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Cross-linking mass spectrometry identifies new interfaces of Augmin required to localise the γ-tubulin ring complex to the mitotic spindle

Jack W. C. Chen1, Zhuo A. Chen2, Kacper B. Rogala3, Jeremy Metz1, Charlotte M. Deane3, Juri Rappsilber2,4,* and James G. Wakefield1,6*

ABSTRACT

The hetero-octameric protein complex, Augmin, recruits γ-Tubulin ring complex (γ-TuRC) to pre-existing microtubules (MTs) to generate branched MTs during mitosis, facilitating robust spindle assembly. However, despite a recent partial reconstitution of the human Augmin complex in vitro, the molecular basis of this recruitment remains unclear. Here, we used immuno-affinity purification of in vivo Augmin from Drosophila and cross-linking/mass spectrometry to identify distance restraints between residues within the eight Augmin subunits in the absence of any other structural information. The results allowed us to predict potential interfaces between Augmin and γ-TuRC. We tested these predictions biochemically and in the Drosophila embryo, demonstrating that specific regions of the Augmin subunits, Dgt3, Dgt5 and Dgt6 all directly bind the γ-TuRC protein, Dgp71WD, and are required for the accumulation of γ-TuRC, but not Augmin, to the mitotic spindle. This study therefore substantially increases our understanding of the molecular mechanisms underpinning MT-dependent MT nucleation.

KEY WORDS: γ-TuRC, Augmin, Drosophila, Microtubule, Mitosis, Spindle

INTRODUCTION

Since its discovery in Drosophila (Goshima et al., 2007, 2008; Hughes et al., 2008), the Augmin complex has radically changed our understanding of microtubule (MT) generation during mitosis. Augmin amplifies MT number during mitosis and without it, the density of MTs within the mitotic spindle is dramatically reduced, such that chromosome alignment and mitotic progression are perturbed (Uehara et al., 2009; Lawo et al., 2009; Wainman et al., 2009; Meireles et al., 2009; Bucciarelli et al., 2009; Ho et al., 2011; Petry et al., 2011; Hotta et al., 2012; Hayward et al., 2014). Each of the eight proteins that constitute Augmin localise to MTs (Goshima et al., 2007; Hughes et al., 2008) and, in humans, the HAUS6 (FAM29A) subunit has been shown to associate with NEDD1, part of the MT nucleating γ-Tubulin ring complex (γ-TuRC) (Zhu et al., 2008; Teixidó-Travesa et al., 2010). Moreover, removal of Drosophila Augmin, through RNAi, mutation or immunodepletion, removes the fraction of γ-TuRC normally present on the spindle, without affecting centrosomal levels (Goshima et al., 2007, 2008; Wainman et al., 2009); a phenotype similar to that seen upon loss of the NEDD1 homologue, Dgp71WD (Reschen et al., 2012). The current model is therefore that Augmin acts as a molecular linker between an existing MT and a γ-TuRC, allowing the nucleation of new MTs from the walls of pre-existing ones; a hypothesis supported by observations in Drosophila, Xenopus and plants (Kamasaki et al., 2013; Petry et al., 2013; Liu et al., 2014). However, the relationship between Augmin structure and function is still poorly understood, due both to its multi-subunit complexity and to the very limited homology of Augmin between species; only four of the eight Augmin subunits are conserved between humans and invertebrates at the primary structure level (Dgt6/HAUS6, Dgt4/HICE1/HAUS8, Dgt3/HAUS3 and Dgt5/HAUS5); and even within these, the homology is restricted (Uehara et al., 2009; Duncan and Wakefield, 2011). Although a recent in vitro partial reconstitution of human Augmin identified direct interactions between specific subunits (Hsia et al., 2014), it also highlighted the limitations of a ‘bottom-up’ in vitro reconstitution approach to understanding Augmin function; and the structural integrity of the full complex and its relationship to mitotic function remains unclear.

Here we took an alternative, in vivo-driven approach; using cross-linking/mass spectrometry (CLMS) (Rappsilber, 2011) of Augmin, purified directly and endogenously from Drosophila embryos, to predict the orientation of the subunits within the complex and the likely interfaces that facilitate interaction with γ-TuRC. Validation of these predictions using both direct protein-protein assays and through injecting domains of subunits into Drosophila embryos, identified multiple subunit interfaces required to recruit γ-TuRC to the mitotic spindle. This study therefore highlights both the complexity of regulating MT-dependent MT nucleation in the cell and the predictive power of CLMS.

RESULTS AND DISCUSSION

We have previously shown that transgenic flies expressing a GFP-tagged variant of the Drosophila Augmin subunit, Msdl, rescue the female sterility and mitotic spindle defects associated with a mutation in the msdl gene (Wainman et al., 2009). We subjected extracts from syncytial Drosophila embryos expressing Msdl-GFP to GFP-TRAP-A-based immuno-affinity purification, to isolate
intact Augmin (Fig. 1A). Mass spectrometry confirmed 56–84% coverage of each of the 8 Augmin subunits (Msd1, Msd5, Wac, Dgt2-Dgt6), demonstrating the ability of Msd1-GFP to co-precipitate all other Augmin subunits (Table 1). All subunits of Augmin, apart from Msd1, were quantified at approximately equal abundance (Fig. 1B). The presence of approximately three-fold greater Msd1 is likely a consequence of its role as ‘bait’ protein in this methodology, as sucrose gradient density centrifugation of Msd1-GFP extracts demonstrated two populations of Msd1-GFP of sizes corresponding to monomeric and Augmin-incorporated (not shown). Thus, in agreement with previous qualitative observations (Goshima et al., 2008), Drosophila Augmin possesses a subunit stoichiometry of 1:1.

