

Truncated RASSF7 promotes centrosomal defects and cell death

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HIGHLIGHTS

- The coiled-coil of rassf7 is responsible for its centrosomal localisation.
- The RA domain does not play a key role in the localisation of rassf7.
- C-terminal truncation of rassf7 drives centrosome defects and cell death.
- A similar truncation has been identified in a human tumour sample.
- We propose that RASSF7 might act as an oncogene in a small subset of tumours when truncated in this way.

KEYWORDS

RASSF7; centrosome; mitosis; oncogene; *Xenopus*; cell death

ABSTRACT

RASSF7 protein localises to the centrosome and plays a key role in mitosis. Its expression is also increased in a range of tumour types. However, little is known about the molecular basis of RASSF7's function and it is not clear if it acts as an oncogene in the cancers where its levels are elevated. Here, we carry out the first analysis of the domains of rassf7, focusing on which of them are responsible for its localisation to the centrosome. Constructs were generated to allow the expression of a series of truncated versions of rassf7 and the level of centrosomal localisation shown by each protein quantified. This analysis was carried out in *Xenopus* embryos which are a tractable system where rassf7 localisation can easily be studied. Our data shows that the coiled-coil domain of rassf7 is required and sufficient to direct its centrosomal localisation. The RA domain did not appear to have a role in mediating localisation. Surprisingly, removal of the extreme C-terminus of the protein caused rassf7 to accumulate at the centrosome and drive centrosome defects, including accumulation of the centrosomal protein γ -tubulin and an amplification of the number of γ -tubulin foci. These effects required the centrosomal localisation mediated by the coiled-coil domain. Later in development cells expressing this truncated rassf7 protein underwent cell death. Finally, analysis of a database of tumour sequences identified a mutation in RASSF7 which would cause a similar C-terminal truncation of the protein. Based on our data this truncated protein might drive centrosomal defects and we propose the hypothesis that truncated RASSF7 could act as an oncogene in a small subset of tumours where it is mutated in this way.

INTRODUCTION

Mitosis is a spectacular part of the cell cycle and it is over one hundred years since errors in this process were first proposed to be linked to the origin of cancers (Boveri, 1902). In animal cells centrosomes play an essential role during mitosis, they act to nucleate and anchor microtubule minus ends in a process that requires γ -tubulin (Luders and Stearns, 2007) and provide a platform for a range of mitotic signalling proteins (Basto and Pines, 2007). Defective regulation of centrosomal proteins has been linked to cancer formation (de Carcer and Malumbres, 2014; Satinover et al., 2004), making studying their function important to provide a better understanding of their role in mitosis and cancer formation.

One protein which localises to the centrosome and has a key role in regulating mitosis is RASSF7. There are ten RASSF family members which can be divided into 2 groups, the classical RASSFs (RASSF1-6) have a C-terminal Ras Association (RA) domain, while the N-terminal RASSFs (RASSF7-10) have their RA domains at the opposite end of the protein (Sherwood et al., 2008; Sherwood et al., 2010). Several of the classical RASSF proteins are tumour suppressors with established roles in a range of biological processes, including microtubule stability, cell cycle control, apoptosis, immune system regulation and nuclear transport (Richter et al., 2009; Volodko et al., 2014). In contrast, RASSF7 (discussed below) and the other N-terminal proteins are much less well studied (Hesson et al., 2009; Lee et al., 2011; Li et al., 2013; Lock et al., 2010; Underhill-Day et al., 2011; Wang et al., 2014).

RASSF7 was originally identified and called HRC1 in a study which searched for genes that are close to HRAS1 in the genome (Weitzel et al., 1992). Our laboratory then identified it in a microarray screen comparing superficial and deep cells in early *Xenopus* embryos, where it was initially called carcinoma associated protein (Chalmers et al., 2006). RASSF7 has broad expression in early *Xenopus* embryos, with high levels in neural and epidermal tissue (Sherwood et al., 2008). Studies of Human cell lines and mouse embryos also showed it to be expressed in a wide range of cell and tissue types (Recino et al., 2010). Functional analysis demonstrated that RASSF7 is required for mitosis in cells from *Xenopus* embryos, where morpholino knockdown caused mitotic defects (Sherwood et al., 2008). In Human cells shRNA knockdown also caused defects in mitosis, including aberrant microtubule regrowth, a failure in chromosome congression and reduced Aurora B activity at the kinetochores (Recino et al., 2010). It has also been shown that RASSF7 negatively regulates pro-apoptotic JNK signalling by inhibiting the activity of phosphorylated mitogen-activated protein kinase 7 (MKK 7) (Takahashi et al., 2011), but it is not currently clear if the stress and mitotic roles are linked. Consistent with its role in mitosis, both human and *Xenopus* studies showed that RASSF7 localises to centrosomes. The aforementioned studies were performed using fusion proteins in *Xenopus* embryos and by staining for endogenous RASSF7 in human cell lines (Recino et al., 2010; Sherwood et al., 2008).

In contrast to the loss of expression seen with many classical RASSF proteins, microarray studies have shown that RASSF7 expression is up-regulated in pancreatic, islet cell, endometrial, ovarian cell and thyroid cancers (Friess et al., 2003; Li et al., 2013; Logsdon et al., 2003; Lowe et al., 2007; Tan et al., 2009) and the increase in expression may be driven in some of these tumours by the fact that RASSF7 is up-regulated by hypoxia (Camps et al., 2008; Liang et al., 2009; Recino et al., 2010). Despite an increase in expression in many tumours it is not clear whether RASSF7 is acting as an oncogene and contributing to tumour formation or whether its increased expression in tumours is simply a consequence of tumour formation. To begin to understand if RASSF7 might act as an oncogene we are working on establishing the molecular basis for its function.

