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Human RASSF7 regulates the microtubule cytoskeleton and is required for spindle formation, Aurora B activation and chromosomal congression during mitosis

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Short title: Human RASSF7 regulates microtubule dynamics during mitosis

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Abbreviations used: COBRA, Combined bisphite restriction analysis; H a E, Haematoxylin and Eosin; PLK 1, Polo Like Kinase 1; RASSF, Ras association domain family; small interfering RNA, siRNA; short hairpin RNA, shRNA.

SUMMARY

RASSF7, a member of the N-terminal Ras association domain family, has increased expression in various cancers and, based on our previous work in *Xenopus* embryos, may be a regulator of mitosis. In the present study we address, for the first time, the role of human RASSF7 in mitosis. We demonstrate that RASSF7 is expressed in a broad range of different cell types and that this expression could be enhanced following exposure to hypoxia. Knocking down RASSF7 in human cell lines inhibited cell growth and induced defects in mitosis, including aberrant spindle formation and a failure in chromosomal congression. In order to understand the molecular basis of the defects in more detail, we analysed the activity of mitotic signalling proteins and found that activation of Aurora B did not occur in cells in which RASSF7 was knocked down. We also show that endogenous RASSF7 protein localises to the centrosome and demonstrate using microtubule regrowth assays that RASSF7 is an important regulator of microtubule dynamics. Based on these observations, we propose that, due to its key role in regulating the microtubule cytoskeleton, RASSF7 is required for mitosis in human cells.

INTRODUCTION

RASSF7, previously known as HRC1 [1] and C11orf13, is a member of the N-terminal Ras association domain family (RASSF) family [2], a group of four proteins (RASSF7-10) which are structurally distinct from the classical RASSF proteins (RASSF1-6) [3, 4]. There is emerging evidence to suggest that at least two members of the N-terminal RASSF family may be tumour suppressors. *RASSF8* has reduced expression in lung cancers and knocking down its expression causes an increase in growth [5-7] and *RASSF10* expression is epigenetically inactivated in leukaemias and thyroid cancers [8, 9]. In contrast, microarray studies have demonstrated that *RASSF7* expression is upregulated in a range of cancers [10-14], but little is known about its function. Our previous work showed that knocking down the expression of *Xenopus rassf7* in embryos, caused a failure in spindle formation, nuclear fragmentation and apoptosis [15]. This suggests that RASSF7 is required for mitosis, a complex process involving microtubule nucleation from the spindle pole and the stable attachment of microtubules to the kinetochore [16-18]. However, RASSF7 function has not been studied in species other than *Xenopus* and the role that it plays in mitosis remains unknown. In the present paper we study the function of human RASSF7 and establish why it is required for mitotic progression. Our data shows that knocking down RASSF7 causes a reduction in cell growth and multiple defects in mitosis including a failure in spindle formation, Aurora B activation and chromosomal congression. In addition we show that RASSF7 localises to the centrosome and that it plays a crucial role in regulating microtubule growth. These results indicate that RASSF function is crucially important for mitosis because it regulates microtubule growth from the centrosome.

EXPERIMENTAL

Cell culture

Cell culture medium and reagents were purchased from Sigma. H1792 and HeLa cells were maintained at 37°C, 5% CO₂ in DMEM, with 10% Fetal Bovine Serum, 1% penicillin/streptomycin, 1% glutamine. Mitotic cell enrichment and the microtubule depolymerisation and regrowth assays were carried out as described [19, 20]. To expose cells to hypoxic conditions, cultures were placed in a humidified 37°C multigas incubator with 5% CO₂/95% nitrogen for 3 hours. Residual O₂ levels

were monitored regularly and found to be consistently less than 0.1%. Control cultures, maintained under oxic conditions (21% O₂), were processed in parallel.

Cloning, transfection and gene silencing

The coding sequence of human RASSF7 (image clone: 40032773) was cloned into the expression vector pcDNA3-DEST53 (Invitrogen) using Topo and Gateway cloning (Invitrogen) to generate a GFP fusion protein. This was transfected into HeLa cells using Lipofectamine (Invitrogen). H1792 cells were transiently transfected with siRNA oligos targeting RASSF7, RASSF7 KD1 (Ambion oligo ID 13242), RASSF7 KD2 (Ambion oligo ID 13338) or a control oligo targeting luciferase (MWG) using Oligofectamine (Invitrogen). The soft agar colony forming assay was conducted as described [7]. shRNA silencing of gene expression was achieved using RASSF7 (code: KHO9867N) and scrambled control shRNA. pGeneClip/Neo vectors from SuperArray Bioscience. 2x10⁵ HeLa cells were seeded one day prior to transfection. Transfected cells were selected for 14 days in 1.2 mg/ml G418 (Sigma) and subsequently counted and analysed. The experiment was repeated as described above, except that the seeding of 2x10⁵ HeLa cells was carried out post selection.