To obtain structural information on the relationship between Augmin subunits, we subjected purified Augmin on beads to chemical cross-linking using bis(sulfo)succinimidyl) suberate (BS3), followed by trypsin digestion and mass spectrometry (see the Materials and Methods). We then identified cross-linked peptides between and within Augmin subunits. No cross-links were identified between Augmin proteins and proteins co-purified on GFP-TRAP-A beads, suggesting that these additional proteins bind non-specifically to the GFP-TRAP-A beads, rather than being Msd1-GFP/Augmin interacting proteins (not shown).

Our IP-CLMS analysis identified 77 intra-protein linkages, and 59 inter-protein linkages within Augmin at 5% FDR (Table 2). A predicted molecular topology of Drosophila Augmin was constructed from this data, revealing a set of potential inter-connections between the eight Augmin subunits, where seven subunits interact with two or more others (Fig. 1C). The structural restraints suggest a ‘core’ of interactions centred around the C-termini of Dgt5, Dgt3 and Wac, the N-terminus of Dgt6 and the Dgt2, Msd1 and Msd5 subunits. Such a complex network of interactions provides a molecular explanation for the reported inter-dependence of these subunits, in terms of Augmin stability (Goshima et al., 2008; Meireles et al., 2009): removal of one of these core subunits could theoretically lead to the complex instability observed in vivo. (Fig. 1C). This predicted topology differs in some aspects with the recently proposed in vitro reconstituted network of human Augmin subunits (Hsia et al., 2014). In that in vitro-driven approach, human Dgt4 (HAUS8, previously known as HICE1) was placed within a central dimer, together with hDgt6 (HAUS6), interacting with the four non-conserved subunits to constitute a
In contrast, our in vivo-driven CLMS map suggests Dgt4, with only a single, weak predicted interaction, lies on the outside of core Augmin. However, in both studies, Dgt3/HAUS3 and Dgt5/HAUS5 appear to have structurally distinct properties to the rest of Augmin.

Our analysis also identified 10 parallel cross-links along the length of the N-termini of Dgt3 and Dgt5 (∼aa 75-350), suggesting the possibility of a hetero-dimeric sub-complex along this interface. Moreover, an additional six interactions were identified between Dgt6 and Dgt3/5. As the human homologue of Dgt6 (HAUS6) has previously been shown to interact with the NEDD1 subunit of γ-TuRC, we hypothesised that these regions of Dgt3 and Dgt5 might function co-operatively with Dgt6 to recruit Drosophila γ-TuRC through the NEDD1 homologue, Dgp71WD.

Initially, to test this hypothesis, we subjected the N-terminal sequences of Dgt3 and Dgt5 to de novo structural bioinformatics predictions (Fig. 2A). This was consistent with a model in which the N-termini of Dgt3 and Dgt5 form coiled coils; when the structural restrictions from our cross-linking experiment are applied, a hetero-dimeric parallel combination was found along two extended regions covering most of the ∼300 length of the interacting polypeptides (Fig. 2A). In contrast, the C-terminus of Dgt6 (∼aa 300-654) is

### Table 1. The identities and amounts of the proteins present in the purification of Msd1-GFP from Drosophila embryo extracts, identified through LC-MS/MS

| Protein ID | Protein name | No. of identified peptides | Sequence coverage (%) | Mr mass (kDa) | Intensity  |
|------------|--------------|---------------------------|-----------------------|--------------|-----------|
| 1 FBpp0084783 | Dgt6 | 30 | 58.1 | 72.826 | 6.11E+09 |
| 2 FBpp0087059 | Dgt5 | 30 | 46.9 | 77.977 | 5.05E+09 |
| 3 FBpp0085720 | betaTub56D | 19 | 57.3 | 50.147 | 4.72E+09 |
| 4 FBpp0271922 | Dgt3 | 26 | 54 | 65.822 | 4.33E+09 |
| 5 FBpp0078869 | Dgt2 | 14 | 66.2 | 25.844 | 3.83E+09 |
| 6 FBpp0081082 | alphaTub84D | 15 | 45.8 | 49.89 | 3.35E+09 |
| 7 FBpp0288410 | Wac | 8 | 51.5 | 19.053 | 2.98E+09 |
| 8 FBpp0072608 | Msd5 | 10 | 58.9 | 28.461 | 2.85E+09 |
| 9 FBpp0070610 | Dgt4 | 7 | 53.7 | 21.425 | 2.60E+09 |
| 10 FBpp0083683 | T-cp1 | 24 | 58.9 | 59.566 | 2.56E+09 |
| 11 FBpp0083611 | PyK | 22 | 54.2 | 57.44 | 2.13E+09 |
| 12 FBpp0032514 | Hsc70-4 | 31 | 59.4 | 71.131 | 2.05E+09 |
| 13 FBpp0072571 | Msd1 | 6 | 42 | 15.669 | 1.93E+09 |
| 14 FBpp0073002 | Tcp-1beta | 26 | 52.7 | 58.246 | 1.48E+09 |
| 15 FBpp0075649 | I(1)G0156 | 16 | 49.6 | 40.844 | 1.32E+09 |
| 16 FBpp0087287 | Cctgamma | 26 | 59.7 | 59.394 | 1.16E+09 |
| 17 FBpp0076122 | alphaTub67C | 14 | 40.5 | 51.18 | 1.15E+09 |
| 18 FBpp0076652 | Yp3 | 17 | 54.5 | 46.101 | 1.11E+09 |
| 19 FBpp0071359 | Yp2 | 18 | 60.6 | 49.66 | 1.06E+09 |
| 20 FBpp0305828 | ATPsyn-beta | 19 | 60.7 | 54.682 | 1.01E+09 |

The most abundant 20 proteins are shown. Augmin subunits are highlighted in bold.

