse forming aggregates. Since it has not been possible to determine the exact centrosome number and ploidy in the same cells, we cannot exclude the possibility that *mast* mutant cells over-replicate their centrosomes. Nevertheless, these abnormal centrosome aggregates do nucleate MT asters but are rarely able to organise bipolar spindles. However, the circular organisation of condensed chromosomes located around a large centrosome from which MTs are nucleated suggests that chromosomes do interact with these MT asters.

Spindle checkpoint in mast mutant neuroblasts

Mutations in *mast* cause mitotic arrest, however many cells progress into subsequent cell cycles and become polyploid. To determine whether *mast* mutant cells are able to activate the spindle checkpoint, we have stained *mast* mutant cells with antibodies against the checkpoint protein Bub1 (Basu *et al*., 1999) and α-tubulin to visualise the spindle (Fig. 9). In wild type controls (Fig. 9A-C), Bub1 localises to centromeres during prophase and prometaphase and is virtually absent during metaphase. However, condensed mitotic chromosomes in mutant cells that show monopolar spindles (Fig.9D and E) or that are highly polyploid (Fig. 9F), consistently show strong Bub1 accumulation at the centromere. Furthermore, incubation of $mast^{P1}$ mutant cells in colchicine results in a significant prometaphase arrest (>98%) and no

premature sister chromatid separation is observed (data not shown). These results suggest that *mast* mutant cells have an active spindle checkpoint response.

Discussion

We have identified a new *Drosophila* gene, *multiple asters* (*mast*), which encodes an essential protein required for the organisation and function of the mitotic spindle. Mast is a MAP that appears to define a new family of proteins conserved from yeast to man. Mutations in *mast* cause severe alterations in chromosome segregation. The Mast protein shows co-localisation with interphase MTs and during mitosis associates to centrosomes, the mitotic spindle, centromeres and the spindle midzone. We propose that the Mast protein is required for accurate centrosome separation and organisation of the bipolar spindle during mitosis.

Mast is part of a new family of proteins

Database searches and phylogenetic analysis have shown that Mast is part of a new conserved family of proteins that so far contains two human, three *C. elegans* and two yeast members. Mast is more closely related to the humans and the three *C. elegans* than either to the *S. pombe* or the *S. cerevisiae* proteins. Indeed, extensive identity is observed between the *Drosophila*, human and *C. elegans* members throughout the protein sequence. Interestingly, when the EGFP-Mast fusion protein is expressed in HeLa cells it shows a pattern of cell cycle-dependent localisation like in *Drosophila* cells. Furthermore, antibodies raised against one of the human proteins also show a very similar immunolocalisation pattern as described for Mast (Maiato and Sunkel, unpublished observations). The strong sequence conservation together with the localisation data suggests that the human and *Drosophila* proteins might be functionally related.

Mast binds microtubules and localises to multiple compartments of the mitotic apparatus

Sequence analysis of the Mast protein revealed that it shares conservation to both bovine and mouse MAP4. The homology is restricted to a domain rich in proline and basic residues thought to be involved in MT binding (Aizawa *et al*., 1991). This domain falls outside the conserved regions CR-1, CR-2 and CR-3. Although we have not determined experimentally whether this region of the protein is responsible for MT binding, our results indicate that Mast co-sediments with polymerised MTs in a salt-stable complex. The immunolocalisation and transfection studies provided further evidences that Mast is a MAP since the protein shows clear co-localisation with both interphase and spindle MTs. In agreement, Stu1p was also demonstrated to interact with MTs (Pasqualone and Huffaker, 1994). However, Mast also localises to other compartments of the mitotic apparatus even in the presence of MT poisons. The presence of two putative $p34^{cdc2}$ phosphorylation sites suggests that Mast might undergo specific postranslational modifications during G2/M transition allowing it to reach specific mitotic structures. A number of MAPs have been shown to be phosphorylated upon entry into mitosis allowing for modifications in MT dynamics to take place (Verde *et al*., 1992).

Mast shares limited homology with members of the dis1-TOG family, however, like Mast, all these proteins localise either to centrosomes and/or spindle MTs during mitosis. In some respects, Mast shows patterns of localisation closer to those of ZYG-9, $p93^{dis1}$ and XMAP215. These three proteins co-localise with interphase MTs and during mitosis, ZYG-9 and $p93^{dis1}$ localise to the centrosome in the absence of MTs. Nevertheless, during late stages of mitosis, Mast shows strong localisation to the spindle midzone, like Msps and ch-TOG, two other members of the dis1-TOG family. Mast and ZYG-9, however, do show some unique features since both proteins remain localised to the centromeres of mitotic chromosomes when cells are incubated in the presence of MT depolymerising agents. The

localisation of Mast to MTs, centrosomes, centromeres and the spindle midzone suggests very strongly that this protein might play a role in the regulation of MTs as it has been shown *in vitro* and in cell free extracts for some members of the dis1-TOG family (Tournebize *et al*., 2000).

Mast is required for centrosome segregation and bipolar spindle organisation

Genetic analysis of the *mast* locus shows that this protein is required for the organisation of the mitotic spindle. Mutant neuroblasts show highly abnormal mitotic figures, rarely organise bipolar spindles and most of them contain one or more MTOCs of irregular size and shape. Detailed analysis of the abnormal MTOCs indicates that these are mostly composed of multiple CNN positive ring-like structures that are present in all normal centrosomes. Therefore, it is unlikely that mutations in *mast* cause an abnormal organisation of the centrosome itself. Furthermore, the abnormal MTOCs observed in *mast* mutant cells contain many CNN positive ring-like structures, suggesting that centrosome replication is not affected. One possible interpretation of our data could be that in mutant cells centrosomes do segregate but at a later stage collapse forming a monopolar configuration as was shown for mutations in KLP61F (Sharp *et al*., 1999). However, in strong mutant alleles multiple MTOCs of irregular size are associated with MT asters that are found dispersed throughout the cell. In this context, mutations in *mast* display a phenotype similar to *zyg-9* that shows numerous cytoplasmic clusters of short MTs during meiosis (Kemphues *et al*., 1986). However, ZYG-9 is thought to be required for the organisation of long MTs during meiosis and mutations in *mast* do not appear to cause a significant reduction in MTs length (see Fig. 8). Nevertheless, the morphology of MTs in *mast* mutant cells is not normal, appearing to be much more irregular in shape. Furthermore, disruption of *STU1* in *S. cerevisiae* causes severe defects in spindle assembly (Pasqualone *et al*., 1994). Accordingly, the highly abnormal pattern of centrosome

segregation observed as a result of mutations in *mast* could be due to abnormal MT organisation.

Mast and the control of mitotic progression

If Mast is essential for normal centrosome segregation and bipolar spindles are rarely observed, it might be expected that the spindle checkpoint (Nicklas, 1997) would prevent these cells from advancing into a new cycle of proliferation. However, despite the increase in mitotic index, mutations in *mast* cause the formation of highly polyploid cells. Mutant cells respond to the spindle checkpoint when arrested with colchicine in prometaphase, since premature sister chromatid separation is never observed. Furthermore, mutant cells with abnormal spindle morphology and highly condensed chromosomes show strong accumulation of the spindle checkpoint protein Bub1. This staining pattern is similar to that observed in chromosomes of cells arrested in prometaphase after MT depolymerisation (Basu *et al*., 1999), suggesting that in *mast* mutant cells the interactions that occur between MTs and chromosomes are unable to inactivate the spindle checkpoint, leading to a prolonged prometaphase arrest. However, since highly polyploid cells are formed, these cells must have undergone multiple cell cycles in the absence of chromosome segregation and cytokinesis. Therefore, it is most likely that after some time, these cells adapt, either become insensitive or override the spindle checkpoint and progress into a new cycle of proliferation. Indeed, the length of time different cell types remain in M phase in the presence of microtubule inhibitors varies widely (Kung *et al*., 1990).