Immunoblotting

Immunoblotting was carried out and quantitated using an Optichem detector with associated software (Ultra Violet Products) as described [21]. After transfer, the membranes (Whatman) were blocked in 5% milk powder in Tris-Buffered Saline Tween-20. Antibodies: rabbit anti RASSF7 (AVIVA ARP34390_T100), mouse β Tubulin (Sigma T4026), mouse anti phospho Histone H3 (Abcam 14955). The anti RASSF7 antibody produced a band at 34 KDa as predicted.

Immunofluorescence

Cells were grown on coverslips, fixed and permeabilised at -20°C in methanol for 10 minutes and incubated in blocking solution (phosphate-buffered saline, 1% bovine serum albumin and 3% normal goat serum). Primary antibodies were: rabbit anti Pericentrin (Covance PRB-4325), mouse anti γ Tubulin (Sigma T6557), mouse anti RASSF1A (eBioscience 14-6888). The rabbit anti RASSF7 (AVIVA ARP34390_T100) required antigen retrieval to unmask the epitope, this involved EDTA treatment (1 mM, pH: 8.0) for 1.5 hours at 37°C prior to blocking. For mouse anti α Tubulin (Sigma T9026), mouse anti Aurora A (BD biosciences 610938), mouse anti Aurora B (BD biosciences 611082), rabbit anti phospho Aurora (Cell Signalling 2914), rabbit anti phospho Polo Like Kinase 1 (Biolegend 618601), mouse anti INCENP (Upstate 05-940), anti rabbit phospho CENP-A (Millipore 04-792) and rabbit anti active caspase 3 (Abcam ab13847) cells were fixed in 4% paraformaldehyde and permeabilised in 0.1% Triton X-100 at room temperature. Incubation with relevant secondary antibodies and DAPI (1 μ g/ml, Sigma) followed. Cells were then mounted in mowiol (Sigma), visualised using a Zeiss LSM 510 confocal and images captured and processed using LSM image browser software.

Promoter methylation analysis

Combined bisphite restriction analysis (COBRA) [8] was used to determine the methylation status of the 5' CpG island associated with the *RASSF7* gene. Primers used for methylation analysis were: TTTAGGAGYGGGGTTAGATATTTATTT and TTAACCTACCTCTATCACRCCCCTCCC.

Mouse in situ hybridization

In situ hybridisation was carried out as previously described [7]. The mouse *Rassf7* probes were generated using a pCMV.sport6 vector (image clone: 4206694) and an SP6 (sense) or T7 polymerase (anti sense) following digestion with *Stu*I or *Ahd*I enzymes, respectively. Haematoxylin-eosin staining was performed on paraffin sections according to standard methods.

Statistical analysis

Means and standard deviations were calculated and plotted using Microsoft Excel. Each experiment was repeated at least three times. $p < 0.05$ was considered significant. Statistical analysis was carried out using unpaired t tests.

RESULTS AND DISCUSSION

We begun by establishing which tissues and cell types express RASSF7. *In Situ* hybridisation was carried out on mouse embryos and adult organs and *Rassf7* was found to be expressed in all tissues examined, however, the level of expression varied, with particularly high levels seen in the lung and the brain (Fig. 1A-C). Immunoblotting of lysates from mammalian and human cell lines showed a major band which migrated at the predicted size of RASSF7 (34 KDa) (Fig. 1D) and this band is specific for RASSF7 because it was reduced by two different knockdown approaches (Fig. 2A+B). The immunoblotting showed that RASSF7 was expressed in all cell lines tested (Fig. 1D). Consistent with the fact that RASSF7 is expressed in a number of different cancerous cell lines, and in contrast to other RASSF proteins [3, 4], we found no evidence of *RASSF7* gene silencing by promoter methylation in 57 cancer cell lines (Supplementary Fig. S1A and data not shown). *RASSF7* mRNA is upregulated by hypoxia [22, 23] which, given the hypoxic nature of solid tumours, might explain the increase in *RASSF7* expression seen in tumour cells [10-14]. Western blotting demonstrated that RASSF7 protein levels are also increased by hypoxic insult (Fig. 1E), showing that RASSF7 protein is likely to be increased by the hypoxic environment found in solid tumours.