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Fig. 2. Bioinformatic and in vitro validation of the relationships between Dgt3, Dgt5, Dgt6 and the γ-TuRC subunit, Dgp71WD. (A) Top two panels show potential coiled coil formation of Dgt3 and Dgt5 as predicted by Multicoll2 (blue) and Marcoil (green) (see Materials and Methods). Both proteins are likely to form two separate segments of homo-dimeric coiled coils. Bottom panel shows Dgt3 and Dgt5 and alignment of the cross-links identified by CLMS. Coiled-coil segments are coloured in cyan, and cross-linked residues in red. Imposition of a short ‘loop’ between the two coiled-coil regions in Dgt5 bring all positional restrictions between Dgt3 and Dgt5 into alignment, strengthening the hypothesis that these proteins form a hetero-dimeric coiled coil. (B) Purified GST-Dgp71WD on glutathione beads incubated with His-Dgt3N, His-Dgt5N, His-Dgt6C and His-GFP, singly and in combination. His-Dgt5N and His-Dgt6C associate strongly with GST-Dgp71WD. His-Dgt3N associates weakly when incubated singly, but increases affinity in the presence of His-Dgt5N. His-GFP provides a negative control and does not associate with GST-Dgp71WD.
predicted to be disordered, with no structural homology to known protein folds (not shown). We next bacterially expressed and purified His-tagged versions of the N-terminal half of Dgt3 (aa 1-350), Dgt5 (aa 1-450), and the C-terminal portion of Dgt6 (aa 298-654) and tested their ability to interact with a GST-tagged variant of the γ-TuRC subunit, Dgp71WD (Reschen et al., 2012). While GST-Dgp71WD did not interact with a control His-tagged protein (GFP), it was able to sequester all three Augmin polypeptides (Fig. 2B; data not shown). Interestingly, Dgt3N interacted only weakly with GST-Dgp71WD on its own but consistently showed greater affinity in the presence of Dgt5N, further supporting the notion that the N-termini of Dgt3 and Dgt5 act co-operatively in vivo (Fig. 2B).

To functionally validate the hypothesis that these regions of Dgt3 and Dgt5 have a role in recruiting γ-TuRC to pre-existing MTs, in addition to Dgt6, we injected bacterially expressed, MBP-tagged purified Dgt3N, Dgt5N and Dgt6C into Drosophila syncytial embryos expressing GFP transgenes. Injection of 5 mg/ml BSA,
Table 2. The identities and linkage (cross-link) sites within and between Augmin subunits, as identified by CLMS.