Materials and methods

Genetic variants

mast^{P1} was initially described as the *v40*^{1} allele of the gene *v40* (Fedorova *et al*., 1997). It has a P{1ArB} insertion in position 78C1-C2. Two other mutant alleles, *mast*^{P2} and *mast*^{P3}, EP(3)3515 and EP(3)3403, respectively, were obtained from BDGP. *mast*^{P4} was obtained by mobilisation of the P-element of *mast*^{P1} as described below. Df(3L)31A, a deficiency for the region 78A-78E, was obtained from Bloomington *Drosophila* Stock Centre. All lines were balanced over TM6B. Oregon-R strain was used as wild-type control. All stocks were grown at 25°C under standard conditions and media.

Mobilisation of the P-element of mast^{P1} *and generation of mast*^{P4}

mast^{P1} / TM6, *Tb, Hu*, *e* females were crossed with Δ(2-3), *Sb, e* / TM6, *Ubx, e* males. Resulting Δ(2-3), *Sb, e* / *mast*^{P1} males were individually crossed with *red*^{1}, *mbc*^{C1}, *e*^{1} / TM3, *Sb*^{1}, *ry*^{1}, *e*^{1} females (Umea). Excision of the P insertion was recognised by the loss of *ry*$^{+}$. From 35,000 chromosomes scored, we obtained 39 lines that had lost the insertion and were viable, fertile and had no mitotic phenotype. Among the non-viable *ry*$^{-}$ lines, we identified one line, *mast*^{P4}, in which homozygous died as late larvae/pupae and cytological analysis revealed to have very severe mitotic defects.

Cytological analysis

The analysis of the mitotic phenotype was done according to González and Glover (1993). For quantification of the mitotic parameters, the number of cells present in 50 randomly chosen optic fields (100x) per brain were counted. Five brains were scored for each allele. All the calculations were based on the sum of total number of cells present in the five brains scored.

Molecular analysis

Genomic fragments adjacent to P element insertion were cloned by plasmid rescue and inverse PCR and sequenced. Three cDNA clones were identified in the BDGP database: LD11488 (0 to 24 hours mixed stage embryonic library), LP08134 (larval-early pupal library) and GH26741 (adult head library). The genomic sequence (AC014071), from Celera Genomics, includes the complete *mast* gene. The LD11488 cDNA clone was completely sequenced with ^{T7}Sequencing Kit^{TM} (Pharmacia). This cDNA is 5938 bp long, with a 769 bp 5' untranslated region and a 3' untranslated region of 679 bp.

Database searches and sequence analysis

BLAST (Altschul *et al.,* 1997) searches were performed in non-redundant GenBank CDS translations, PDB, SwissProt, SPupdate and PIR databases. All the unpublished protein sequences that were used in this study can be found under the following accession numbers: CeC07H6.3 (CE01153), SpStu1p (CAA15921), At-TOG (AAD15450), SpAlp4p (CAA22843). Phylogenetic analysis and unrooted dendrogram elaboration were performed using PHYLIP (Retief, 2000).

Antibodies and western analysis

In order to express a segment of the Mast protein in *E. coli*, the LD11488 cDNA was digested with Bgl II and Sac I. The resulting 1281 bp fragment was subcloned in the pQE-32 vector (Qiagen), obtaining plasmid pQE-Mast1. The recombinant-Mast1 protein was purified from inclusion bodies isolated from *E. coli* transformed with pQE-Mast1 after IPTG induction and used for immunisation in rabbits (Diagnostics Scotland - Edinburgh, UK). The polyclonal Rb726 serum was subsequently immunopurified (IP726α) against the recombinant protein

immobilised in nitrocellulose (according to Sambrook *et al*., 1989). For western blotting, total protein extracts from embryos, brains, testes and ovaries were prepared in SDS-PAGE sample buffer as previously described (Sunkel *et al*., 1995), separated in 7-12% SDS-PAGE and blotted to nitrocellulose. Membranes were incubated with IP726α (1:100) or with an anti-α-tubulin monoclonal antibody (Amersham) (1:500) and detection was done with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Vector, UK) (1:1000). The signal was developed with ECL Chemiluminescent Detection System (Amersham, UK).

Immunofluorescence in S2 culture cells

Drosophila S2 cells were grown in Schneider's Medium (Gibco BRL) with 5 % fetal bovine serum, at 25 ºC. For microtubule depolimerisation, cells were incubated with 30 μM colchicine (Sigma, USA) for 8 or 16 hours. Cells were spun against a slide at 1000 rpm for 5 min, fixed in 3.7 % formaldehyde in PHEM (Pipes 60 mM; Hepes 25 mM, pH 7.0; EGTA 10 mM; $MgSO_4$ 4 mM) for 12 min and washed 3× 5 min with PBS. Blocking was performed in PBS-TF (1% Triton X-100, 10 % FBS in PBS) with 0.5 mg/ml RNase, for 1h at RT. Slides were then incubated with IP726α and a mouse anti-α-tubulin antibody (Amersham) in PBS-TF, at a 1:10 and 1:200 dilution, respectively, for 45 min at RT and washed in PBS. FITC-α-rabbit conjugated IgG (Vector, UK) and CY5-α-mouse conjugated IgG (Jackson Laboratories, USA) were used as secondary antibodies, diluted 1:1000 and 1:200, respectively. Cells were washed as before, DNA stained with propidium iodide and the preparation mounted in Vectashield (Vector, UK). Preparations were observed with a Bio-Rad MRC600 confocal microscope and images processed with Photoshop 5.5 (Adobe Systems).

Cell transfection and GFP analysis

EGFP-Mast constructs were done by linker-mediated subcloning. A linker corresponding to the 5' end of the *mast* coding sequence including a 5' protruding extremity compatible with Bgl II and a 3' protruding end compatible with Bst XI was produced by the annealing of complementary oligonucleotides. A 5128 bp Bst XI - Xho I *mast* cDNA fragment was ligated in the presence of the linker to the plasmids pMTEGFP-C1 (T. Megraw, unpublished) or pEGFP-C1 (Clontech), both digested with Bgl II and Sal I, resulting in plasmids pMTEGFP-Mast and pEGFP-Mast. These plasmids were used to transfect, respectively, *Drosophila* S2 and human HeLa cells. Cells were transfected using 1 μg of plasmid, prepared by QIAGEN midiprep (Qiagen), and the FuGENE^{TM} 6 Transfection Reagent (Boehringer Mannheim). After 24 hours of growth, expression of EGFP-Mast in S2 cells was induced from the metallothionein promoter by 1.0 mM of $CuSO_4$. Significant expression was detected 9 hours after induction. Cells were spun, fixed and washed as described above. The expression of EGFP-Mast in transfected HeLa cells is constitutive and detected soon after transfection. Cells were grown for 24 hours, washed 2× 5min with PBS, fixed in 3 % formaldehyde in PBS for 10 min, and washed as above. S2 and HeLa preparations were mounted in Vectashield (Vector, UK) after DNA staining with propidium iodide, observed with a Bio-Rad MRC600 confocal microscope and images processed as above.

Chromosome isolation

S2 cells were arrested in mitosis with 30 μM colchicine (Sigma, USA) for 16 hours. Chromosomes were then prepared as previously described (Bousbaa *et al.*, 1997). Immunofluorescence was performed as above, using IP726α and a monoclonal anti-Polo antibody (Logarinho and Sunkel, 1998).

Microtubule binding assays

Microtubules were purified from 0-3-hours-old *Drosophila* embryos as described by Saunders *et al*. (1997). Samples of the various steps were collected and 30 μg of total protein of each extract was separated by SDS-PAGE and stained with coomassie blue or subjected to western blot and probed with IP726α or an anti-α-tubulin monoclonal antibody (Amersham) as described above.