Several members of the RASSF family are believed to be tumour suppressors and act to restrain growth [3, 4]. Consistent with this idea, knockdown of RASSF6 or RASSF8 causes an increase in the anchorage independent growth of the human lung adenocarcinoma cell line H1792 [7, 24]. To establish if RASSF7 functions in a similar way we analysed the effect of RASSF7 knockdown on anchorage independent growth. RASSF7 expression was knocked down in cells transfected with RASSF7 KD 2 siRNA (Fig. 2A). In contrast, RASSF7 KD 1 did not efficiently knockdown RASSF7 and this siRNA was used as an additional negative control along with the standard control siRNA (Fig. 2A). H1792 cells showed significantly lower colony-forming activity in soft agar ($p < 0.01$) after RASSF7 KD 2 siRNA treatment as both the size and the number of colonies was reduced compared to the controls (Fig. 2A). These findings show that, unlike RASSF6 and RASSF8 which act to restrain growth, RASSF7 is essential for anchorage-independent growth. The fact that RASSF7 is required for growth may explain why its expression is not silenced in the cancer cell lines analysed above.

To address why RASSF7 is required for growth we carried out shRNA knockdowns in HeLa cells. The alternative knockdown strategy ensures the specificity of the phenotype, while HeLa cells are easier to analyse than H1792 cells. Consistent with the data in H1792 cells, shRNA knockdown of RASSF7 in HeLa cells caused a reduction in cell number (Fig. 2B). This could be caused by increased apoptosis, however the percentage of active Caspase 3 positive RASSF7 KD cells was not significantly different to controls (Supplementary Fig. S2). To establish if the reduction in cell number was caused by a defect in mitosis, analogous to that seen in *Xenopus*, knockdown cells were examined and mitotic aberrations in metaphase RASSF7 knockdown cells were clearly evident. Loss of RASSF7 resulted in a failure in chromosomal congression, demonstrated by an increase in the number of mitotic cells which had failed to align their chromosomes (63% vs 18% in controls, $n=300$, $p < 0.01$) (Fig 2C), an increase in metaphase cells with lagging chromosomes (21.5% vs 6% in controls, $n=300$, $p < 0.01$) (Fig. 2C, white arrows) and a decrease in metaphase cells with

correctly aligned DNA (15.7% vs 75.7% in controls, $n=300$, $p < 0.001$). Spindle defects were also found in RASSF7 deficient cells, with spindles showing less pronounced polarisation towards the DNA and a more radial organisation of microtubules (Fig. 2C, red arrows). Finally, there was a small increase in the rate of multi-polar spindles (32.6% vs 23.7% in controls, $n=300$, $p < 0.05$) (Fig. 2C, arrowheads).

In order to understand the molecular basis for these mitotic defects we examined the affect of RASSF7 knockdown on established regulators of mitosis. Analysis of the activation of Polo Like Kinase 1 (PLK 1) [25], using an antibody which recognises the active form, showed similar staining in RASSF7 knockdown and control cells (Fig. 3A). The localisation of RASSF1A, which is required for progression through mitosis [26-28], to the centrosome appeared the same in knockdown and control cells (Fig. 3B). The localisation and activation of Aurora A and Aurora B [29], was also examined (Fig. 3C,D). An antibody to Aurora A and Aurora B and a phospho specific antibody which recognises the active form of Aurora A and B enzymes were used. Aurora A was present at the centrosome in RASSF7 (Fig. 3C, arrows) and Aurora B was present at the kinetochores (Fig. 3D, arrowheads). The active Aurora staining was present at the centrosomes (Fig. 3C+D, arrows) but strongly reduced at the kinetochore (Fig. 3C+D, arrowheads) in the RASSF7 knockdowns, with only 9.3% of cells showing strong Aurora B staining compared with 83.8% in the controls ($n= 240$, $p < 0.001$), suggesting a failure in Aurora B activation. This loss appeared to be restricted to metaphase as the activation of Aurora B during cytokinesis appeared normal (Supplementary Fig. S3A). To confirm the loss of Aurora B activity the phosphorylation of an Aurora B target CENP-A [30] was analysed. Phospho CENP-A levels were reduced with only 22% of cells showing strong staining compared with 93.4% of control cells ($n=210$, $p < 0.001$), providing further evidence that Aurora B is not activated normally in the absence of RASSF7 (Fig. 3E). The loss of Aurora B activity is likely to contribute to the mitotic defects seen in RASSF7 knockdowns and consistent with this hypothesis inhibiting Aurora B causes chromosomal congression defects similar to those in RASSF7 knockdown cells [31, 32].