| Protein 1 name | Linkage site 1 (aa) | Linked residue 1 | Protein 2 name | Linkage site 2 (aa) | Linked residue 1 | MS2 count | Note          |
|----------------|---------------------|------------------|----------------|---------------------|------------------|-----------|---------------|
| Dgt2           | 193                 | K                | Dgt3           | 508                 | K                | 6         | between proteins |
| Dgt2           | 55                  | K                | Dgt5           | 154                 | K                | 2         | between proteins |
| Dgt2           | 214                 | K                | Dgt5           | 653                 | K                | 2         | between proteins |
| Dgt2           | 160                 | K                | Dgt5           | 124                 | K                | 1         | between proteins |
| Dgt2           | 193                 | K                | Dgt6           | 545                 | T                | 1         | between proteins |
| Dgt2           | 193                 | K                | Msd1           | 113                 | K                | 1         | between proteins |
| Dgt2           | 193                 | K                | Msd1           | 25                  | K                | 1         | between proteins |
| Dgt2           | 193                 | K                | Wac            | 132                 | K                | 4         | between proteins |
| Dgt2           | 214                 | K                | Wac            | 146                 | K                | 3         | between proteins |
| Dgt2           | 214                 | K                | Wac            | 143                 | K                | 2         | between proteins |
| Dgt2           | 193                 | K                | Wac            | 140                 | K                | 4         | between proteins |
| Dgt2           | 217                 | K                | Wac            | 143                 | K                | 3         | between proteins |
| Dgt2           | 193                 | K                | Wac            | 143                 | K                | 2         | between proteins |
| Dgt2           | 217                 | K                | Wac            | 146                 | K                | 4         | between proteins |
| Dgt3           | 72                  | K                | Dgt5           | 73                  | K                | 10        | between proteins |
| Dgt3           | 72                  | K                | Dgt5           | 71                  | K                | 6         | between proteins |
| Dgt3           | 119                 | K                | Dgt5           | 124                 | K                | 8         | between proteins |
| Dgt3           | 119                 | K                | Dgt5           | 116                 | K                | 5         | between proteins |
| Dgt3           | 249                 | K                | Dgt5           | 286                 | K                | 5         | between proteins |
| Dgt3           | 249                 | K                | Dgt5           | 287                 | K                | 1         | between proteins |
| Dgt3           | 230                 | K                | Dgt5           | 277                 | K                | 1         | between proteins |
| Dgt3           | 324                 | K                | Dgt5           | 383                 | K                | 2         | between proteins |
| Dgt3           | 322                 | K                | Dgt5           | 378                 | K                | 3         | between proteins |
| Dgt3           | 336                 | K                | Dgt5           | 378                 | K                | 1         | between proteins |
| Dgt3           | 308                 | K                | Dgt5           | 154                 | K                | 1         | between proteins |
| Dgt3           | 318                 | Y                | Dgt5           | 378                 | K                | 1         | between proteins |
| Dgt3           | 324                 | K                | Dgt6           | 561                 | K                | 1         | between proteins |
| Dgt3           | 324                 | K                | Dgt6           | 390                 | K                | 1         | between proteins |
| Dgt3           | 324                 | K                | Dgt6           | 555                 | K                | 2         | between proteins |
| Dgt3           | 165                 | S                | Msd1           | 113                 | K                | 3         | between proteins |
| Dgt3           | 334                 | S                | Msd1           | 137                 | S                | 1         | between proteins |
| Dgt3           | 308                 | K                | Wac            | 132                 | K                | 3         | between proteins |
| Dgt4           | 97                  | K                | Msd5           | 174                 | K                | 2         | between proteins |
| Dgt5           | 315                 | K                | Dgt6           | 352                 | K                | 1         | between proteins |
| Dgt5           | 98                  | K                | Dgt6           | 353                 | K                | 1         | between proteins |
| Dgt5           | 98                  | K                | Dgt6           | 143                 | K                | 1         | between proteins |
| Dgt5           | 98                  | K                | Dgt6           | 360                 | K                | 1         | between proteins |
| Dgt5           | 100                 | K                | Dgt6           | 353                 | K                | 1         | between proteins |
| Dgt5           | 413                 | S                | Msd1           | 113                 | K                | 1         | between proteins |
| Dgt5           | 625                 | K                | Wac            | 132                 | K                | 5         | between proteins |
| Dgt5           | 632                 | K                | Wac            | 140                 | K                | 1         | between proteins |
| Dgt5           | 632                 | K                | Wac            | 146                 | K                | 3         | between proteins |
| Dgt5           | 606                 | S                | Wac            | 132                 | K                | 2         | between proteins |
| Dgt6           | 71                  | K                | Dgt6           | 71                  | K                | 1         | between proteins |
| Dgt6           | 270                 | K                | Msd1           | 78                  | K                | 4         | between proteins |
| Dgt6           | 82                  | K                | Msd1           | 113                 | K                | 1         | between proteins |
| Dgt6           | 237                 | K                | Msd1           | 25                  | K                | 1         | between proteins |
| Dgt6           | 270                 | K                | Msd1           | 33                  | S                | 1         | between proteins |
| Dgt6           | 285                 | T                | Msd1           | 78                  | K                | 1         | between proteins |
| Dgt6           | 265                 | K                | Msd1           | 45                  | K                | 1         | between proteins |
| Dgt6           | 190                 | K                | Msd5           | 17                  | K                | 1         | between proteins |
| Dgt6           | 190                 | K                | Msd5           | 26                  | K                | 1         | between proteins |
| Dgt6           | 462                 | K                | Msd5           | 88                  | Y                | 1         | between proteins |
| Dgt6           | 71                  | K                | Msd5           | 87                  | K                | 3         | between proteins |
| Dgt6           | 143                 | K                | Msd5           | 87                  | K                | 2         | between proteins |
| Dgt6           | 71                  | K                | Msd5           | 86                  | K                | 2         | between proteins |
| Msd1           | 113                 | K                | Msd1           | 113                 | K                | 4         | between proteins |
| Msd1           | 25                  | K                | Msd1           | 25                  | K                | 4         | between proteins |
| Msd1           | 113                 | K                | Msd5           | 226                 | S                | 1         | between proteins |
| Dgt2           | 209                 | T                | Dgt2           | 217                 | K                | 3         | within protein |
| Dgt2           | 212                 | S                | Dgt2           | 217                 | K                | 1         | within protein |
| Dgt2           | 51                  | K                | Dgt2           | 55                  | K                | 1         | within protein |
| Dgt2           | 101                 | S                | Dgt2           | 192                 | T                | 1         | within protein |
| Dgt3           | 230                 | K                | Dgt3           | 324                 | K                | 2         | within protein |
| Dgt3           | 508                 | K                | Dgt3           | 522                 | K                | 1         | within protein |
| Dgt3           | 210                 | K                | Dgt3           | 324                 | K                | 2         | within protein |
| Dgt3           | 118                 | T                | Dgt3           | 336                 | K                | 1         | within protein |
| Dgt3           | 271                 | S                | Dgt3           | 273                 | T                | 2         | within protein |
| Dgt4           | 97                  | K                | Dgt4           | 105                 | K                | 2         | within protein |