Here we focus on determining which domains of RASSF7 are required to localise it to the centrosome. *Xenopus* embryos were used as they are a tractable, in vivo, vertebrate model of development and disease with good community resources and are well suited for carrying out studies of protein function (Bartlett and Weeks, 2008; Conlon et al., 2012; Karpinka et al., 2014; Pearl et al., 2012). Our data shows that the coiled-coil domain of RASSF7 is sufficient and required for its centrosomal localisation, whereas the RA domain does not appear to have a role in the localisation of the protein. Surprisingly, removing the C-terminal domain of RASSF7 resulted in a large increase in levels of RASSF7 at the centrosome. Expressing this truncated construct also caused centrosomal defects including a striking increase in the size and number of the foci positive for the centrosomal protein γ -tubulin. Mutating the coiled-coil abrogated these effects, arguing they require RASSF7 to localise at the centrosomes. Cells expressing this truncated version of RASSF7 ultimately underwent cell death later in development and an increase in embryo death was also observed. Finally, we analysed sequences of RASSF7 from a large database of human cancer samples and identified a mutation predicted to result in a similar truncation from a renal clear cell carcinoma. Our data shows that C-terminally truncated RASSF7 promotes centrosomal defects and we speculate that RASSF7 could be oncogenic in a small subset of tumours which harbour such truncating mutations.

MATERIALS AND METHODS

DNA Sequences, Plasmids and database/bioinformatics Analysis

Xenopus RASSF7 pBlueScript SK(-) clone (X1095b08) was previously cloned into the gateway system to generate an N-terminal GFP-rassf7 fusion pCS2 construct (Sherwood et al., 2008). A pCS2 GFP construct was also used as previously described (Chalmers et al., 2005). The sequence of the *Xenopus* RASSF7 protein was obtained from NCBI: Ras-association domain family 7 [*Xenopus laevis*] (ABR21988.1). The Simple Modular Architecture Research Tool (SMART : <http://smart.embl-heidelberg.de/>) was used to identify potential protein domains and this information was then used to inform design of the GFP-rassf7 truncation constructs and the coiled coil mutations which were generated as described below.

Mutations in RASSF7 from human cancer samples were identified using cBioPortal from the Cancer Genomics website (<http://www.cbioportal.org/public-portal/index.do>) which contained data from 89 cancer genomic studies covering a total of 20,958 tumour samples (August-Nov 2014). Sequence of the human protein was obtained from the NCBI (Ras-association domain containing protein 7 isoform 1 [Homo sapiens]:NP_003466.1) and SMART was then used to predict the effect of the mutations on the expected domains produced by human RASSF7 from these cancer samples.

Primer Design and Site-directed Mutagenesis

The QuickChange site-directed mutagenesis kit (Agilent Technologies) was used to make plasmids coding for a series of truncated versions of GFP-rassf7 (Figure 1), by mutating the original GFP-rassf7 CS2 plasmid. Primers were designed according to the kit guidelines and PCR was carried out following the protocol supplied by the manufacturer. Following mutagenesis all constructs were sequenced to confirm the mutations were made correctly.

The following PCR primers were used:

GFP-rassf7 (RA+A+CC) forward:	5'-CTCAGGCAGTGTAACCTCCAATAATTTATCCTTCAGACAGG-3';
reverse:	5'-CCTGTCTGAAGGATAAATTATTGGAGGTTACTGCTGAG-3';
GFP-rassf7 (RA+A) forward:	5'-GCTTGAGAAGAGATGTTTTAGGAGGATGAACTGCAAAGG-3';
reverse:	5'-CCTTTGCAGTTCATCCTCCTAAAACATCTCTTCTCCAAGC-3';
GFP-rassf7 (RA) forward:	5'-GAGGCGCACAGGACCATGATTAGCTGAACGTCCATC-3';
reverse:	5'-GATGGACGTTACGTAATCATGGTCCTGTGCGCCTC-3';
GFP-rassf7 (A+CC+B) forward:	5'-CTCATTCTCGTGTTCATATGAGCTTAGCTGAACGTCCATCCTC-3';
reverse:	5'-GAGGATGGACGTTACGTAAGCTCATATTGAACACGAGGAATGAG-3';
GFP-rassf7 (CC+B) forward:	5'-CCTCATTCTCGTGTTCATATTGGGAGGATGAACTGC-3';
reverse:	5'-GCAGTTCATCCTCCAATTGAACACGAGGAATGAGG-3';
GFP-rassf7 (A+CC) forward:	5'-CTCAGGCAGTGTAACCTCCAATAATTTATCCTTCAGACAGG-3';
reverse:	5'-CCTGTCTGAAGGATAAATTATTGGAGGTTACTGCTGAG-3';
GFP-rassf7 (CC) forward:	5'-CCTCATTCTCGTGTTCATATGTGGGAGGATGAACTGCAAAGGG-3';
reverse:	5'-CCCTTTGCAGTTCATCCTCCACATATTGAACACGAGGAATGAGG-3';
GFP-rassf7 (A) forward:	5'-

GGAGAAGAGATGTTTTGAGAGGATGAACTGCAAAGGGC-3'; reverse: 5'-
 GCCCTTGCAGTTCATCCTCTCAAAACATCTCTTCTCC-3', GFP-rassf7 (B) forward: 5'-
 CCTCATTCTCTCGTGTCAATGCCAGGAATCAGGAGC-3'; reverse: 5'-
 GCTCCTGATTCTGGCATTGAACACGAGGAATGAGG-3'.

To add point mutations to the coiled-coil region hydrophobic residues were identified and mutations made that would replace the hydrophobic amino acid with the hydrophilic amino acid proline. The smart tool was used to select the mutations which would cause the biggest disruption in the coiled coil and these were then made using the the QuickChange kit.

The following PCR primers were used for coiled coil mutations: GFP-rassf7 (RA+A+CC'+B) forward: 5'-GAGGAGTACACCGTGAAAATACAGGAGCTCACTGAGCGG-3' reverse: 3'-CCGCTCAGTGAGCTCCTGTATTTTACGGTGTACTCCTC-5', GFP-rassf7 (RA+A+CC''+B) forward: 5'-GTTTTGGGAGGATGAACCGCAAAGGGAAAGGGCAG-3', reverse: 3'-CTGCCCTTCCCTTTCGGTTCATCCTCCCAAAC-5', GFP-rassf7 (RA+A+CC''' +B) forward: 5'-GGAATCAGGAGCCCGACGAAGTGGACGAAG-3', reverse: 3'-CTTCGTCCACTTCGTCCGGGCTCCTGATTCC-5'. All constructs were sequenced to confirm the mutations were made correctly.