To begin to understand the mechanistic basis for these mitotic defects we analysed the subcellular localisation of RASSF7. *Xenopus* rassf7 fusion proteins localise to the centrosome [15] and a GFP tagged human RASSF7 protein also localised to the centrosome in HeLa cells (Supplementary Fig. S4A). To establish the localisation of endogenous RASSF7, HeLa cells were stained with an anti RASSF7 antibody. This showed strong staining which co-localised with γ Tubulin, a component of the pericentriolar material (Fig. 4A) and this staining was specific as the signal was not present in RASSF7 knockdown cells (Supplementary Fig. S4B). RASSF7 staining was found at the centrosome in interphase cells and throughout all stages of mitosis (Fig. 4A), showing that RASSF7 is not recruited to the centrosome during mitosis, but localises there in dividing and non-dividing cells. The amount of RASSF7 protein was also not increased in mitotic cells (Supplementary Fig. S1B), so despite RASSF7 having a role in mitosis, RASSF7 protein levels do not appear to be regulated during the cell cycle. The localisation of RASSF7 at the centrosome was not affected by microtubule depolymerisation with nocodazole (Fig. 4B), indicating that human RASSF7 is an integral centrosomal component whose localisation is microtubule independent. Our previous data with *Xenopus* rassf7 suggested its localisation was microtubule dependent, this discrepancy could well be due to the use of a fusion protein in the *Xenopus* experiments.

The localisation of RASSF7 at the centrosome and the spindle defects seen in the knockdowns suggest that RASSF7 might have a role in regulating microtubules. To test if RASSF7 is required for microtubule polymerisation, we investigated whether microtubule regrowth was affected by the loss of RASSF7. Cells were treated with nocodazole and subsequent microtubule regrowth examined. The initial nucleation of microtubules in knockdown cells appeared normal (5 minutes) but after 15 minutes 53% of RASSF7 knockdowns cells showed a delay in microtubule regrowth compared with 14% of controls ($n= 100$, $p < 0.001$) (Fig. 4C, arrows). The microtubules in RASSF7

knockdown cells also appeared more bent and looped than controls (Fig. 4C, arrows). A similar delay in microtubule regrowth was seen at 30 minutes. After 60 minutes the number of microtubules in the RASSF7 knockdowns resembled the controls (Fig. 4C), showing that RASSF7 knockdowns eventually produce similar numbers of microtubules to control cells. However, the microtubules still appeared less straight than the control microtubules (Fig. 4C, arrows). This assay demonstrates that RASSF7 is a key regulator of microtubule growth and provides an explanation for the spindle defects seen in mitosis. The broad expression of RASSF7 and its localisation to the centrosomes of interphase cells suggests that RASSF7 may also regulate microtubules in non-dividing cells.

A role in regulating microtubules can explain the defects in spindle formation seen in the knockdowns, but why is RASSF7, which localises at the centrosome, required for the activation of Aurora B at the kinetochores? Aurora B activation requires the recruitment of INCENP [33], but RASSF7 knockdown did not compromise the localisation of this protein (Supplementary Fig. S3B). Interestingly, Aurora B activation also requires contact with microtubules [34, 35]. We propose that the defect in spindle formation seen in the RASSF7 knockdowns might stop microtubules from correctly interacting with Aurora B and cause the failure in Aurora B activation. This argues that the primary role of RASSF7 is in regulating microtubule growth and mis-regulation of this process is responsible for the spindle defects and the loss of Aurora B activation. The aberrant spindle and loss of Aurora B would then both contribute to the dramatic failure in chromosomal congression seen in RASSF7 knockdown cells.

In summary this work provides the first functional analysis of human RASSF7 and shows that down-regulation of RASSF7 impairs cell growth and causes defects in mitosis, including abnormal spindle assembly, loss of Aurora B activation and a failure in chromosomal congression. We propose that these mitotic defects are caused because RASSF7 localises to the centrosome and regulates microtubule dynamics.

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Figure Legends

Figure 1: RASSF7 is expressed in a wide range of cell types. (A) *In situ* hybridisation on E10.5 mouse embryos (22x). (B) *In situ* hybridisation on E15.5 mouse embryos (8x). (C) *In situ* hybridisation on mouse adult tissues (50x). The Haematoxylin and Eosin (H a E) staining shows the tissue architecture. (D) Western blotting shows expression of RASSF7 protein in different mammalian cell lines. (E) RASSF7 expression levels increased in HeLa cells exposed to hypoxic conditions. * $p < 0.05$ vs corresponding controls.