Continued
| Protein 1 name | Linkage site 1 (aa) | Linked residue 1 | Protein 2 name | Linkage site 2 (aa) | Linked residue 1 | MS2 count | Note |
|---------------|---------------------|-----------------|---------------|---------------------|-----------------|-----------|------|
| Dgt5          | 97                  | K               | Dgt5          | 100                 | K               | 8         | within protein |
| Dgt5          | 116                 | K               | Dgt5          | 124                 | K               | 13        | within protein |
| Dgt5          | 98                  | K               | Dgt5          | 102                 | K               | 1         | within protein |
| Dgt5          | 430                 | K               | Dgt5          | 441                 | K               | 2         | within protein |
| Dgt5          | 305                 | Y               | Dgt5          | 315                 | K               | 2         | within protein |
| Dgt5          | 97                  | K               | Dgt5          | 102                 | K               | 1         | within protein |
| Dgt5          | 73                  | K               | Dgt5          | 81                  | S               | 1         | within protein |
| Dgt5          | 71                  | K               | Dgt5          | 97                  | K               | 1         | within protein |
| Dgt5          | 71                  | K               | Dgt5          | 102                 | K               | 1         | within protein |
| Dgt5          | 1                   | M               | Dgt5          | 475                 | S               | 2         | within protein |
| Dgt5          | 1                   | M               | Dgt5          | 15                  | T               | 2         | within protein |
| Dgt6          | 390                 | K               | Dgt6          | 462                 | K               | 2         | within protein |
| Dgt6          | 19                  | K               | Dgt6          | 23                  | K               | 3         | within protein |
| Dgt6          | 362                 | K               | Dgt6          | 390                 | K               | 2         | within protein |
| Dgt6          | 475                 | K               | Dgt6          | 481                 | K               | 1         | within protein |
| Dgt6          | 390                 | K               | Dgt6          | 481                 | K               | 1         | within protein |
| Dgt6          | 573                 | S               | Dgt6          | 589                 | S               | 2         | within protein |
| Dgt6          | 462                 | K               | Dgt6          | 475                 | K               | 7         | within protein |
| Dgt6          | 353                 | K               | Dgt6          | 362                 | K               | 4         | within protein |
| Dgt6          | 23                  | K               | Dgt6          | 143                 | K               | 1         | within protein |
| Dgt6          | 23                  | K               | Dgt6          | 71                  | K               | 2         | within protein |
| Dgt6          | 444                 | K               | Dgt6          | 462                 | K               | 1         | within protein |
| Dgt6          | 555                 | K               | Dgt6          | 589                 | S               | 1         | within protein |
| Dgt6          | 574                 | T               | Dgt6          | 589                 | S               | 2         | within protein |
| Dgt6          | 444                 | K               | Dgt6          | 475                 | K               | 1         | within protein |
| Dgt6          | 71                  | K               | Dgt6          | 82                  | K               | 2         | within protein |
| Dgt6          | 352                 | K               | Dgt6          | 390                 | K               | 1         | within protein |
| Dgt6          | 71                  | K               | Dgt6          | 143                 | K               | 1         | within protein |
| Dgt6          | 422                 | K               | Dgt6          | 462                 | K               | 1         | within protein |
| Dgt6          | 389                 | S               | Dgt6          | 442                 | K               | 1         | within protein |
| Dgt6          | 390                 | K               | Dgt6          | 442                 | K               | 1         | within protein |
| Dgt6          | 362                 | K               | Dgt6          | 364                 | S               | 1         | within protein |
| Msd1          | 44                  | S               | Msd1          | 113                 | K               | 3         | within protein |
| Msd1          | 33                  | S               | Msd1          | 45                  | K               | 2         | within protein |
| Msd1          | 83                  | S               | Msd1          | 113                 | K               | 3         | within protein |
| Msd1          | 33                  | S               | Msd1          | 113                 | K               | 2         | within protein |
| Msd1          | 84                  | S               | Msd1          | 113                 | K               | 3         | within protein |
| Msd1          | 45                  | K               | Msd1          | 113                 | K               | 3         | within protein |
| Msd1          | 25                  | K               | Msd1          | 113                 | K               | 8         | within protein |
| Msd1          | 33                  | S               | Msd1          | 110                 | S               | 2         | within protein |
| Msd1          | 25                  | K               | Msd1          | 33                  | S               | 4         | within protein |
| Msd1          | 25                  | K               | Msd1          | 44                  | S               | 3         | within protein |
| Msd1          | 109                 | S               | Msd1          | 113                 | K               | 5         | within protein |
| Msd1          | 33                  | S               | Msd1          | 78                  | K               | 2         | within protein |
| Msd1          | 110                 | S               | Msd1          | 113                 | K               | 5         | within protein |
| Msd1          | 25                  | K               | Msd1          | 110                 | S               | 3         | within protein |
| Msd1          | 44                  | S               | Msd1          | 48                  | S               | 2         | within protein |
| Msd1          | 25                  | K               | Msd1          | 78                  | K               | 4         | within protein |
| Msd1          | 25                  | K               | Msd1          | 45                  | K               | 7         | within protein |
| Msd1          | 33                  | S               | Msd1          | 100                 | Y               | 1         | within protein |
| Msd1          | 42                  | S               | Msd1          | 48                  | S               | 2         | within protein |
| Msd1          | 45                  | K               | Msd1          | 83                  | S               | 1         | within protein |
| Msd1          | 25                  | K               | Msd1          | 100                 | Y               | 1         | within protein |
| Msd1          | 25                  | K               | Msd1          | 42                  | S               | 1         | within protein |
| Msd1          | 42                  | S               | Msd1          | 113                 | K               | 1         | within protein |
| Msd1          | 100                 | Y               | Msd1          | 110                 | S               | 2         | within protein |
| Msd1          | 33                  | S               | Msd1          | 48                  | S               | 1         | within protein |
| Msd1          | 26                  | K               | Msd5          | 135                 | K               | 4         | within protein |
| Msd5          | 72                  | K               | Msd5          | 86                  | K               | 4         | within protein |
| Msd5          | 135                 | K               | Msd5          | 143                 | K               | 1         | within protein |
| Msd5          | 163                 | T               | Msd5          | 178                 | T               | 2         | within protein |
| Wac           | 132                 | K               | Wac           | 143                 | K               | 5         | within protein |
| Wac           | 132                 | K               | Wac           | 140                 | K               | 7         | within protein |
| Wac           | 140                 | K               | Wac           | 146                 | K               | 2         | within protein |
| Wac           | 132                 | K               | Wac           | 146                 | K               | 1         | within protein |
| Wac           | 143                 | K               | Wac           | 156                 | K               | 1         | within protein |
| Wac           | 131                 | T               | Wac           | 143                 | K               | 1         | within protein |