Immunofluorescence in brains

The whole brain of third instar larva was prepared for immunostaining as previously described (Bonaccorsi *et al.*, 2000). Immunostaining of microtubules and centrosomes was performed with antibodies against α-tubulin (Amersham) and Centrosomin (Heuer *et al*., 1995) diluted 1:200 and 1:500, respectively. The antibodies were visualised with FITC-α-mouse conjugated IgG (Vector, UK) or CY3-α-rabbit conjugated IgG (Jackson Laboratories, USA). To visualise the Bub1 protein we used antibodies as described previously (Basu *et al*., 1999). The preparations were analysed in a ZEISS Axioskop microscope, and images acquired with a SPOT 2 camera (Diagnostic Instruments, USA) and processed as above.

Acknowledgements

We would like to thank T. Megraw for the EGFP plasmids and advice on cell transfection, and T. Kaufman for the anti-CNN antibody. We are grateful to E. Bronze-da-Rocha for help with HeLa cell cultures, all the people from Sunkel's lab for helpful comments and stimulating discussions, M.J. Falcão and T. Lopes for laboratory assistance and to A. Santos for general technical support. C.L. and H.M. hold Ph.D. studentships and P.S. holds a Postdoctoral research fellowship from the PRAXIS XXI program of the FCT of Portugal. H.M. is a student

of the Gulbenkian Ph.D. Program in Biology and Medicine. C.E. Sunkel is financed by grants from FCT of Portugal and the TMR program of the EU.

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Figure Legends

Fig. 1. Cytological analysis and quantification of mitotic phenotypes in *mast* mutant neuroblasts. Third instar larval brains were dissected from wild type (**A** and **G**) or *mast* mutant (**B**-**F** and **H**-**K**) individuals. Wild type cells in metaphase (**A**) or anaphase (**G**) are shown for comparison. *mast* mutant cells show either diploid (**B**) or polyploid (**C**) circular mitotic figures with chromosomes organised with their centromeres facing a central region where the small fourth chromosomes are located. Most cells show highly condensed chromosomes (**B-F, J** and **K**). *mast* mutant cells at anaphase (**H** and **I**) can also be found and occasionally show chromatin bridges and abnormal segregation. In the most severe $mast^{P4}$ allele, cells show extensive polyploidy with most chromosomes organised in a sphere-like conformation (**F**). (**L**) Quantification of mitotic index. (**M**) Quantification of mitotic cells with respect to different stages of mitosis. (**N**) Quantification of the abnormal mitotic parameters in all alleles. Bar corresponds to 5 μm except in figures J and K, that is 50 μm.

Fig. 2. Molecular characterisation and expression analysis of *mast*. (**A**) Molecular map of *mast* locus. Wedges represent the insertion of P{1ArB} element in $mast^{P1}$ (P_1) and P{EP} element in $mast^{P2}$ (P_2) or $mast^{P3}$ (P_3) and black boxes the exons. Bar under map corresponds to the deleted region in $mast^{P4}$ (ΔP_4). Arrows represent the two transcripts corresponding to the short cDNA from adult heads or larva/pupa, and the long cDNA from embryos. The open reading frame is represented by a grey box. Note that neither exon 1 or exon 1' are coding exons. (**B**) Predicted amino-acid sequence of Mast. Black boxes represent HEAT repeats and predicted sites of phosphorylation by $p34^{cdc2}$ are bold underlined. The grey region defines the conserved MAP-4 microtubule-binding domain. (**C**) Developmental expression of Mast.

Protein samples prepared from successive developmental stages of the wild-type strain were loaded in equal amounts (see α-tubulin as control). P: rMast1; E0-2: 0-2 hours embryos; E2-24: 2-24 hours embryos; B: third instar larval brains; T: adult testis; O: adult ovaries. The anti-Mast antibody specifically recognises the recombinant protein and a band of 165 kDa, corresponding to Mast, in all extracts. Note that in early embryo extracts the antibody recognises an additional band of higher molecular weight. (**D**) Expression of Mast in different mutant alleles. Protein samples from brains of wild type or homozygous mutant third instar larvae were loaded in equal amounts (see α-tubulin as control).

Fig. 3. Protein sequence alignment and phylogenetic analysis. (**A**) Multiple sequence alignment of the predicted protein sequences closely related to Mast, including two human (KIAA0622 and KIAA0627), three *C. elegans* (CeC07H6.3, CeR107.6 and CeZC84.3), one *S. pombe* (SpStu1p) and one *S. cerevisiae* (Stu1p), revealed three regions of more significant identity. (**B**) Conserved regions are represented in grey boxes and percentage identity and similarity (in parenthesis) of the most conserved proteins is indicated below. Additionally, a small domain of 18 amino acid residues that is highly conserved between Mast and members of the dis1-TOG family is represented. (**C**) Phylogenetic unrooted tree with all proteins that share significant sequence identity with Mast.

Fig. 4. Immunolocalisation of Mast in S2 *Drosophila* culture cells. Individual images for DNA, Mast and α-tubulin are shown. In the merged images DNA is in blue, Mast in red and α-tubulin in green. Cells in interphase show Mast localised in a punctuate cytoplasmic pattern. At prophase Mast is found at the centrosomes and most of the times is also associated with an unidentified rod-like structure (arrowhead). During metaphase Mast associates with spindle microtubules and it is also concentrated at the centromeres. During anaphase, Mast is found at

the spindle poles, microtubules and at the spindle midzone. At early telophase the whole spindle midzone is labelled and some Mast is still present at the spindle poles and at later stages Mast localises at either side of the midbody even after cytokinesis is almost complete. Bar is 10 μm.

Fig. 5. Transfection of EGFP-Mast in *Drosophila* (S2) and human (HeLa) culture cells. DNA is shown in red and EGFP-Mast in green. During interphase, both S2 and HeLa cells show strong EGFP-Mast signal associated with a fibrillar network that resembles microtubule bundles. In prophase EGFP-Mast is restricted to the centrosomes. At prometaphase and metaphase, EGFP-Mast signal accumulates at the spindle poles, spindle microtubules and the centromeres. During anaphase, spindle poles, microtubules and a more diffused cytoplasmic signal is observed. Finally, at telophase, EGFP-Mast localises to the centrosomes and spindle midzone. Bar is 10 μm.