Figure 2: RASSF7 knockdown caused a reduction in cell number and defects in mitosis (A) RASSF7 siRNA oligonucleotides KD 2, but not KD 1, blocked RASSF7 expression and prevented H1792 cells from forming colonies (arrows). Quantification is based on the number of colonies bigger than 50 μm (** $p < 0.01$ RASSF7 KD2 vs Luciferase KD and RASSF7 KD1). (B) shRNA knockdown of RASSF7 reduced HeLa cell numbers. The difference between control and RASSF7 depleted cells was 2.6 ± 0.6 fold (** $p < 0.001$), based on five independent experiments. This was not due to variations in the selection process as it was observed when cells were selected, replated at the same density and then allowed to grow (1.9 ± 0.4 fold reduction). (C) RASSF7 knockdown HeLa cells exhibit defects in spindle formation (red arrows highlight the more radial microtubules) and chromosomal congression, with an increase in cells which had failed to align their DNA (63% vs 18% in controls, $n=300$, $p < 0.01$) and an increase in cells with lagging chromosomes (21.5% vs 6% in controls, $n=300$, $p < 0.01$, white arrows). There was also a small increase in tripolar spindles (32.6% vs 23.7% in controls, $n=300$, $p < 0.05$, arrowheads). Scale bars 100 μm (A+B), 5 μm (C).

Figure 3: Analysis of mitotic signalling proteins in RASSF7 knockdown HeLa cells. (A) active PLK1 (green) appeared normal. (B) RASSF1A (green) appeared normal. (C+D) Aurora A (red, arrows) and Aurora B (red, arrowheads) appeared to localise normally. Phospho Aurora at the centrosomes (green, arrows) appeared normal but phospho Aurora at the kinetochore (green, arrowheads) was reduced (9.3% of cells showed strong Aurora B staining compared with 83.8% in the controls, $n=240$, $p < 0.001$) suggesting that activation of Aurora B fails. (E) Phospho CENP-A (green, arrow) was reduced (22% of cells showed strong staining compared with 93.4% of control cells, $n=210$, $p < 0.001$). Blue: nuclear staining. Scale bar, 5 μm .

Figure 4: RASSF7 localises to the centrosome and knocking down its expression causes defective microtubule regrowth in HeLa cells. (A) Co-localisation of RASSF7 with the centrosomal marker γ Tubulin (arrows). (B) Microtubules (α Tubulin/green) were disrupted by treatment with nocodazole (300 ng/ml) for 1 hour at 37°C (top panels). This did not affect RASSF7 localisation at the centrosomes which were stained for γ Tubulin (arrows, bottom panels). (C) Microtubule regrowth assay. Microtubules were disrupted with nocodazole (300 ng/ml) for 1 hour and subsequent regrowth monitored at different time points. RASSF7 knockdowns had fewer microtubules (after 15 and 30 minutes) and more bent microtubules (arrows). Quantification showed that after 15 minutes 53 % of knockdown cells had a delay in regrowth compared with 14 % of controls ($n=100$, $p < 0.001$). Blue: nuclear staining. Scale bars 5 μm (A), 10 μm (B+C).