Linkage FDR was set to 5%. 136 linkages were identified for the 8 Augmin subunits. As a control, a search against the 12 most intense co-purified proteins (Table 1) was carried out with the same search parameters. No linkage sites were identified.
or the Augmin subunit Wac, had no effect on mitotic progression, spindle architecture or the spindle localisation of either Msd1-GFP or γ-Tubulin-GFP (Fig. 3A; Movies 1-5). However, injection of any of the three truncated proteins following nuclear envelope breakdown resulted in an *augmin*-like phenotype of long, weak density spindles, which arrested at the metaphase/anaphase transition (Fig. 3A-C; Movies 6-8; Wainman et al., 2009; Hayward et al., 2014). We measured the fluorescence intensity of Msd1-GFP on multiple spindles, in multiple embryos for each condition, and found that, in all cases, it did not significantly change over time (Fig. 3D,E; Movies 9-11). Similarly, injection of the truncated proteins into embryos expressing Dgt5-GFP did not result in loss of Dgt5 from spindle MTs (Movies 12-14). This demonstrates that the *augmin*-like phenotype is not a consequence of disrupting the localisation and function of the Augmin complex, per se. In contrast, the intensity of γ-Tubulin-GFP and Dgp71WD on spindles in each condition reduced over time to apparent near-background levels (Movies 15-20). This was quantified for spindle-associated γ-Tubulin-GFP (Fig. 3D). The measured difference in effect between Msd1-GFP and γ-Tubulin-GFP accumulation on the spindle after subunit injection was most apparent, and statistically significant, when the initial fluorescence intensity and the intensity *~*600 s after injection was compared (Fig. 3E). These results therefore support a model in which injected Dgt3N, Dgt5N or Dgt6C bind directly to Augmin and γ-TuRC.

Overall, the structural, biochemical and cell biological data presented here suggests a complex mechanism by which *Drosophila* Augmin bridges the gap between pre-existing MTs and γ-TuRC, requiring at least three of the eight subunits. It also demonstrates the power of CLMS as a tool with which to provide testable hypotheses regarding the cellular function of protein complexes for which there is little, or no, structural data; expanding the base of CLMS (Chen et al., 2010; Lasker et al., 2012) to a structure investigation method in its own right. Future investigations of purified Augmin, based on the data here, should shed further light on the precise architecture of Augmin and the mode of action by which it facilitates MT-templated MT nucleation.

**MATERIALS AND METHODS**

**GFP-TRAP-A isolation of Augmin**

Flies expressing full-length Msd1-GFP via UASp/maternal-α-Tubulin GAL4 control (Wainman et al., 2009) were maintained according to standard procedures at 25°C. Batches of 0- to 3-h-old embryos laid by cages of 1- to 10-day-old flies were dechorionated, weighed, flash frozen in N2 (l) and stored at −80°C. A total of 8 g of frozen embryos were homogenized in 16 ml C buffer (50 mM HEPES, pH 7.4, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.1% IGEPAL CA-630, Roche protease inhibitors). Extract was clarified through centrifugation at 10,000 g for 10 min, 100,000 g for 30 min, and 100,000 g for a further 10 min. Clarified extract was incubated with 50 μl GFP-TRAP-A beads (Chromotek, Germany) overnight at 4°C to immunoprecipitate Augmin. Msd1-GFP/Augmin-GFP-TRAP-A beads were washed three times with ice-cold C buffer and three times with ice-cold C buffer without IGEPAL CA-630. Based on previous semi-quantitative western blotting (not shown) we estimate 80 μg of Augmin was present in the sample.

**Mass spectrometry sample preparation**

To estimate sample quality and digestion efficiency, 2.5% of total beads were analyzed by LC-MS/MS. This Augmin aliquot was resuspended in 50 μl of 50 mM ammonium bicarbonate. Trypsin was added to a final concentration of 20 ng/μl and samples digested at 37°C with shaking overnight. Supernatant (containing peptides) was collected and acetyfied to pH 3 with 0.1% trifluoroacetic acid. Peptides were subsequently desalted using C18-StageTips (Rappsilber et al., 2003) for mass spectrometric analysis.

The remaining (97.5%) purified Augmin was re-suspended in 200 μl C buffer and cross-linked using 400 μg of bis(sulfosuccinimidyl) suberate (BS3) [i.e. 1:5 protein to cross-linker ratio (g/g)]. The cross-linking reaction was incubated on ice for 2 h with periodic agitation. After removal of supernatant, the beads were incubated with 200 μl of 50 mM ammonium bicarbonate for 30 min on ice with periodic agitation; then, 3 μg trypsin was added and digestion left to occur at 37°C with shaking overnight. After digestion, peptide mixture (in supernatant) was collected and fractionated using SCX-StageTips (Rappsilber et al., 2003) with a small variation to the protocol previously described for linear peptides (Ishihama et al., 2006). In short, the peptide mixture was acetyfied with 2.5% acetic acid to pH3 and was loaded on a SCX-Stage-Tip. The bound peptides were eluted in four steps with buffers (10% v/v ACN, 0.5% v/v acetic acid) containing 50 mM, 100 mM, 200 mM and 500 μM ammonium acetate into four fractions. Cross-linked peptides were expected to be in the three fractions that were eluted with higher ammonium acetate concentrations. Peptides in these three fractions were desalted using C18-StageTips (Rappsilber et al., 2007) prior to mass spectrometric analysis.

**Mass spectrometric analysis**

Samples were analyzed using an LTQ-Orbitrap mass spectrometer (ThermoElectron, Germany) in order to determine composition. Peptides were separated on an analytical column packed with dmel-all-RepresSil-Pur C18-AQ 3 μm; Dr Maisch GmbH, Ammerbuch-Entringen, Germany) in a spray emitter (75 μm inner diameter, 8 μm opening, 250 mm length; New Objectives). Mobile phase A consisted of water and 0.5% acetic acid. Mobile phase B consisted of acetonitrile and 0.5% acetic acid. Peptides were loaded at a flow rate of 0.5 μl/min and eluted at 0.3 μl/min using a linear gradient going from 1% B to 32% B in 55 min followed by a linear increase from 32% to 76% in 5 min. The eluted peptides were directly introduced into the mass spectrometer. MS data was acquired in the data-dependent mold. For each acquisition cycle, the mass spectrometric spectrum was recorded in the orbi-trap with a resolution of 60,000. The 20 most intense ions in the with a precursor charge state 2* or higher were fragmented in the ion-trap by collision-induced disassociation. The fragmentation spectra were then recorded in the LTQ linear ion trap at normal scan rate. Dynamic exclusion was enabled with single repeat count and 60 s exclusion duration.