Embryo fertilisation and cultures

Xenopus laevis eggs were fertilized using standard procedures (Sive et al., 2000), chemically dejellied in (3 % cysteine in 0.1 X Marc's Modified Ringer's solution (MMR), pH 7.9) and transferred to 0.1 X MMR, pH 7.4 (100mM NaCl, 2mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) or by cell number. Embryos were cultured in 0.1 X MMR until the required stage or transferred to injection buffer for microinjection.

RNA Overexpression

The GFP-rassf7 pCS2 plasmid, GFP plasmid and GFP-rassf7 mutant plasmids were used as templates to transcribe mRNA, using the message machine kit (Ambion, Austin, TX). *Xenopus* Embryos were transferred to the injection buffer (1% Ficoll, 0.5X MMR) and 9.2 nl of RNA (1:1 dilution) was injected into one cell of the embryo at the two-cell stage using a Nanoject II injector (Drummand Scientific Company). Embryos were then cultured until stage 10 or stage 30. Once at the required stage, GFP expression was confirmed in whole embryos using a Leica MZFL III microscope (Deerfield, IL) and the expressing embryos fixed in MEMFA (0.1 MOPS, pH 7.4, 2 2 mM EGTA, 1 mM MgSO₄, and 3.7% formaldehyde) for 2 hours at room temperature and where required, processed for immunofluorescence as described below.

Immunofluorescence

For antibody staining embryos were embedded in fish gelatin as described previously (Chalmers et al., 2003), except tadpole-stage (stage 30) embryos, which were incubated in 20% sucrose for 2 h, washed several times in PBS and then embedded in 15% fish gelatin for freezing. The embryos were cryosectioned and antibody stained as described (Chalmers et al., 2003). The following antibodies were used: monoclonal anti- γ -tubulin produced in mouse clone GTU-88 (Sigma; T6557, 1 in 100), polyclonal rabbit anti-active caspase 3 (Abcam; ab13847, 1 in 100) rabbit polyclonal anti-histone H3 phospho S10 (Abcam; ab5168, 1 in 500). The following secondary antibodies were used: anti-mouse Alexa 568 (A-11004, Molecular Probes, Eugene, OR); anti-rabbit Alexa 568 (A-11011, Molecular Probes). All secondary antibodies were used at a 1 in 200 dilution. The nuclear stain, DAPI (Sigma, D9542, 1mg/mL) was used at 1 in 1000 dilution. Stained sections were mounted in Vectasheild (Vector Laboratories, Burlingame, CA) and imaged on a Zeiss LSM META confocal microscope (Thornwood, NY). GFP fluorescence was visualised directly without the use of antibody staining. All fluorescent images were captured in the linear range of the confocal to allow quantification (see below).

Quantification and Statistics

Quantification of cells expressing GFP constructs was carried out by identifying GFP positive cells with a centrosome(s) in the section being analysed (clear γ -tubulin staining adjacent to the nucleus). The area and intensity of the centrosomal GFP was then measured by using the LSM510 Image Browser software (ZEISS) and the integrated density was calculated by multiplying the area by the average intensity across the region of interest. The area, intensity and integrated density of the γ -tubulin, DAPI and nuclear GFP staining was measured in the same way. The centrosome number in GFP positive cells was quantified based on the number of clear γ -tubulin foci, each cell was classified as containing 1, 2 or ≥ 3 centrosomes and each group was displayed as a percentage of the total GFP positive cells. Quantification of mitotic cells (Phospho-H3 positive) or apoptotic cells (active caspase-3) was carried out by calculating the % of GFP positive cells that were also positive for the marker of interest.

Means and SDs were calculated and plotted using GraphPad Prism 6 (San Diego, CA). Statistical analysis was carried out using One-way Anova tests with Bonferroni post-test corrections, $p < 0.05$ was considered significant. Statistical analysis was based on data from at least three independent experiments and with a minimum sample size as described in the figure legends.

RESULTS

The coiled coil, but not the RA domain, is required for centrosomal rassf7 localization

To begin to understand the functional domains within RASSF7 we set out to identify those domains which are responsible for its centrosomal localization. Wild type rassf7 is predicted to have an RA and a coiled-coil domain. In addition, there are two stretches of amino acids that are not predicted to form an identifiable domain by SMART, which we have called the A and B domains (Figure 1). A series of constructs were generated which code for truncated versions of rassf7 fused to GFP (Figure 1) and RNA synthesised from these plasmids was injected into embryos. The amount of centrosomal localisation shown by each truncated protein was then analysed in cells from stage 10 embryos. The localisation of the truncated proteins was compared to GFP-rassf7 which co-localised with the centrosomal protein γ -tubulin (Figure 2A+F). A GFP control which did not show enrichment at the centrosomes was used as a negative control (Figure 2B+F). GFP-rassf7 also showed some nuclear localisation and sometimes some perinuclear or cortical enrichment. However, this staining is also seen in cells expressing the GFP control and so is not specific to the rassf7 protein.

The analysis of centrosomal localisation was initially performed with truncations at the N-terminal end of rassf7. The GFP fusion protein which lacked an RA domain, GFP-rassf7 (A+CC+B), showed a very similar pattern of localisation to full length GFP-rassf7, with clear localisation to γ -tubulin containing structures (Figure 2C+F). A similar distribution was observed when both the A domain and the RA domain were removed, GFP-rassf7 (CC+B) (Figure 2D+F). Further truncation of rassf7 to remove the coiled-coil domain (GFP-rassf7B) resulted in a loss of centrosomal localisation (Figure 2E+F) and a distribution resembling that of the GFP control (Figure 2B). The nuclear localisation, seen with the GFP control and full length GFP-rassf7, was not lost and the ratio between centrosomal GFP and nuclear GFP was reduced (Supplementary Figure 1). This shows that the reduction in centrosomal GFP, seen with GFP-rassf7 (B), cannot be explained by a reduction in RASSF7 levels. These results suggest that the coiled coil, but not the RA and A domains, is required for its centrosomal localisation.