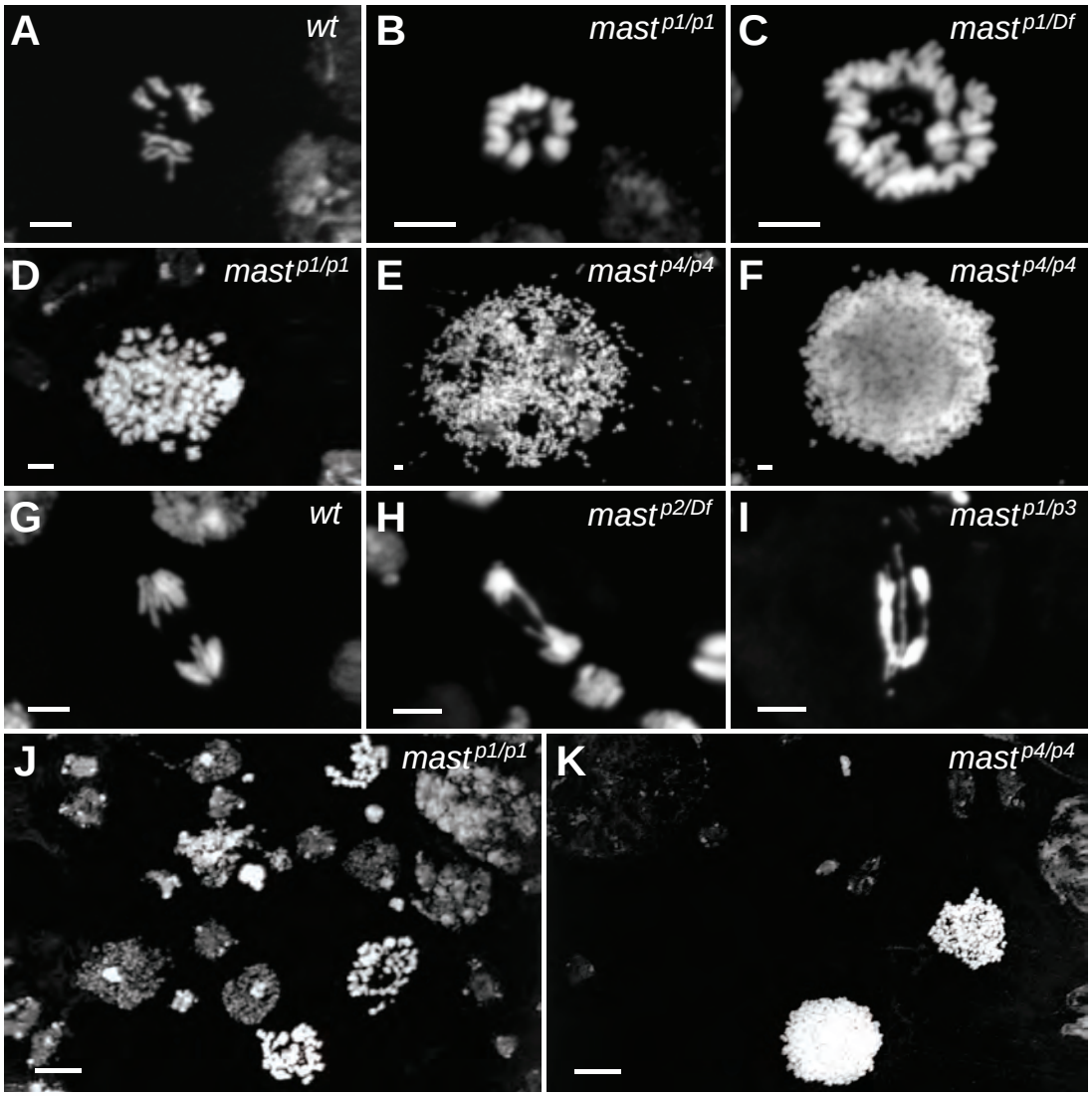
Fig. 6. Mast binds to microtubules in vitro. Microtubules were purified from 0-3 hours-old embryos by sequential rounds of polymerisation and depolymerisation. (1) crude extract, (2) low speed pellet and (3) supernatant. The supernatant was centrifuged at high-speed and the resulting supernatant (4) was incubated on ice to depolymerise microtubules, followed by incubation with taxol and GTP at 20ºC to repolymerise microtubules. After saccarose gradient centrifugation, the soluble material (5) was separated from microtubules and associated proteins (6). Microtubule-associated proteins (7) were extracted from microtubules (8) with 0.5 M NaCl. Samples (30 μg) from each purification stage were separated by SDS-PAGE and the gel stained with coomassie blue (top panel) or immunoblotted with IP726α (middle panel) and anti-α-tubulin antibodies (bottom panel).

Fig. 7. Immunolocalisation of Mast after microtubule depolymerization. S2 cells were grown for 0, 8 or 16 hours in the presence of colchicine. Cells were stained to reveal Mast (red), and α-tubulin (**A-C**) or γ-tubulin (**D**) (green). Isolated chromosomes (**E**) were stained to reveal Mast (red) and Polo (green). DNA is shown in blue. Control cells (**A**) show a well-organised bipolar spindle and Mast localisation to the spindle poles and the centromeres. In cells incubated in colchicine for short (8 h, **B**) or longer periods (16 h, **C** and **D**), microtubules depolimerise and Mast remains associated to both centrosomes (arrows) and centromeres. Mast staining co-localises with γ-tubulin at the centrosomes (**D**) and with Polo at the centromeres (**E**). Bar is 5 μm.

Fig. 8. Centrosomes and mitotic spindles in *mast* mutant cells. Squashed preparations of brains isolated from either wild type (**A**) or various *mast* mutant allelic combinations (**B-G**), were incubated with an anti-CNN antibody to reveal the centrosome, anti-α-tubulin antibody to visualise the microtubules and DAPI to stain DNA. The first column shows a magnified view of the CNN staining of centrosomes shown in the second column (**A-F**). In the last column individual images of CNN (red), α-tubulin (green) and DNA (blue) have been merged. (**A**) During metaphase, wild-type neuroblasts show a normal bipolar spindle organised from ring-like CNN positive structures. (**B**) Most *mast* mutant cells show abnormal spindle organisation even if a polyploid cell is able to organise a bipolar structure. The spindle poles are highly unequal in size and show many ring-like structures tightly associated. (**C**) Monopolar spindle in a *mast* mutant cell organised from two associated ring-like structures. (**D**) Monopolar spindle in a *mast* mutant cell organised by an aggregate of CNN positive centrosomal material that shows no clear internal organisation. (**E**) Mutant cell with highly

condensed chromosomes organised into a ball-like structure around a mass of centrosomal material. Note the extensive microtubule network emanating from the single large centrosome. (**F**) Mutant cell with highly condensed chromosomes organised around several CNN staining aggregates from which asters are irradiated. Note, in the magnified view of the centrosome, two pairs of ring-like structures resembling normal centrosomes. (**G**) Highly polyploid cell exhibiting a large number of centrosomal aggregates within the chromosome mass, as well as, multiple cytoplasmatic CNN positive aggregates. Note that all CNN positive aggregates are associated with microtubule asters. Bar is 2 μm in the "CNN zoom 3.5x" column and 5 μm in all other figures.