SCX-Stage-Tip fractions were analyzed using same LC-MS/MS system as described above however with a high-high strategy. Peptides were loaded at a flow rate of 0.5 μl/min and eluted at 0.3 μl/min using a linear gradient going from 3% B to 35% B in 130 min followed by a linear increase from 35% to 80% in 5 min. The eluted peptides were directly introduced into the mass spectrometer. MS data was acquired in the data-dependent mold. For each acquisition cycle, the mass spectrometric spectrum was recorded in the orbi-trap with a resolution of 100,000. The eight most intense ions in the with a precursor charge state 3* or higher were fragmented in the ion-trap by collision-induced disassociation. The fragmentation spectra were then recorded in the orbi-trap at a resolution of 7500. Dynamic exclusion was enabled with single repeat count and 60 s exclusion duration.

**Identification of proteins in the purified Augmin sample**

The raw mass spectrometric data of the purified Msd1-GFP/Augmin-GFP-TRAP-A beads sample was processed into peak list using MScove module from Proteowizard (v.3.0.3414) (Kessner et al., 2008). Database search was conducted using Mascot (v. 2.4) (Matrix Sciences). Specified database search parameters were: MS accuracy, 6 ppm; MS/MS accuracy, 0.5 Da; enzyme, trypsin; variable modification, oxidation on methionine; database, dmel-all-translation-r5.48 database (FlyBase); protein FDR, 1%. Protein abundance in the sample was estimated based on PAI value (Ishihama et al., 2008). The top 20 identified proteins (based on abundance) are listed in Table 1.

**Identification of cross-linked peptides**

The raw mass spectrometric data files of SCX fractions of cross-linked Msd1-GFP/Augmin were processed into peak lists using MaxQuant version 1.2.2.5 (Cox and Mann, 2008) with default parameters, except ‘Top MS/MS Peaks per 100 Da’ was set to 20. The peak lists were searched against the sequences of the eight Augmin subunits, using Xi software (ERI, Edinburgh) for identification of cross-linked peptides. Search parameters...
were as follows: MS accuracy, 6 ppm; MS/MS accuracy, 20 ppm; enzyme, trypsin; specificity, fully tryptic; allowed number of missed cleavages, four; cross-linker, BS3; fixed modifications, carboxymethylation on cysteine; variable modifications, oxidation on methionine. The linkage specificity for BS3 was assumed to be for lysine, serine, threonine, tyrosine and protein N-terminus. Linkage FDR was set to 5%. As a control, a search against the 20 most abundant proteins identified from the purified Msd1-GFP/Augmin-GFP-TRAP samples (including 8 Augmin subunits and the 12 most intense co-purified proteins; Table 1) was carried out with the same search parameters.

Protein expression and purification
pGEX-Dgp71WD was a gift from Jordan Raff (University of Oxford, UK). pQE80-His-GFP was obtained from Steven Porter (University of Exeter, UK). pRSETA-Dgt3N, pRSETA-Dgt5N, and pRSETA-Dgt6N were created using the GeneArt service (Life Technologies). pRSETA-Dgt3N constituted the Dgt3N construct (see following section). Bacteria expressing His-tagged Dgt3N, Dgt5N, and Dgt6C were incubated in Buffer B (PBS adjusted to 900 mM NaCl, 0.5% Tween 20, 0.2 mg/ml lysozyme, 100 mM imidazole, and 0.1% IGEPAL CA-630). His-tagged GFP was purified as described (Trigg et al., 2011) and Marcoil (based on window-less HMMs, optimised sliding window method with Hidden Markov Model (HMM) approaches: DISOPRED2 (Ward et al., 2004) and PrDOS (Ishida and Kinoshita, 2007). PrDOS judges disorder by local amino acid sequence (sliding window), using support vector machine learning, and also by template prediction based on conservation of disorder in related protein families (subject to availability of high-resolution structural data). DISOPRED2 identifies ordered residues in a similar fashion – based on prior knowledge of crystal structures (missing residues in electron density), and also through a local sequence profile classification using neural networks (sliding window).

Drosophila stocks
The Msd1-GFP flies have been previously reported (Wainman et al., 2009). To follow Dgt5 localisation in vivo, full-length dgt5 was cloned into the Gateway expression vector pPWG (Drosophila Genome Resource Center) via pENTR/D/TOPO. The plasmid was injected into w1118 embryos by BestGene, Inc. In both cases, expression was driven in the female germline using the Maternal-α-Tubulin VP16GAL4 line (Bloomington Stock Center, Indiana University, USA). Flies expressing α-Tubulin-GFP were obtained from the Bloomington Stock Center. Flies expressing γ-Tubulin-GFP, under the control of the Ncd promoter, were a gift from Sharyn Endow. Flies expressing Dgp71WD under the control of the Polyubiquitin promoter were a gift from Jordan Raff (University of Oxford, UK).

Drosophila embryo microinjections and imaging
Drosophila embryos 1- to 2-h-old were harvested from 1- to 5-day-old adults. Embryos were manually dechorionated and mounted on 22×50 cm coverslips with heptane glue. The embryos were covered with 1:1 mixture of halocarbon oil 700 and halocarbon oil 27 (Sigma). Images were acquired with Visitron Systems Olympus IX81 microscope with a CSO-X1 spinning disk using a UPlanS APO 1.3 NA (Olympus) 60× objective. Images were acquired at 10 s intervals, in which five stacks 1 μm apart were taken with coarse focus, and 10 µm exposure at 10% laser power for all genotypes, except embryos expressing Dgp71WD-GFP, where 20% laser power was used. His-tagged Wac, Dgt3N, Dgt5N, and Dgt6N were buffer-exchanged with injection buffer (100 mM HEPES pH 7.4, 50 mM KCl) and concentrated with 30 kDa size-exclusion columns (Amicon). Protein concentration was measured by Bradford assay. Proteins were injected at 5 µl/µl. Embryos were injected using Eppendorf Inject Man NI 2 and Femtotips II needles (Eppendorf).