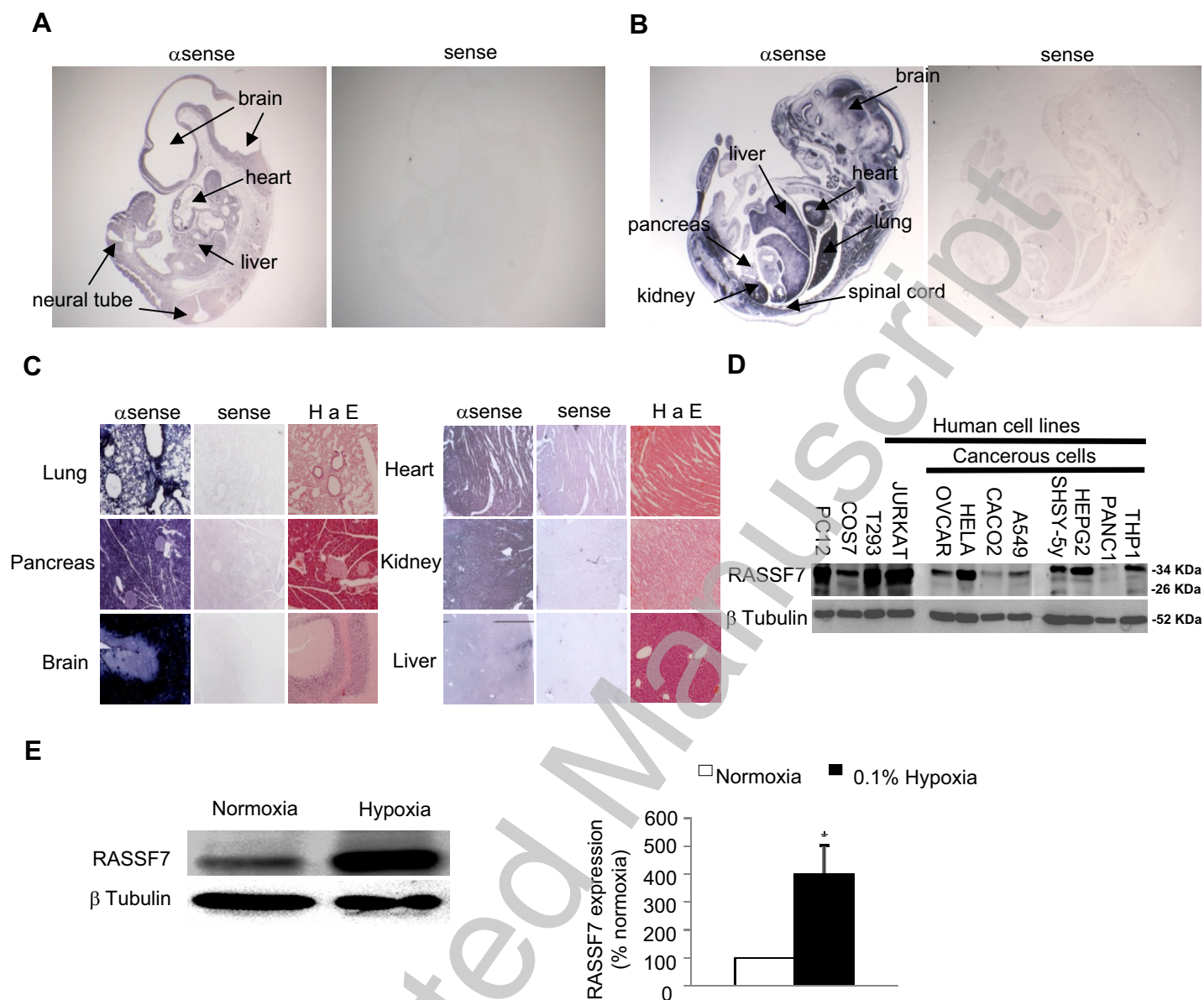


Figure 1 Recino *et al.*

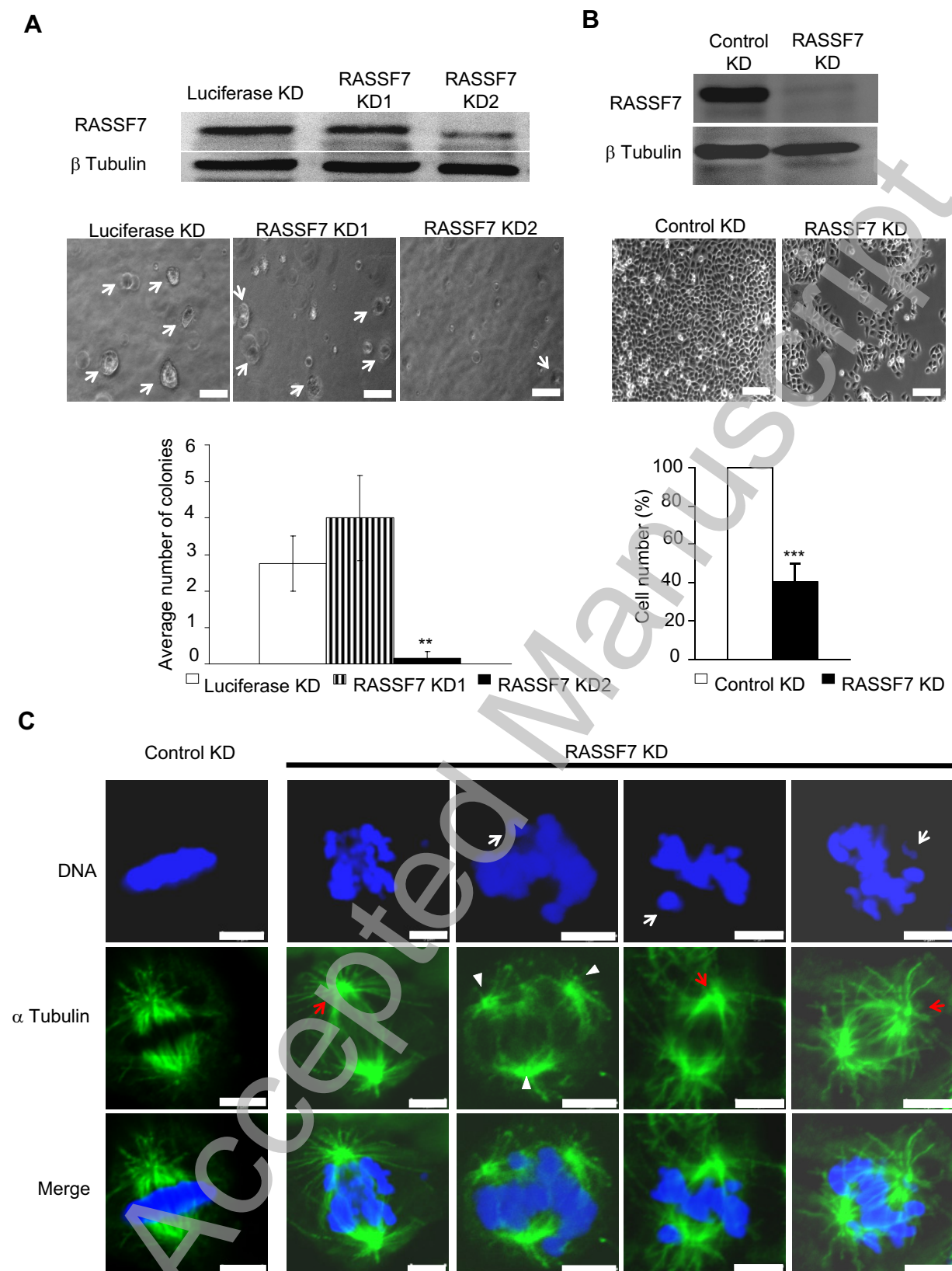