Fig. 9. Immunolocalisation of a spindle checkpoint protein in *mast* mutant cells. Wild type (**A**-**C**) or *mast* mutant (**D**-**F**) neuroblasts were stained with antibodies against Bub1 (red) and α-tubulin (green). DNA is shown in blue. In wild type cells, Bub1 accumulates at the centromeres during prophase (**A**) and prometaphase (**B**) and is severely reduced as the chromosomes align in the metaphase plate (**C**). In *mast* mutant cells, Bub1 shows strong accumulation at the centromeres of chromosomes in monopolar (**D** and **E**) and polyploid (**F**) cells. Bar is 5 μm.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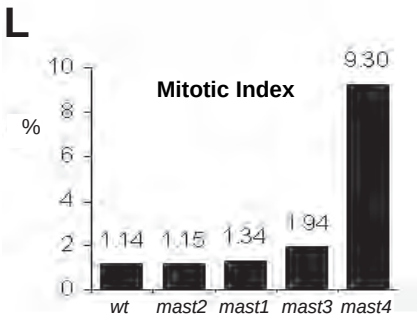
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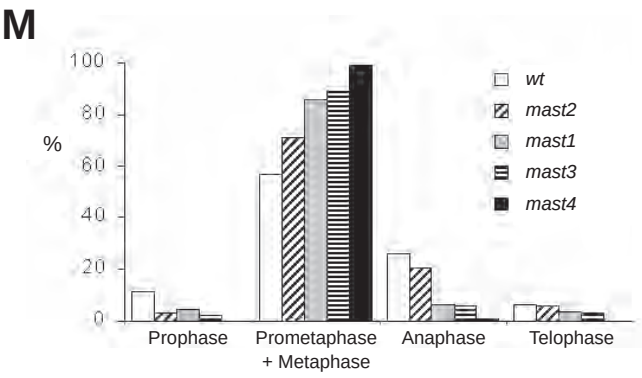
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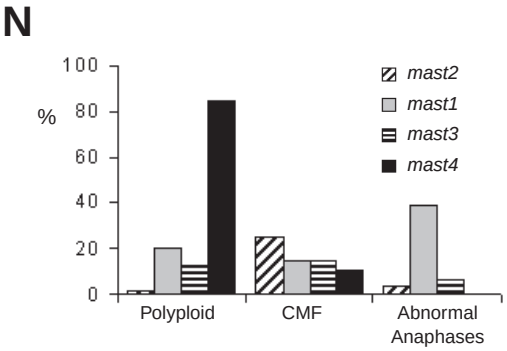
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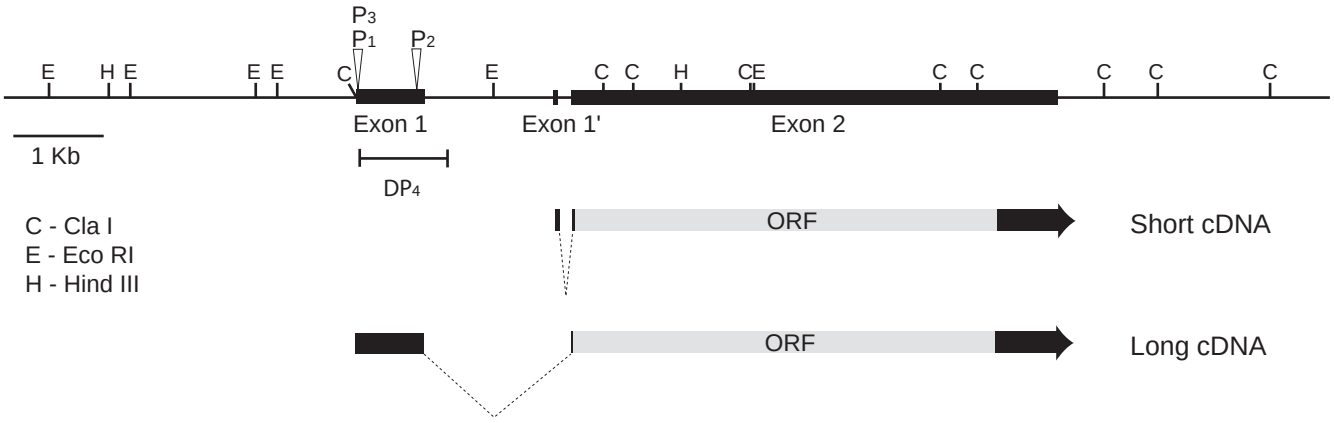

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ATGEFPTNLCAIKILLEVTEHHGSEITDAHLDIVFPNLARSADDTQSMVRKAAVFCIVKL 1440
YFVLGEEKVKPKLSVLNPSKVRLLNVYIEKQRNCISGGGSSTKNSSAASSS
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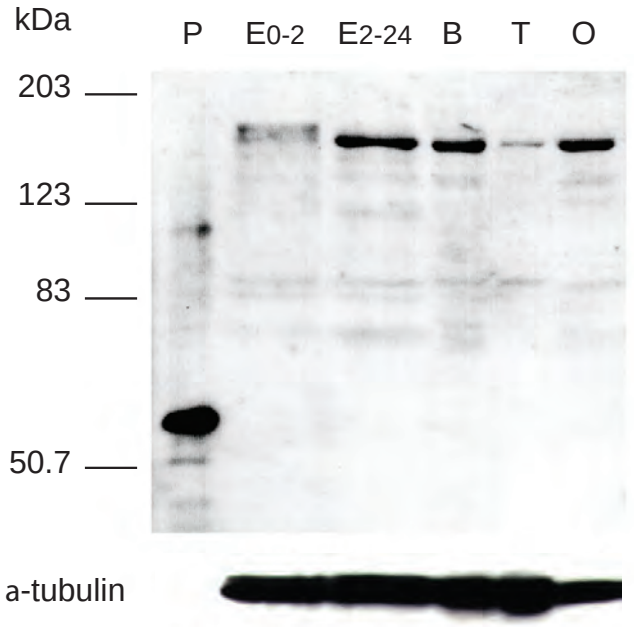
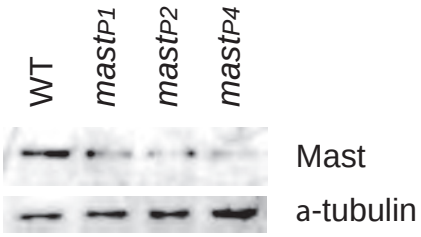
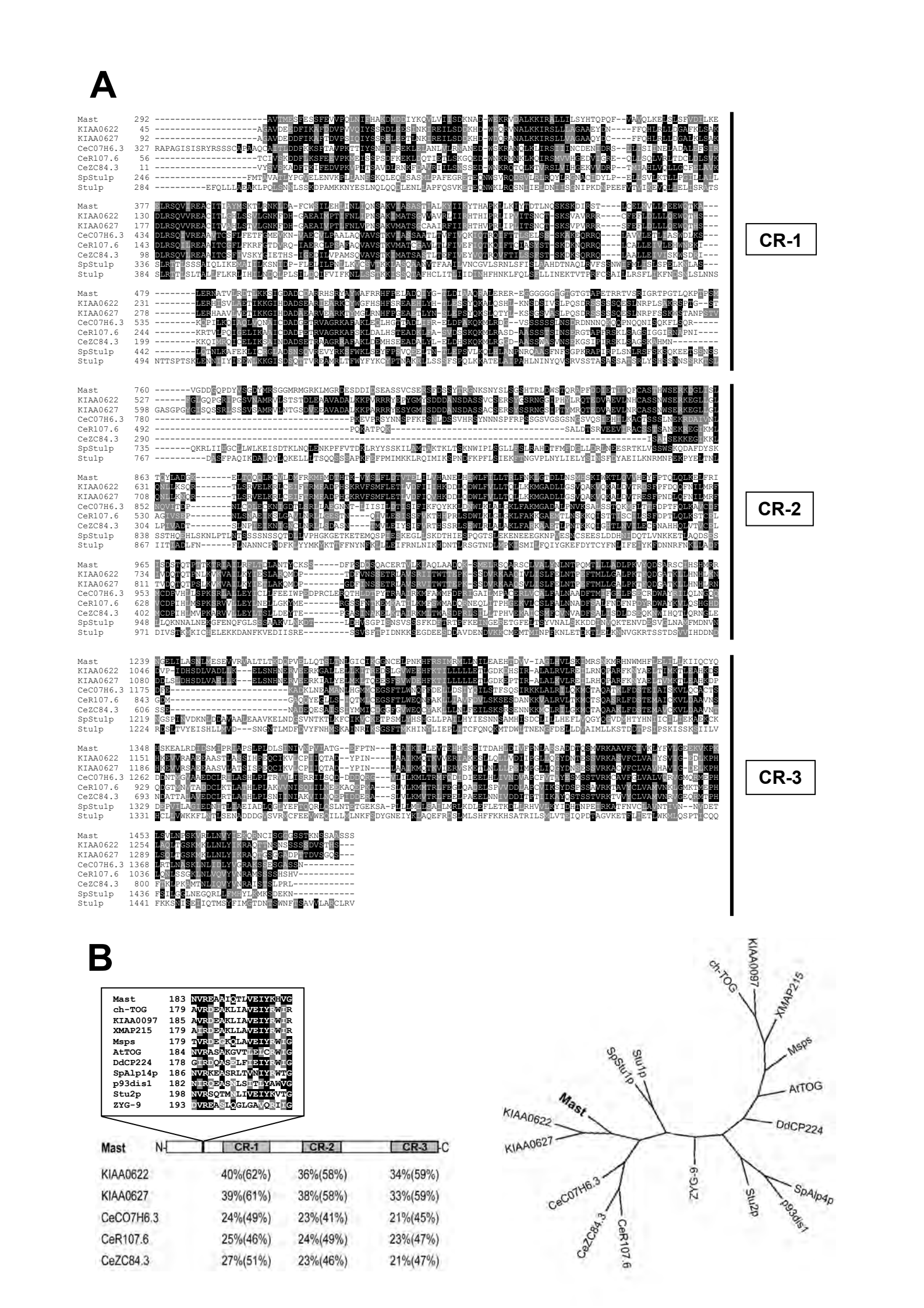
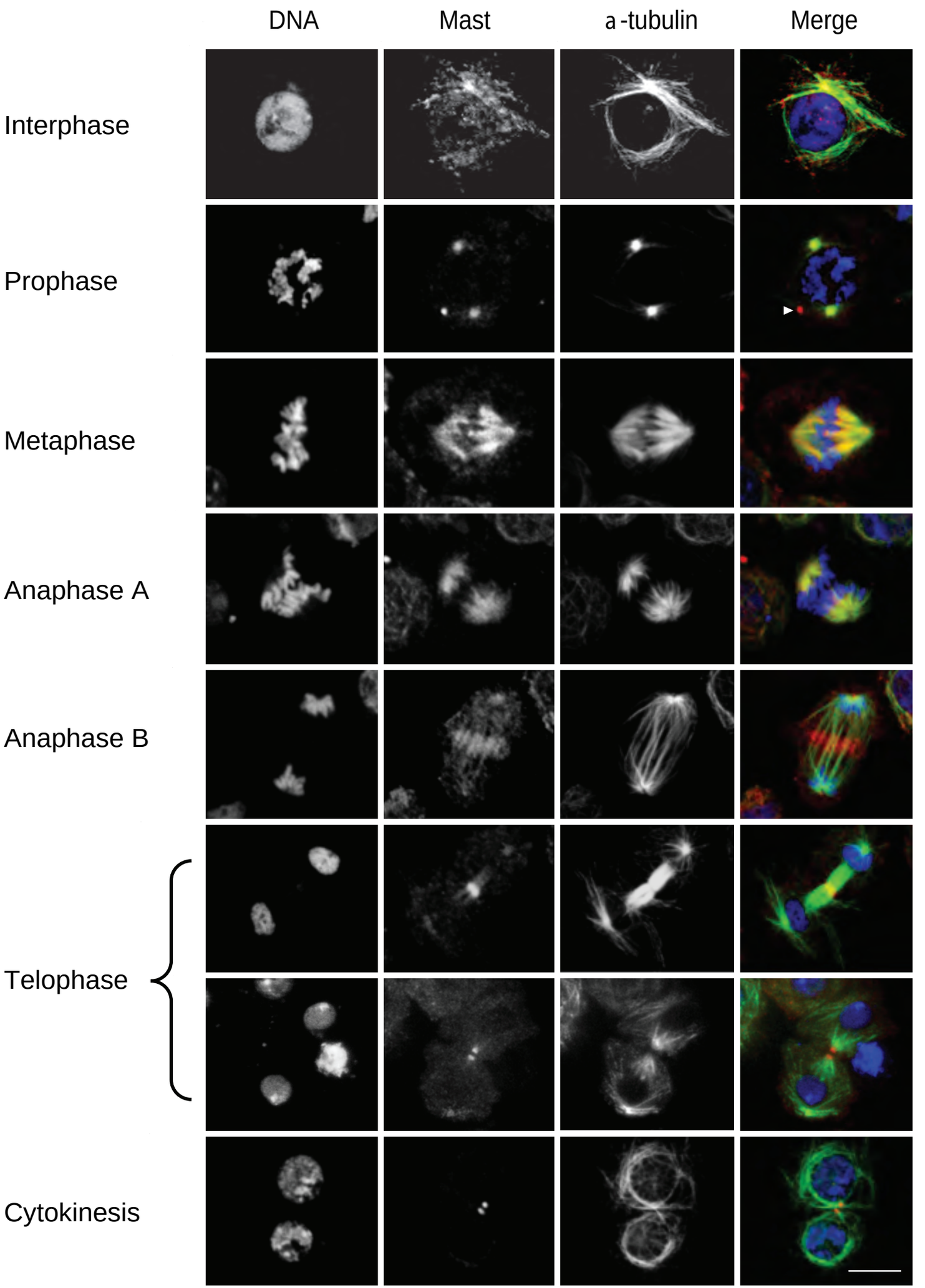
C