The amount of γ -tubulin staining was also measured following expression of these proteins. There was no significant difference between cells expressing GFP, GFP-rassf7 and the three truncated proteins analysed (Figure 1G), so expression of these constructs did not lead to significant changes in γ -tubulin staining.

Removal of the B domain led to accumulation of truncated rassf7 at the centrosomes and increased levels of γ -tubulin staining

Having studied N-terminal truncations, we next investigated truncations at the C-terminal end of rassf7. Removal of the B domain, GFP-rassf7 (RA+A+CC), did not disrupt the ability of the protein to localise at the centrosome (Figure 3A), in fact there was a striking increase in GFP signal that overlapped with the γ -tubulin (Figure 3A+F). This effect was so pronounced it was possible to see GFP containing punctae in whole mount embryos (Supplementary Figure 2C). Quantification revealed that there was a greater than fivefold increase in the amount of GFP that overlapped with the γ -tubulin

containing puncta (Figure 3D). In contrast GFP localisation in the nucleus was reduced and the ratio between centrosomal GFP and nuclear GFP was greatly increased (Supplementary Figure 1). Therefore, the increase in centrosomal rassf7 cannot be explained by an increase in RASSF7 levels. Surprisingly, cells expressing GFP-rassf7 (RA+A+CC) also had a fourfold increase in the amount of γ -tubulin staining in comparison to controls (Figure 3A+E), suggesting that expression of this truncated version of rassf7 can effect centrosomal morphology.

Removal of the coiled-coil domain and the B domain, GFP-rassf7 (RA+A), caused a loss of co-localisation with γ -tubulin (Figure 3B+D), consistent with the coiled-coil domain being required for centrosomal rassf7 localisation. The RA domain on its own, GFP-rassf7 (RA), also failed to co-localise with γ -tubulin (Figure 3C+D). In both cases a reduction in the ratio between centrosomal GFP and nuclear GFP was also observed (Supplementary Figure 1) and following expression of these two constructs, GFP-rassf7 (RA+A) and GFP-rassf7 (A), there was no effect on γ -tubulin staining levels (Figure 3E).

To further assess the requirement of the coiled coil for localisation a series of point mutations were made which replace hydrophobic amino acids with prolines. These are predicted to disrupt the three dimensional architecture of the coiled coil (Figure 3A). The triple mutation, which is predicted to cause almost complete loss of coiled coil structure, showed significantly less centrosomal localisation than wildtype rassf7 (Figure 3D+E), consistent with the coiled-coil domain being required for the localisation of rassf7. Expression of these mutations did not affect the gamma tubulin staining (Figure 4F).

The coiled-coil domain is sufficient for centrosomal rassf7 localisation

The final set of constructs to be investigated allowed expression of the internal domains of rassf7. GFP-rassf7 (A+CC) lacks the N-terminal RA domain and the C-terminal B domain, but was still able to localise to the centrosomes (Figure 5A). Similar to rassf7 which lacked just the B-domain, there was also an increase in GFP that overlapped with the γ -tubulin, more γ -tubulin staining (Figure 5D+E) and an increase in the ratio between centrosomal GFP and nuclear GFP (Supplementary Figure 1).

The coiled-coil of rassf7 alone, GFP-rassf7 (CC), was able to localise to centrosomes (Figure 5B) at a level comparable to full length GFP-rassf7 (Figure 5B+D). This shows that the coiled-coil domain is sufficient for centrosomal rassf7 localisation, building on observations described above showing the coiled-coil is required to localise rassf7 at the centrosome. Expression of the coiled-coil of rassf7 did not result in increased γ -tubulin signal (Figure 5B+E). This argues that the combination of coiled-coil and the A domain are required for the aberrant accumulation at centrosomes of both rassf7 and γ -tubulin. The final truncated protein to be expressed was GFP-rassf7 (A), which failed to localize to the centrosomes (Figure 5C+D) and showed a reduction in the ratio between centrosomal GFP and nuclear GFP (Supplementary Figure 1). It did not have any effect on centrosomal morphology (Figure 4C+E).

The results from all constructs are summarised in Table 1. The key findings are that the coiled-coil domain is sufficient and required for centrosomal rassf7 localisation. In contrast, the RA domain does not seem to be responsible for rassf7's centrosomal localisation. Constructs which lacked the B

domain, but contained the A domain and the coiled-coil, showed aberrant accumulation at the centrosomes and resulted in an abnormal increase of γ -tubulin staining at centrosomal punctae.

GFP-rassf7 (RA+A+CC) promoted amplification of the γ -tubulin foci and increased numbers of mitotic cells

To investigate the aberrant centrosomal morphology observed in cells expressing C-terminally truncated rassf7 in more detail, an analysis of the number of γ -tubulin foci per cell was carried out. Cells expressing GFP or wild-type GFP-rassf7 had one or two γ -tubulin foci depending on their cell cycle stage (Figure 6A+B+D). However, in cells expressing GFP-rassf7 (RA+A+CC) there were often three or more γ -tubulin foci (Figure 6C+D).

Given these apparent abnormalities in the centrosomes (increased size and number of γ -tubulin foci) we investigated the functional consequences of these defects by quantifying the proportion of cells in mitosis. This was achieved by staining for Phospho-H3 (Figure 7). GFP-rassf7 (RA+A+CC) expressing cells had more Phospho-H3 positive cells than cells expressing GFP or GFP-rassf7 (Figure 7D). We hypothesise that the centrosomal abnormalities caused by expression of GFP-rassf7 (RA+A+CC) lead to a delay in progression through mitosis and an accumulation of mitotic cells.

GFP-rassf7 (RA+A+CC) injected embryos showed increased embryo and cell death

To establish whether embryos which express GFP-rassf7 (RA+A+CC) can survive we cultured injected embryos and monitored rates of embryo death. Uninjected, GFP injected and GFP-rassf7 injected embryos showed some death during development (Figure 8A). However, the rate of embryo death was significantly increased in embryos injected with GFP-rassf7 (RA+A+CC) (Figure 8A).

