Orbit/CLASP determines centriole length by antagonising Klp10A in *Drosophila* spermatocytes

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Summary Statement:

A microtubule polymerising factor, Orbit/CLASP, localises on centrioles and determines centriole length by antagonising Klp10A in *Drosophila* spermatocytes.
ABSTRACT

After centrosome duplication, centrioles elongate before the M phase. To identify genes required for this process and understand the regulatory mechanism, we investigated the centrioles in *Drosophila* premeiotic spermatocytes, expressing fluorescently tagged centrioles. We demonstrated that an essential microtubule polymerisation factor, Orbit/CLASP, accumulated at the distal end of centrioles and was required for the elongation. Conversely, a microtubule severing factor, Klp10A, shortened the centrioles. Genetic analyses revealed that these two proteins functioned antagonistically for determining centriole length. Furthermore, Cp110 in the distal tip complex was closely associated with the factors involved in centriolar dynamics at the distal end. We observed loss of centriole integrity, including fragmentation of centrioles and earlier separation of the centriole pairs in *Cp110* null mutant cells either overexpressing Orbit or harbouring *Klp10A* depletion. Excess centriole elongation in the absence of the distal tip complex resulted in the loss of centriole integrity, leading to the formation of multipolar spindle microtubules emanating from centriole fragments, even when they are unpaired. Our findings contribute to understanding the mechanism of centriole integrity, leading to chromosome instability in cancer cells.
INTRODUCTION

The centrosome plays an indispensable role as the major microtubule organising centre (MTOC) in a cell. During mitosis, centrosomes duplicated in the S phase move apart from each other and reach the opposite poles of the cell. Each centrosome is involved in the assembly of spindle poles, which enables construction of the bipolar spindle microtubule structure (Vitre and Cleveland, 2012). A centrosome consists of two components: a pair of centrioles and the surrounding pericentriolar matrix (PCM). After replication of the centrioles, the longer centriole (mother centriole) engages with the shorter one (daughter procentriole) in a V-shape. When a cell enlarges in the G2 phase, the short daughter procentriole undergoes elongation to a certain length before the subsequent M phase. A single centriole consists of microtubule doublet or triplet, which is equivalent to the cytoplasmic microtubule. Several factors localised on centrioles have been shown to be involved in the centriole elongation process (Delgehyr et al., 2012, Mottier-Pavie and Megraw, 2009, Saurya et al., 2016, Schmidt et al., 2009). The most critical step in centrosome duplication is the duplication of centrioles, which requires stringent regulation. However, the entire mechanism underlying regulation of centriole elongation and the regulatory factors required for the process are not known. Among the centriole-associated proteins, those belonging to the kinesin-13 family are known to act as microtubule-severing kinesins (Ems-McClung and Walczak, 2010). Klp10A, a Drosophila member of the family, has been shown to play an indispensable role in the regulation of centriole elongation (Delgehyr et al., 2012). Based on these observations, we speculated that some of the factors regulating microtubule length
might overlap with those required for centriole elongation. Possibly, the production of centrioles of specific length requires a balance between polymerisation and depolymerisation of the triplet microtubules. However, the main factor(s) counteracting Klp10A and promoting centriole elongation remain to be identified.

Another characteristic complex containing Cp110 is localised at the distal tip of the centriole, where it regulates the accessibility of the distal end to the shrinking and hypothetical lengthening factors (Chen et al., 2002, Nigg and Raff, 2009), thereby regulating centriole elongation at this end (Chen et al., 2002, Schmidt et al., 2009, Spektor et al., 2007). In the absence of Klp10A, the longer centrioles harbour incomplete nine-fold symmetry at their ends in Drosophila cultured cells and tend to undergo fragmentation (Delgehyr et al., 2012). Importantly, Cp110 depletion differentially affects centriole elongation in a species- and/or cell type-specific manner. In Drosophila S2 cultured cells, Cp110 depletion resulted in centriole length diminution. This effect was rescued by simultaneous depletion of Klp10A (Delgehyr et al., 2012). In contrast, Cp110 depletion resulted in centriole elongation in mammalian cells (Schmidt et al., 2009). The centriolar microtubules were dramatically elongated in somatic cells such as wing discs and larval brain cells in the Cp110 null mutant, while subtle elongation of the structure was observed in the premeiotic spermatocytes of the mutant (Franz et al., 2013).