Figure 2 Recino *et al.*

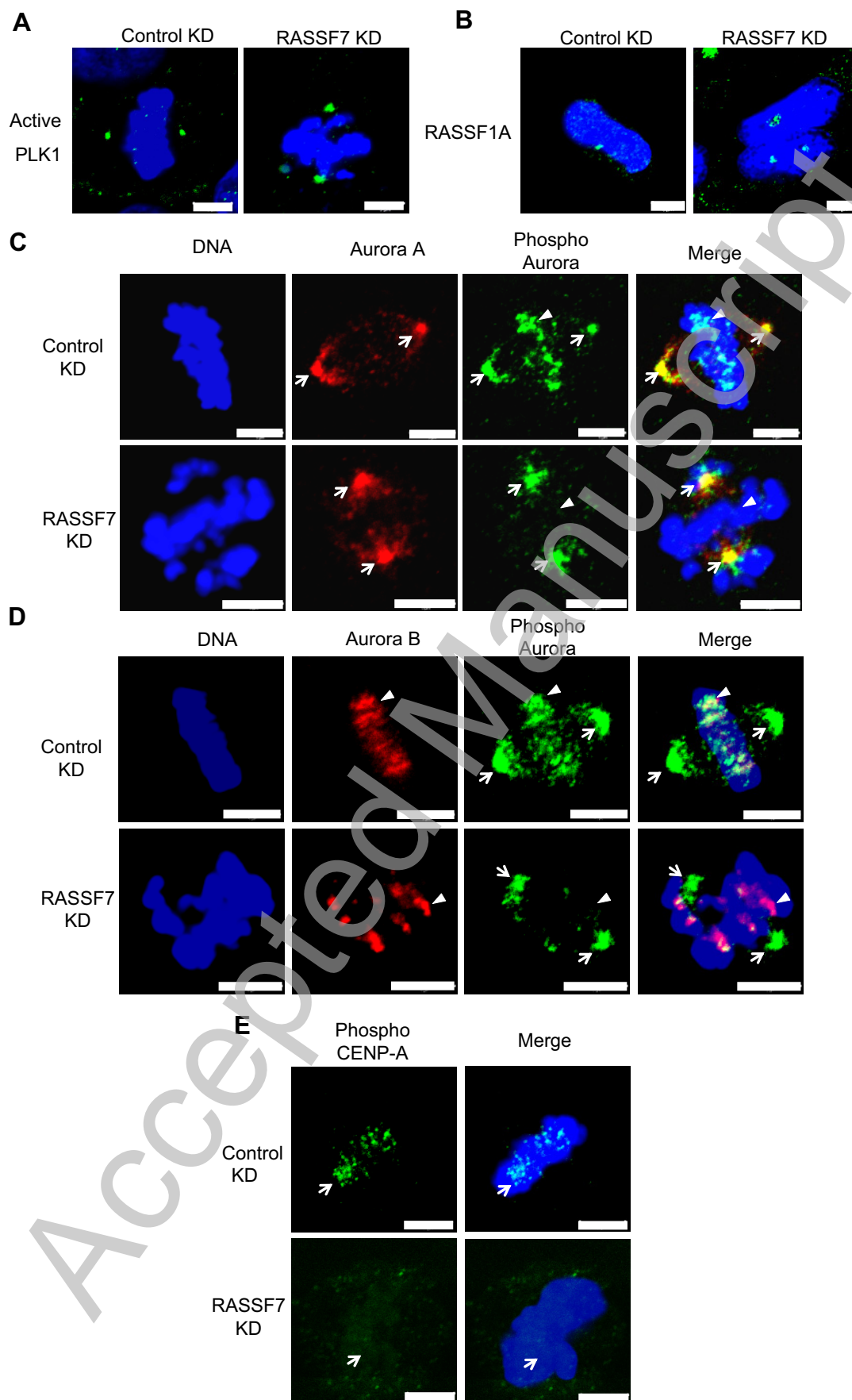