Some stage 30 embryos expressing GFP-rassf7 (RA+A+CC) did survive (Figure 8A+B), allowing us to study the subcellular localization of rassf7 at this stage. GFP and GFP-rassf7 in stage 30 embryos showed similar sub-cellular distributions to stage 10, with cytoplasmic and centrosomal localisation respectively (Figure 8C+D). However, GFP-rassf7 (RA+A+CC) did not co-localise with γ -tubulin in stage 30 embryos (Figure 8D). Where the GFP-rassf7 (RA+A+CC) was expressed the cells did not appear to have clear γ -tubulin foci, and where γ -tubulin foci were observed there did not appear to be expression of the GFP fusion protein. This led to the question of whether there was increased programmed cell death associated with this apparent loss of γ -tubulin containing punctae.

Apoptotic cells in stage 30 embryos were identified by staining for active caspase-3 (Figure 9). GFP and GFP-rassf7 expressing control cells showed low levels of active caspase-3 staining (Figure 9A+B+D). In contrast, a high percentage of GFP-rassf7 (RA+A+CC) expressing cells were positive for active caspase-3 (Figure 9C+D). This data suggests that early centrosome defects may lead to later centrosome loss and apoptosis.

Mutating the coiled coil of GFP-rassf7 (RA+A+CC) reduces its ability to promote centrosomal defects

Expression of GFP-rassf7 (RA+A+CC) caused early centrosomal defects and later cell and embryo death. A key question is whether these defects are mediated by truncated rassf7 at the centrosome or in other cellular compartments, for example the nucleus. To address this question we made the triple coiled coil mutations, which showed reduced centrosomal localisation (Figure 4), in the GFP-rassf7 (RA+A+CC) construct (Figure 10A). This would produce a protein with the same truncation but with reduced ability to bind the centrosome and so show if loss of centrosomal localisation would reduce the centrosomal defects and cell death seen previously.

The GFP-rassf7 (RA+A+CC'') protein showed less centrosomal localisation than either the wildtype RASSF7 or the truncated GFP-rassf7 (RA+A+CC), which accumulates at the centrosome (Figure 10B-D). Unexpectedly the mutated and truncated construct showed a distinctive punctate nuclear localisation (Figure 10D), which was not observed with the other constructs.

Cells expressing GFP-rassf7 (RA+A+CC'') showed reduced centrosomal defects when compared to GFP-rassf7 (RA+A+CC) positive cells, they did not have enlarged centrosomes (Figure 10D) or duplicated centrosomes (Figure 10E). This suggests that the early centrosome defects require the increased centrosomal localisation seen with GFP-rassf7 (RA+A+CC). At later stages cells expressing GFP-rassf7 (RA+A+CC'') did show increased amounts of cell death, comparable to that seen in cells expressing GFP-rassf7 (RA+A+CC) (Supplementary figure 3). The death seen with GFP-rassf7 (RA+A+CC'') might mean that cell death is not linked to the centrosomal localisation of this truncated protein, but it might also be that the extra nuclear localisation seen with this construct promotes death by an alternative route. Therefore, it is not currently possible to say if the cell death requires the centrosomal localisation seen with GFP-rassf7 (RA+A+CC).

Analysis of RASSF7 mutations in human cancers

Truncated rassf7 which lacks its B-domain can drive centrosome abnormalities (enlarged and increased numbers of γ -tubulin foci), something which is often seen in cancer cells. This prompted us to investigate whether mutations might produce similar truncations of RASSF7 in human cancer samples.

The cBioPortal database was used to search sequence data from over eighty different cancer studies and more than 20,000 patient samples (Figure 11A). RASSF7 was found to be amplified in a number of tumours, consistent with previous reports of increased RASSF7 expression. Twenty-three cancers which had mutations in RASSF7 were also identified (Figure 11B). The mutations were mainly missense mutations, but there were two nonsense mutations and one deletion that would be predicted to truncate RASSF7 (Figure 11B). Two of the nonsense mutations are predicted to produce proteins that are truncated in or near the RA domain (Figure 11B2+3). Our data suggests that these are unlikely to localise to the centrosome and may be hypomorphs. The nonsense mutation which is at R285 would be predicted to produce a protein that would be truncated at the end of the coiled-coil (Figure 11B1). This resembles GFP-rassf7 (RA+A+CC) (Figure 1), and raises the possibility that in this cancer truncated RASSF7 might have accumulated at the centrosome and driven centrosome defects

DISCUSSION

RASSF7 is a key regulator of mitosis and possible oncogene, but the molecular basis of its function remains to be elucidated. Here, we show that the coiled coil domain is sufficient and required for its centrosomal localisation. In contrast, the RA domain of rassf7 does not appear to play a key role in mediating its localisation. Removing the B domain of RASSF7 caused it to accumulate at γ -tubulin foci and cause accumulation of abnormally high levels of γ -tubulin. Expression of the truncated protein also increased the number of γ -tubulin foci per cell, elevated the number of cells in mitosis by a process which appears to require the protein to localise at the centrosome. Ultimately expression of truncated rassf7 resulted in cell and embryo death. Finally, analysis of sequences from human tumours identified a similar truncated version of RASSF7 from a clear renal cell carcinoma, suggesting that RASSF7 may promote centrosome defects and act as an oncogene in a small subset of tumours with such mutations.

Our data show that the coiled coil domain is key in driving the localisation of rassf7 to the centrosome. Previous proteomic characterisation of centrosomes found that a high percentage of centrosomal proteins contain a coiled-coil (Andersen et al., 2003) and many centrosome proteins with coiled-coils have been studied. These include, pericentrin (Doxsey et al., 1994), Ninein (Bouckson-Castaing et al., 1996), Hice1 (Wu et al., 2008), TACC (Gergely et al., 2000a; Gergely et al., 2000b), Centriolin (Gromley et al., 2003), Cep135 (Gromley et al., 2003), C-Nap1 (Fry et al., 1998) and Pix1/2 (Hames et al., 2008). Previous work has also shown that coiled coil domains can mediate centrosome localisation. For example, TACC proteins have a C-terminal coiled-coil domain which is required for interactions with the centrosomes (Gergely et al., 2000b). Therefore, our results with the coiled-coil of rassf7 support the idea that coiled-coils are required for the localisation of a number of important centrosomal proteins.