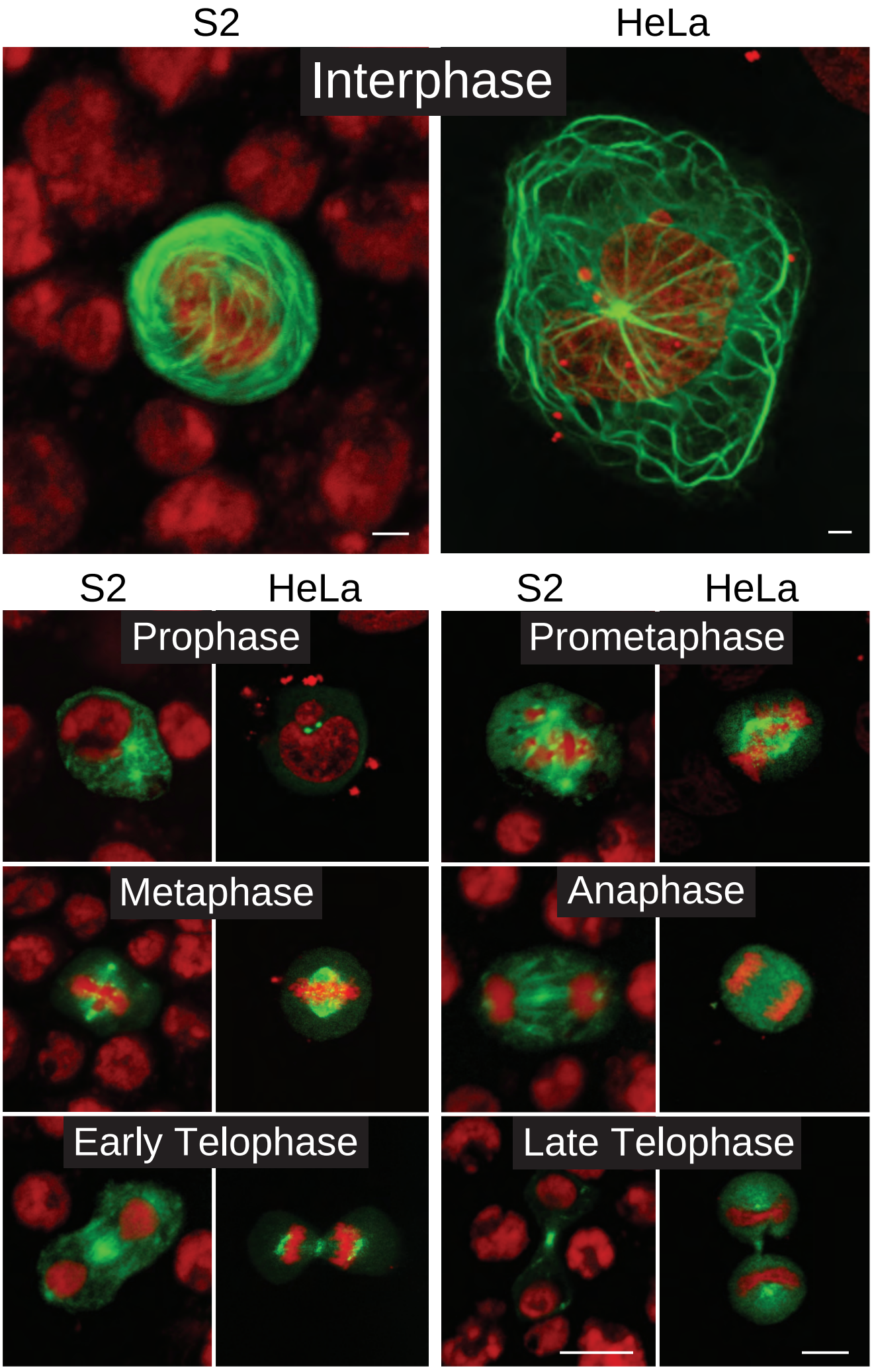


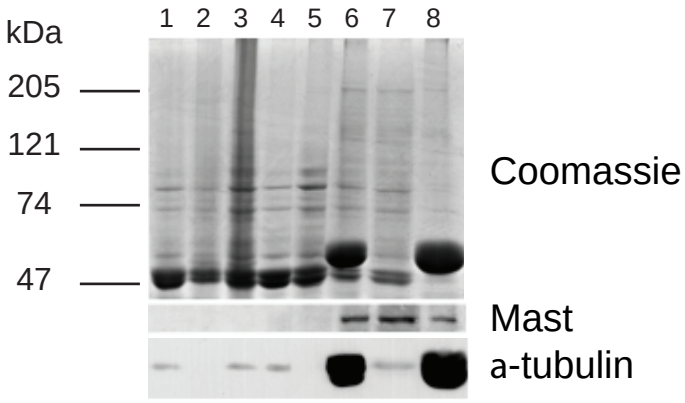
D

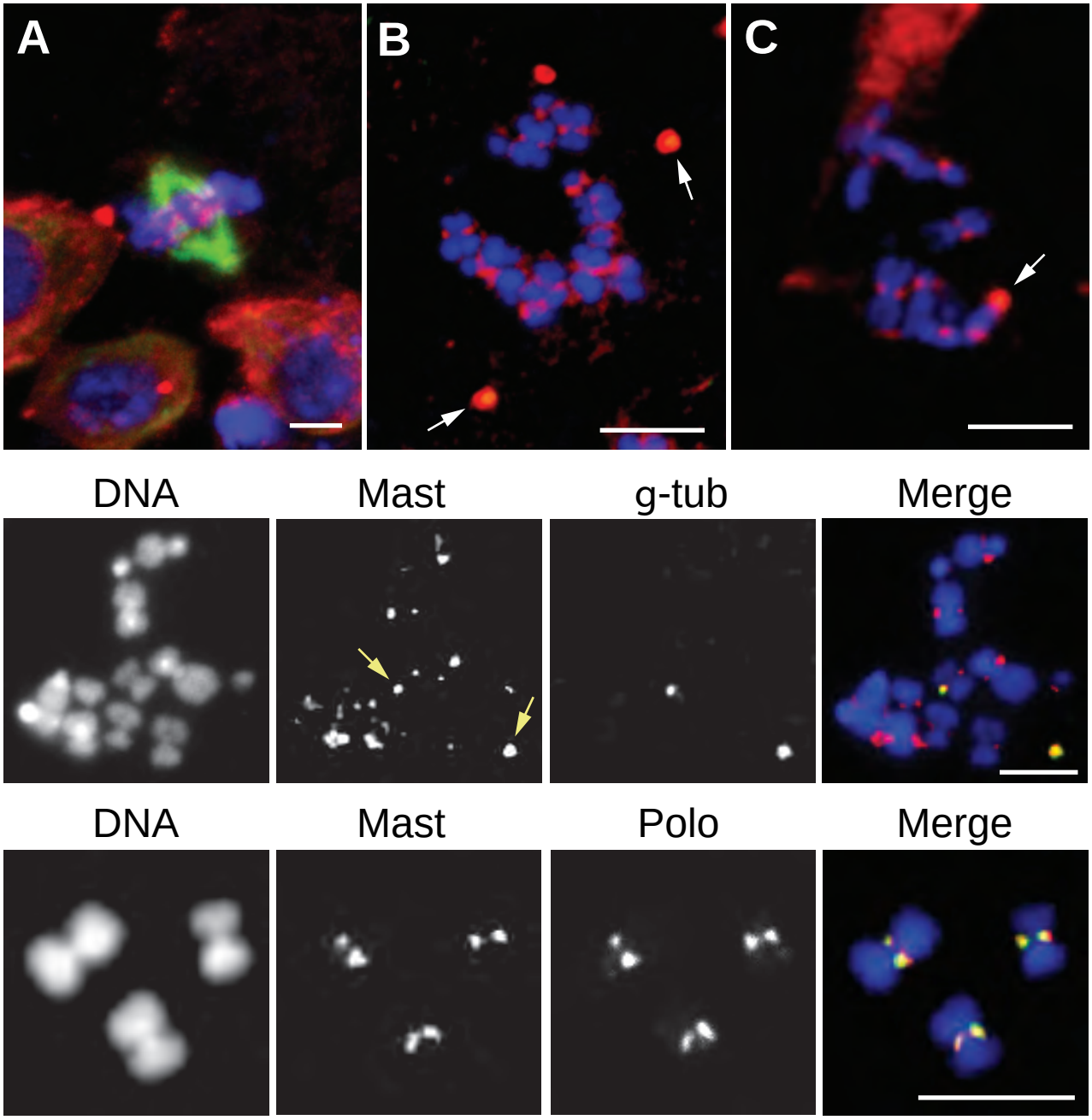


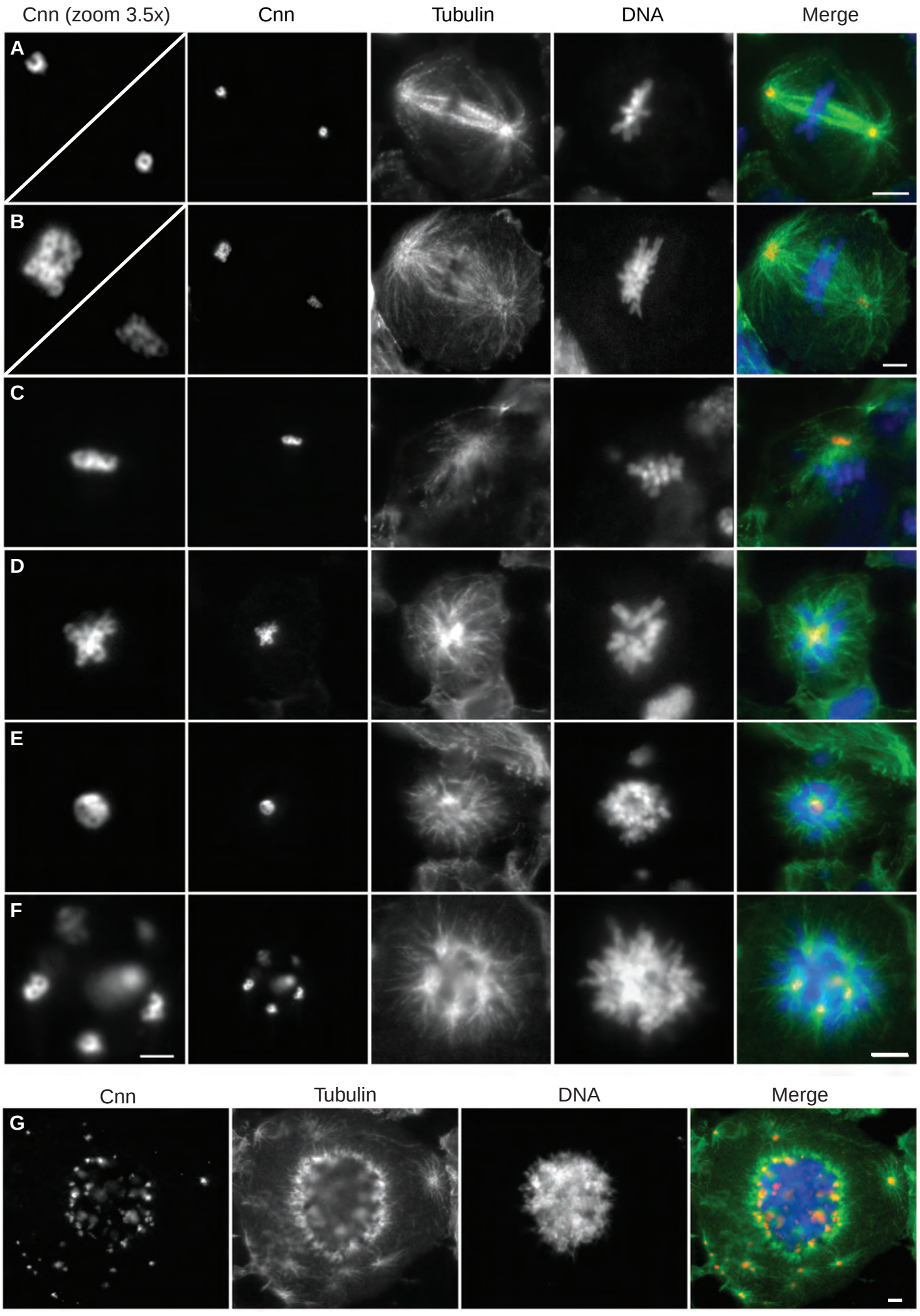

A
CR-1
CR-2
CR-3
B
Mast 183 NVREAAIQTIVEIYKHVG
ch-TOG 179 AVREEAKLIAVEIYRWIR
KIAA0097 185 AVRDEAKLIAVEIYRWIR
XMAP215 179 AIRDEAKLLAVEIYRWIR
Msps 179 TVREEGKQLAVEIYRWIG
AtTOG 184 NVRASAKGVTLELCRWIG
DdCP224 178 GIRDQASSLFIEIYRWIG
SpAlp14p 186 NVRKEASRLTVNIYRWTG
p93dis1 182 NIRCEASNLSITIYAWVG
Stu2p 198 NVRSQTMNLIVEIYKVTG
ZYG-9 193 DVREASLQGLGALQRIIG
Mast N- CR-1 CR-2 CR-3 -C
KIAA0622 40%(62%) 36%(58%) 34%(59%)
KIAA0627 39%(61%) 38%(58%) 33%(59%)
CeCO7H6.3 24%(49%) 23%(41%) 21%(45%)
CeR107.6 25%(46%) 24%(49%) 23%(47%)