Figure 3 Recino *et al.*

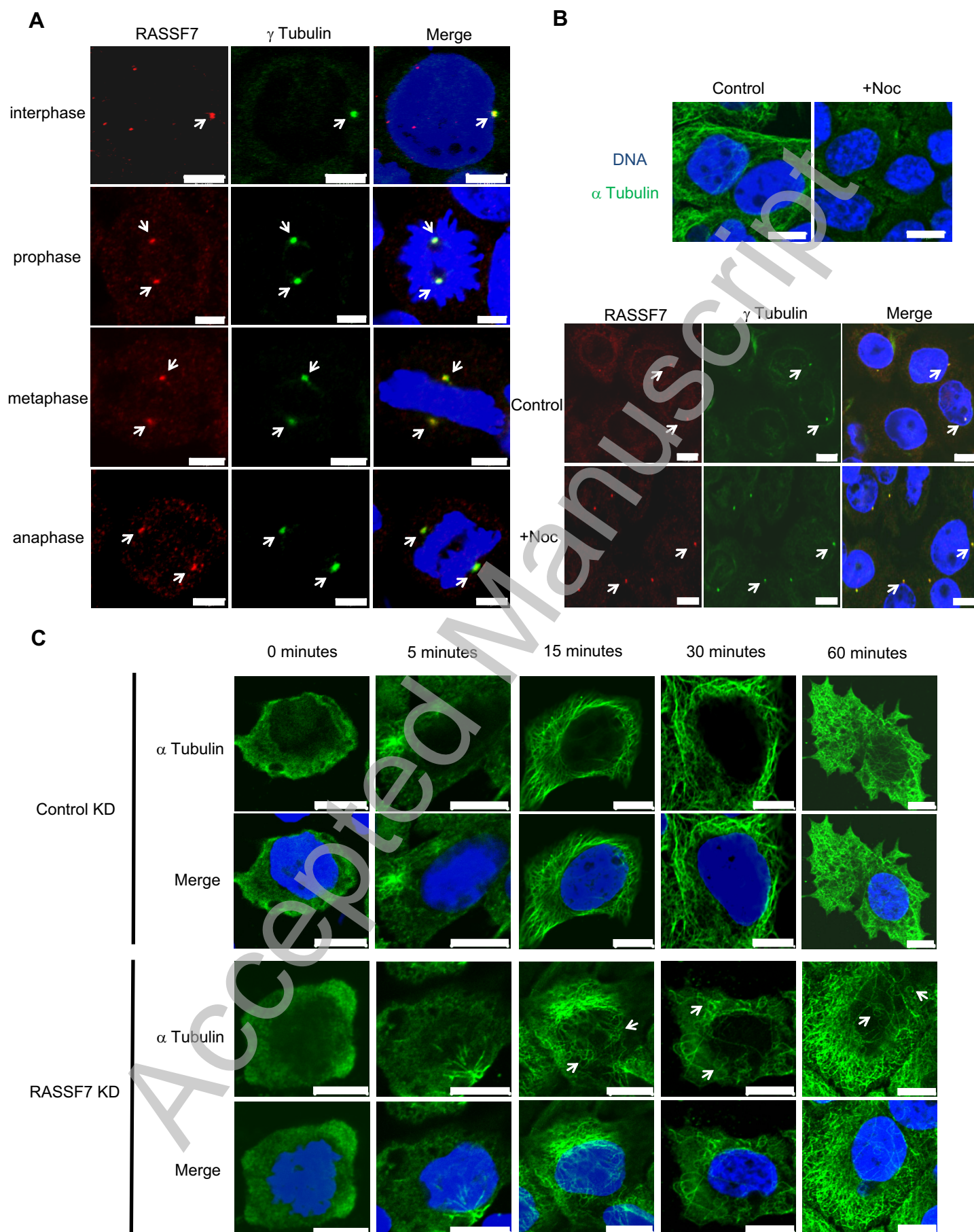


Figure 4 Recino *et al.*