The other domains of rassf7 did not appear to be required for its centrosomal localisation. These included the RA domain, making it likely that the role of this domain is to interact with effector molecules, potentially recruiting them to the centrosome where they may regulate mitosis. RASSF7 has been shown to bind NRas (Takahashi et al., 2011), HRas and KRas (Chan et al., 2013) in pull down experiments with tagged proteins, although it remains to be confirmed that RASSF7 is binding endogenous Ras family members. Recent proteomic studies have shown that RASSF7 can interact with a number of other cellular proteins, including ASPP and PP1 family members (Hauri et al., 2013). dRASSF8, the *Drosophila* homologue of RASSF7 and RASSF8, can also bind dASPP although dRASSF8 protein localises at epithelial junctions rather than the centrosomes (Langton et al., 2009). Future work will need to establish if the RA, and also the A domain, are bringing these or other potential effectors to the centrosomes to regulate mitosis.

Removing the B domain of rassf7 had a striking effect on its localisation, with a far greater amount of rassf7 colocalising with γ -tubulin. There are a number of possible models to explain this result. The B domain might act as an auto-inhibitory domain that binds to the coiled coil of rassf7 in a regulated way and while in a closed conformation blocks the coiled coil from binding centrosomal proteins. Removing the B domain could then cause unregulated accumulation of rassf7 at the centrosome. Another possibility is that the B domain acts to control the turnover of rassf7 at centrosomes in some way and there are predicted phosphorylation sites for a number of mitotic kinases in the B domain that could potentially regulate rassf7 turnover. Finally, increased localisation could be driven by loss

of nuclear localisation making more rassf7 available for centrosome binding or greater delivery to the centrosome, perhaps, by microtubules.

The expression of RASSF7 has been shown to be increased in a number of tumour types (Friess et al., 2003; Li et al., 2013; Logsdon et al., 2003; Lowe et al., 2007; Tan et al., 2009), but it is not clear if RASSF7 can act as an oncogene and promote tumour formation. Our previous work shows that overexpression of wildtype RASSF7, carried out for localisation and rescue experiments, did not have a clear effect on HeLa cells or *Xenopus* embryos (Recino et al., 2010; Sherwood et al., 2008). In our current study expressing tagged full length rassf7 did not drive alterations in centrosome size, number, mitotic cells or cell death. This suggests that increased wild type RASSF7 may not act in an oncogenic role, although RASSF7 can also protect against stress induced cell death, an aspect that we have not investigated here (Takahashi et al., 2011). In contrast to the wildtype protein, expression of truncated rassf7 which lacked the B domain caused elevated amounts of γ -tubulin staining at foci and an increased number of γ -tubulin foci per cell. This phenotype resemble the enlargement and amplification of centrosomes that can occur in cancer cells (Godinho and Pellman, 2014; Nigg, 2006) and recent evidence suggests that centrosome amplification can drive cellular invasion (Godinho et al., 2014). Interestingly, we found a mutation in RASSF7 from a renal clear cell carcinoma that would produce a similar truncation. Our data suggests the protein produced following this mutation could drive centrosome defects, which would be consistent with RASSF7 acting as an oncogene in this tumour. This oncogenic role would require the cancer cells to also have defects in apoptosis as the truncated protein also promoted cell death which could counter a role in promoting tumour formation. We have currently found one tumour with this type of mutation, from a database containing sequences from 80 studies and over 20,000 patient samples, suggesting that it may only be a small subset of tumours where mutated RASSF7 functions in this way. However, as sequences from more and more tumours become available it will be interesting to see whether additional tumours with C-terminal RASSF7 truncations are discovered.

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GFP domain construct	Centrosomal localization	Increased γ-tubulin staining
GFP-RASSF7 (WT)	✓	×
GFP-RASSF7 (RA+A+CC)	✓	✓
GFP-RASSF7 (RA+A)	×	×
GFP-RASSF7 (RA)	×	×
GFP-RASSF7 (A+CC+B)	✓	×
GFP-RASSF7 (CC+B)	✓	×
GFP-RASSF7 (A+CC)	✓	✓
GFP-RASSF7 (CC)	✓	×
GFP-RASSF7 (A)	×	×
GFP-RASSF7 (B)	×	×

Table 1. The ability of truncated rassf7 proteins to localise at the centrosome and promote increased γ -tubulin staining.

FIGURE LEGENDS –

Figure 1. GFP-rassf7 truncation series. A series of constructs were produced that allow expression of truncated versions of rassf7 fused to GFP. GFP-rassf7 (WT), GFP-rassf7 (RA+A), GFP-rassf7 (RA+A+CC), GFP-rassf7 (RA), GFP-rassf7 (A+CC+B), GFP-rassf7 (CC+B), GFP-rassf7 (A+CC), GFP-rassf7 (CC), GFP-rassf7 (A), GFP-rassf7 (B).

Figure 2. The coiled-coil, but not the RA domain, is required for centrosomal rassf7 localisation. Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP-rassf7 (green) co-localised with γ -tubulin (red) (arrows) as previously shown (Sherwood *et al.*, 2008). GFP-rassf7 showed some nuclear localisation and sometimes some perinuclear or cortical enrichment. However, this staining is also seen with the GFP control and so is not specific to the rassf7 protein. **(B)** GFP negative control, GFP (green) and γ -tubulin (red, arrows). GFP showed nuclear localisation and some perinuclear or cortical enrichment, but lacked the centrosomal enrichment seen with GFP-rassf7. **(C)** GFP-rassf7 (A+CC+B) (green) co-localised with γ -tubulin (red). **(D)** GFP-rassf7 (CC+B) (green) co-localised with γ -tubulin (red). **(E)** GFP-rassf7 (B) (green) showed reduced co-localisation with γ -tubulin (red). In A-E arrows highlight potential areas for co-localisation and scale bars=10 μ m. **(F)** The integrated density of GFP fluorescence at the γ -tubulin foci for the GFP-rassf7 fusion proteins and the positive and negative controls (GFP-rassf7 and GFP). Integrated density of GFP-rassf7 (B) was similar to negative control. **(G)** Integrated density of γ -tubulin. Expression of full length or truncated GFP-rassf7 proteins did not affect the integrated density of the γ -tubulin staining. Based on at least three independent experiments, n>100 cells, *p<0.05.