CeZC84.3 27%(51%) 23%(46%) 21%(47%)
KIAA0097
ch-TOG
XMAP215
Msps
AtTOG
DdCP224
SpAlp4p
p93dis1
Stu2p
ZYG-9
CeR107.6
CeZC84.3
CeCO7H6.3
KIAA0627
KIAA0622
Mast
SpStu1p
Stu1p

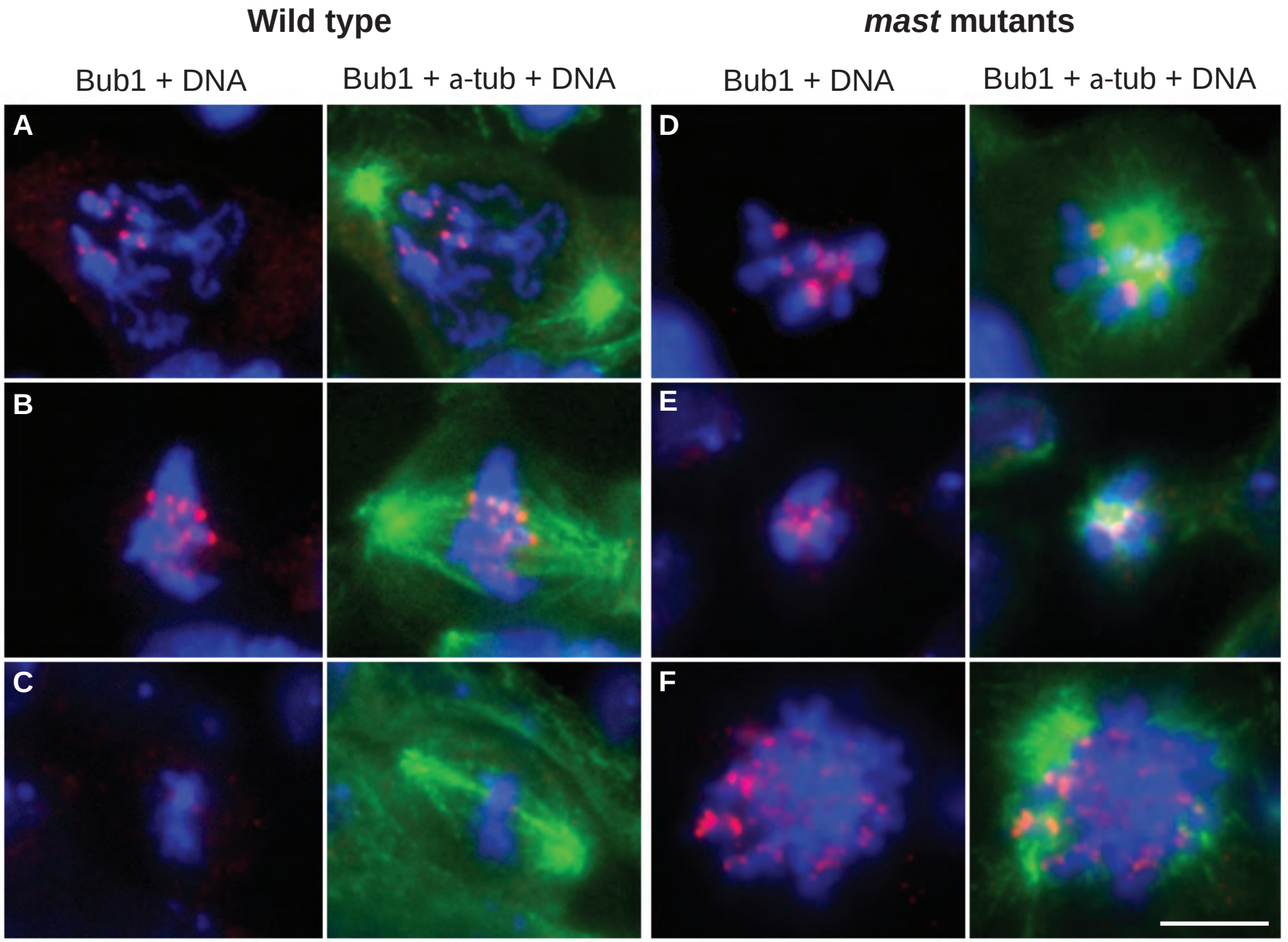

DNA
Mast
a-tubulin
Merge
Interphase
Prophase
Metaphase
Anaphase A
Anaphase B
Telophase
Cytokinesis


S2
HeLa
Interphase
S2
HeLa
Prophase
S2
HeLa
Prometaphase
Metaphase
Anaphase
Early Telophase
Late Telophase


1 2 3 4 5 6 7 8
kDa
205
121
74
47
Coomassie
Mast
a-tubulin


A
B
C
DNA
Mast
g-tub
Merge
D
DNA
Mast
Polo
Merge
E


Cnn (zoom 3.5x)
Cnn
Tubulin
DNA
Merge
A
B
C
D
E
F
Cnn
Tubulin
DNA
Merge
G


Wild type
mast mutants
Bub1 + DNA
Bub1 + a-tub + DNA
Bub1 + DNA
Bub1 + a-tub + DNA
A
B
C
D
E
F