Bioinformatics
Potential coiled-coil formation was assessed by two independent algorithms: MultiCoil2 (a modern algorithm that combines probabilistic sliding window method with Hidden Markov Model (HMM) approaches) (Trigg et al., 2011) and Marcoil (based on window-less HMMs, optimised for the simultaneous recognition of domains of different lengths) (Delorenzi and Speed, 2002).

Image analysis
Image processing and analysis were undertaken using Fiji software. The five stacks taken for each time-frame were combined under maximum projection. For each of the BSA, Dgt3N, Dgt5N, and Dgt6N injections, 3-6 embryos at cycle 10 and between 24 and 47 spindles were selected for length measurements. The distances from the centrosome pairs were measured...
when the metaphase spindles reached a maximum length, determined visually (Fig. 3C). For each of the Dg3N, Dg5N, and Dg6C injections, three embryos expressing γ-Tubulin-GFP, and three embryos expressing Msd1-GFP were selected for fluorescence intensity measurements, as follows: Photobleaching was accounted for using the ratio method as described in (Phair et al., 2003) and used in the widely-used ImageJ bleach correction plug in (Miura et al., 2014). Each spindle intensity measurement was normalised by dividing by a nearby background value. The resulting value was converted to a percent of maximum above background so that the relative fluorescence decrease between γ-Tubulin-GFP and Msd1-GFP could be compared. A significant decrease of fluorescence intensity was determined by comparing the values between γ-Tubulin-GFP and Msd1-GFP 600 s post initial measurement. A two-tailed Mann Whitney U test was performed on the distributions of the resulting percentage changes using the Scipy Python library (Jones et al., 2001) to produce P values for significance levels as indicated in Fig. 3.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.G.W.; Methodology: J.W.C.C., Z.A.C., K.B.R., J.M., Software: K.B.R., J.M.; Validation: Z.A.C., K.B.R., J.M.; Formal analysis: J.W.C.C., Z.A.C., K.B.R., J.M.; J.G.W.; Investigation: J.W.C.C., Z.A.C., K.B.R., J.M.; Writing - original draft: J.W.C.C., J.G.W.; Writing - review & editing: J.W.C.C., Z.A.C., K.B.R., J.M., C.M.D., J.R., J.G.W.; Supervision: C.M.D., J.R., J.G.W.; Project administration: J.G.W.; Funding acquisition: C.M.D., J.R., J.G.W.

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Data availability
The data of the cross-linking/mass spectrometry analysis are available via ProteomeXchange with identifier PXD006246.

Supplementary information
Supplementary information available online at http://bio.biologists.orglookup/doi/10.1242/bio.022905supplemental

References
Buccirelli, E., Pellacani, C., Naim, V., Palena, A., Gatti, M. and Somma, M. P. (2009). Drosophila Dg6 interact with Ndc80, Msps/XMAP215, and gamma-tubulin to promote kinetochore-driven MT formation. Curr. Biol. 19, 1839-1845.

Chen, Z. A., Jawhari, A., Fischer, L., Buchen, C., Tahir, S., Kamenski, T., Rasmussen, M., Lariivi, L., Bukowski-Wills, J.-C.,Nilges, M. et al. (2010). Architecture of the RNA polymerase II-TFIIF complex revealed by cross-linking and mass spectrometry. EMBO J. 29, 717-726.

Cox, J. and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367-1372.

Delorenzi, M. and Speed, T. (2002). An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18, 617-625.

Duncan, T. and Wakefield, J. G. (2011). 50 ways to build a spindle: the microtubule arrays in Arabidopsis. Chromosome Res., 19, 321-333.

Goshima, G., Wollman, R., Goodwin, S. S., Zhang, N., Scholey, J. M., Vale, R. D. and Stuurman, N. (2007). Genes required for mitotic spindle assembly in drosophila S2 cells. Science 316, 417-421.

Goshima, G., Mayer, M., Zhang, N., Stuurman, N. and Vale, R. D. (2008). Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle. J. Cell Biol. 181, 421-429.

Hayward, D., Metz, J., Pellacani, C. and Wakefield, J. G. (2014). Synergy between multiple microtubule-generating pathways confers robustness to centrosome-driven mitotic spindle formation. Dev. Cell 28, 81-93.

Ho, C.-M., Hotta, T., Kong, Z., Zeng, C.-J., Sun, J., Lee, Y.-R. and Liu, B. (2011). Augmin plays a critical role in organizing the spindle and phragmoplast microtubule arrays in Arabidopsis. Plant Cell 23, 2606-2618.
microtubule generation for mitotic progression and cytokinesis in human cells. Proc. Natl. Acad. Sci. USA 106, 6998-7003.

Wainman, A., Buster, D. W., Duncan, T., Metz, J., Ma, A., Sharp, D. and Wakefield, J. G. (2009). A new Augmin subunit, Mad1, demonstrates the importance of mitotic spindle-templated microtubule nucleation in the absence of functioning centrosomes. Genes Dev., 23, 1876-1881.

Ward, J. J., McGuffin, L. J., Bryson, K., Buxton, B. F. and Jones, D. T. (2004). The DISOPRED server for the prediction of protein disorder. Bioinformatics 20, 2138-2139.

Zhu, H., Copinger, J. A., Jang, C.-Y., Yates, J. R., Ill and Fang, G. (2008). FAM29A promotes microtubule amplification via recruitment of the NEDD1-gamma-tubulin complex to the mitotic spindle. J. Cell Biol. 183, 835-848.