Figure 3. Removal of the B domain led to accumulation of truncated rassf7 at γ -tubulin foci and increased γ -tubulin staining. Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP-rassf7 (RA+A+CC) (green) co-localised with γ -tubulin (red). There appeared to be increased localisation and also enlargement of the γ -tubulin staining. The nuclear localisation seen with the wildtype construct and the GFP control was also reduced. **(B)** GFP-rassf7 (RA+A) showed reduced co-localisation with γ -tubulin (red), but the protein still showed the nuclear localisation seen with full length rassf7 and the GFP control. **(C)** GFP-rassf7 (RA) (green) did not show co-localisation with γ -tubulin (red), but did still show the nuclear localisation seen with the wildtype construct and the GFP control. In panels A-C arrows highlight potential areas for co-localisation and scale bars=10 μ m. **(D)** Integrated density of GFP fluorescence at the γ -tubulin foci. GFP-rassf7 (RA+A+CC) showed an increased integrated density when compared to wild type. **(E)** Integrated density of the γ -tubulin spot. Cells expressing GFP-rassf7 (RA+A+CC) showed an increased integrated density when compared to cell expressing wild type rassf7. Based on at least three independent experiments, n>100 cells, *p<0.05.

Figure 4. Mutations in the coiled coil domain reduced the centrosomal localisation of GFP-rassf7.

Constructs were made that contained mutations in the coiled-coil region, embryos were then microinjected, with RNA made from these constructs, at the two-cell stage, cultured until (stage 10), fixed, sectioned and stained with a centrosome marker (γ -tubulin /red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** The predicted structure of the coiled-coil domain mutations; GFP-rassf7 (WT), GFP-rassf7 (RA+A+CC'+B) with one point mutation, GFP-rassf7 (RA+A+CC''+B) with two point mutations and GFP-rassf7 (RA+A+CC''' +B) with three point mutations. **(B)** GFP-rassf7 (RA+A+CC'+B) (green) co-localised with γ -tubulin (red). **(C)** GFP-rassf7 (RA+A+CC''+B) (green) co-localised with γ -tubulin (red). **(D)** GFP-rassf7 (RA+A+CC''' +B) (green) showed decreased co-localisation with γ -tubulin (red). In panels A-C arrows highlight potential areas for co-localisation. All three mutant proteins showed the nuclear localisation seen with the wildtype construct and the GFP control. Scale bars=10 μ m. **(E)** Integrated density of the GFP fluorescence at the γ -tubulin foci. GFP-rassf7 (RA+A+CC''' +B) showed reduced centrosomal localisation when compared to the wild type protein. **(F)** Integrated density of the γ -tubulin spot. Mutations did not affect the integrated density of the γ -tubulin staining. Based on at least three independent experiments, n>100 cells, *p<0.05.

Figure 5. The coiled-coil domain is sufficient for the centrosomal localisation of rassf7.

Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP-rassf7 (A+CC) (green) co-localised with γ -tubulin (red). Accumulation at the centrosome and enlargement of γ -tubulin staining was observed. The nuclear localisation seen with the wildtype construct and the GFP control was also reduced. **(B)** GFP-rassf7 (CC) (green) co-localised with γ -tubulin (red) and showed the nuclear localisation seen with full length rassf7 and the GFP control. **(C)** GFP-rassf7 (A) (green) did not show co-localisation with γ -tubulin (red), but the protein still showed the nuclear localisation seen with full length rassf7 and the GFP control. In panels A-C arrows highlight potential areas for co-localisation and scale bars=10 μ m. **(D)** Integrated density of the GFP at the γ -tubulin foci. GFP-rassf7 (A+CC) showed a significantly increased integrated density compared to wild type rassf7. GFP-rassf7 (CC) was similar to wild type rassf7 and GFP-rassf7 (A) was similar to the GFP negative control. **(E)** Integrated density of the γ -tubulin foci. GFP-rassf7 (A+CC) showed an increase in the integrated density of γ -tubulin when compared to the wild type rassf7. The other constructs did not affect the integrated density of γ -tubulin. Based on at least three independent experiments, n>100 cells, *p<0.05.

Figure 6. Expression of GFP-rassf7 (RA+A+CC) caused increased numbers of γ -tubulin foci. Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP (green) and γ -tubulin (red). **(B)** GFP-rassf7 (green) and γ -tubulin (red). **(C)** GFP-rassf7 (RA+A+CC) (green) and γ -tubulin (red). Cells expressing GFP-rassf7 (RA+A+CC) showed increased numbers of γ -tubulin foci. In A-C arrows highlight example cells and scale bars=10 μ m. **(D)** Centrosome number for GFP, GFP-rassf7 and GFP-rassf7 (RA+A+CC) injected cells. Unlike controls, GFP-rassf7 (RA+A+CC) injected cells frequently had more than two γ -tubulin foci. Based on at least three independent experiments, n>100 cells.

Figure 7. GFP-rassf7 (RA+A+CC) injected cells accumulate in mitosis. Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a mitosis marker (Phospho-H3/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP (green) injected cells were stained for Phospho-H3 (red). **(B)** GFP-rassf7 (green) injected cells were stained for Phospho-H3 (red). **(C)** GFP-rassf7 (RA+A+CC) (green) injected cells were stained for Phospho-H3 (red). The number of Phospho-H3 positive cells increased when compared to GFP and GFP-rassf7 expressing cells. In panels A-C arrows highlight example nuclear regions and all bars=10 μ m. **(D)** The percentage of GFP positive cells that are Phospho-H3 positive. Based on at least three independent experiments, n>100 cells, *p<0.05.

Figure 8. GFP-rassf7 injected embryos at tadpole stages. Embryos were microinjected with RNA at the two-cell stage, cultured until tadpole stages (stage 30), fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** Embryo survival for uninjected, GFP injected, GFP-rassf7 injected and GFP-rassf7 (RA+A+CC) injected embryos. Based on at least three independent experiment, n>60 embryos. **(B)** Wholemout stage 30 embryos expressing GFP, GFP-rassf7 and GFP-rassf7 (RA+A+CC). **(C)** GFP (green) expressing cells stained with γ -tubulin (red) from stage 30 embryos. **(D)** GFP-rassf7 (green) expressing cells stained with γ -tubulin (red) from stage 30 embryos. **(E)** GFP-rassf7 (RA+A+CC) (green) expressing cells stained with γ -tubulin (red) at stage 30. Unlike the situation at stage 10, the GFP-rassf7 (RA+A+CC) fluorescence (green) did not co-localise with γ -tubulin (red) and γ -tubulin foci appeared to have been lost from GFP-rassf7 (RA+A+CC) expressing cells. In panels C-E arrows highlight possible regions where co-localisation might occur and all scale bars=10 μ m.

Figure 9. GFP-rassf7 (RA+A+CC) injected cells undergo increased rates of apoptosis. Embryos were microinjected with RNA at the two-cell stage, cultured until tadpole stages (stage 30), fixed, sectioned and stained with an apoptosis marker (Active caspase-3/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP injected cells (green) were stained with an antibody against active caspase-3 (red). **(B)** GFP-rassf7 injected cells (green) were stained with an antibody against active caspase-3 (red). **(C)** GFP-rassf7 (RA+A+CC) injected cells (green) were stained an antibody against active caspase-3 (red). These cells showed increased levels of active caspase-3 (red) positive nuclei compared to controls. In panels A-C arrows highlight GFP positive cells. All bars=10 μ m. **(D)** The

% percentage of GFP positive cells which were active caspase-3 positive (Based on at least three independent experiments, n>100 cells, *p<0.05).

Figure 10. Mutating the coiled coil domain reduced the centrosomal defects seen in cells expressing GFP-rassf7 (RA+A+CC). Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10, fixed, sectioned and stained with a centrosomal marker (γ -tubulin/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** Predicted structure of GFP-rassf7 (RA+A+CC'') which lacks the B domain and has been mutated at three sites to disrupt the coiled coil. **(B)** GFP-rassf7 (green) co-localised with γ -tubulin (red). **(C)** GFP-rassf7 (RA+A+CC) (green) co-localised with γ -tubulin (red). Accumulation at the centrosome and enlargement of γ -tubulin staining was also seen. **(D)** GFP-rassf7 (RA+A+CC'') (green) did not co-localise with γ -tubulin (red), but accumulated as distinct puncta within the nucleus. Enlargement of γ -tubulin staining was not seen. In panels B-D arrows highlight the potential area for co-localisation and scale bars=10 μ m. **(E)** Centrosome number for GFP, GFP-rassf7, GFP-rassf7 (RA+C+CC) and GFP-rassf7 (RA+C+CC'') injected cells. GFP-rassf7 (RA+C+CC'') injected cells did not have the additional γ -tubulin foci frequently seen in GFP-rassf7 (RA+A+CC) injected cells. Based on at least three independent experiments, n>100 cells.

Figure 11. RASSF7 mutations in human cancer samples. **(A)** Alterations in RASSF7 were identified in cancer studies using the cBioPortal for cancer genomics website which contained data from 89 cancer genomic studies covering a total of 20,958 tumour samples. **(B)** Mutations in the RASSF7 (red: nonsense mutations or deletions, green: missense mutations). The predicted structure of RASSF7 for the three nonsense mutations. Mutation 1, R285*. Mutation 2, S97*. Mutation 3, Q63fs. The predicted structure of the R285* mutation reassembles that of GFP-rassf7 (RA+A+CC).

Supplementary Figure 1. Ratio of GFP intensity at the Y-tubulin foci vs GFP intensity in the nuclei.

The ratio of intensity for GFP at the Y-tubulin foci vs GFP intensity at the nuclei was calculated for each construct. Constructs which showed reduced centrosomal staining had reduced Y-tubulin to nuclei ratio. Constructs with increased centrosomal localisation showed increased Y-tubulin to nuclei ratio. The change in ratio shows that the changes in localisation seen with the constructs cannot be explained by changes in protein expression levels of the constructs. Based on at least three independent experiments and n>100 cells.

Supplementary Figure 2. Wholemount images of stage 10 embryos expressing truncated RASSF7.

Embryos were microinjected with RNA at the two-cell stage, cultured until stage 10 and imaged as wholemounts. Right panels show GFP fluorescence in green. **(A)** GFP expression in a stage 10 embryo **(B)** GFP-rassf7 expression in a stage 10 embryo **(C)** GFP-rassf7 (RA+A+CC) expression in a stage 10 embryo. The localisation of this truncated protein is more punctate than the control proteins.

Supplementary Figure 3. Mutating the coiled coil domain did not stop increased rates of apoptosis seen after expression of truncated rassf7.

Embryos were microinjected with RNA at the two-cell stage, cultured until tadpole stages, fixed, sectioned and stained with an apoptosis marker (Active caspase-3/red) and a nuclear marker (DAPI/blue). GFP fluorescence is shown in green. **(A)** GFP-rassf7 injected cells (green) were stained with an antibody against active caspase-3 (red). **(B)** GFP-rassf7 (RA+A+CC) injected cells (green) were stained an antibody against active caspase-3 (red). These cells showed increased levels of active caspase-3 (red) positive nuclei compared to cells expressing GFP-rassf7. **(C)** GFP-rassf7 (RA+A+CC'') injected cells (green) were stained an antibody against active caspase-3 (red). These cells showed increased levels of active caspase-3 (red) positive nuclei compared to cells expressing GFP-rassf7, showing that point mutations in the coiled coil did not stop the ability of this construct to promote apoptosis. Arrows highlight GFP positive cells. All bars=10µm.