The premeiotic spermatocyte in Drosophila is a good model for investigating centrosomes and centrioles. Drosophila spermatogenesis involves four mitotic and two meiotic cycles for the formation of haploid spermatids (See Fig. S1,
Fuller, 1993; Inoue et al., 2012; Tanabe et al., 2017; White-Cooper, 2004). A same spermatocyte cyst, each of the 16 cells undergoes synchronous cell growth, which can be divided into S1 stage corresponding to S phase, and subsequent five stages S2 to S6 before initiation of meiosis I. Especially the centrioles can be studied more easily in this cell type (Fuller 1993, Riparbelli et al., 2012, Persico et al., 2019), since these organelles dramatically elongate until the onset of meiosis (see Fig. S1C, Riparbelli et al., 2012) and the centriole cylinder is composed of microtubule triplets (Gottardo et al., 2015). In early spermatocytes that possess a pair of centrioles initially, centrioles duplicate at S1 stage. As primary spermatocytes enter in the growth phase, centrioles migrate toward surface where they assemble the primary cilium at the distal end of basal body (Fig. S1B). At the beginning of meiotic division I, centrioles move close to the nucleus with their associated “membrane pocket” on the distal end of cilium-like region (CLR) (Fig. S1C). Between the CLR and the basal body, there is the transition zone (TZ) that plays an important role in elongating primary cilium of spermatocyte (Vieillard et al., 2016). Centrioles are no longer duplicated between two meiotic divisions. Primary spermatocytes hold two pairs of centrioles composed of nine triplet microtubules and engaged by a cartwheel structure at the proximal ends (Fig. S1C). The centriole pair is disengaged during prophase II, consequently singlet centrioles organize centrosomes of secondary spermatocytes.

Previous studies have shown that Orbit/CLASP is essential for microtubule polymerisation, as it adds tubulin dimers to the plus end of the microtubules (Inoue et al., 2000, Inoue et al., 2004, Lemos et al., 2000, Maiato et al., 2003). However, its role
in centriole elongation has not been examined. Hence, in this study, we aimed to investigate whether Orbit/CLASP was involved in centriole elongation in the mature premeiotic spermatocytes before male meiosis. As Orbit antagonises Klp10A, a severing factor determining the length of spindle microtubules in cultured Drosophila cells (Laycock et al., 2006), we assessed whether Orbit was also involved in centriole length regulation.

In addition, we highlighted the importance of these regulators of centriole dynamics and the distal end capping proteins in the centriole elongation process using Drosophila spermatocytes. We also discussed the importance of regulating the elongation of duplicated centrioles to a certain length for proper chromosome inheritance during male meiotic divisions.

RESULTS

Differential distribution of several centriole-associated proteins along the centrioles in Drosophila premeiotic spermatocytes

To understand the mechanism via which centrioles of specific lengths are generated, we used Drosophila primary spermatocytes for observing centrioles before meiosis, and for identifying factors involved in the elongation processes. Initially, we performed immunostaining of premeiotic spermatocytes using an antibody against centriolar protein Asl, to observe centrioles from the S1 phase to prophase I during the growth stage before male meiosis. Conventional fluorescence microscopy revealed that a pair of centrioles gradually elongated to attain the length of the mature centrioles during the
growth stage (Fig. 1A). Hence, we next visualised them using four markers, Asl, Ana1, γ-tubulin, and PACT, which are the centriole-associated proteins. And we measured the average length of the organelles from the S3 stage, when prominent cell growth has been initiated, to the initiation of meiosis I (Fig. 1B). In each case, the centrioles observed using these markers elongated to approximately 1.1 µm on average before and/or at the beginning of meiosis I. It was twice as long as the length at the S3 stage. This encouraged us to investigate the mechanism of centriole elongation in premeiotic spermatocytes. To confirm centriole elongation in spermatocytes, we observed the cells using structure illumination microscopy (SIM). First, we observed the distribution of four known centriole-associated proteins, Asl, PACT, and β- and γ-tubulin on two pairs of centrioles in the premeiotic spermatocyte in the mature stage, by expressing the fluorescently tagged proteins or immunostaining with respective antibodies. We observed the spermatocytes expressing GFP-β-tubulin and mRFP-PACT (Fig. S2A). The mRFP-PACT–expressing cells were investigated through immunostaining for γ-tubulin (Fig. S2B) or Asl (Fig. S2C). These four proteins localised on the centrioles, although their distributions differed (Fig. S2A-A”, S2B-B”, and S2C-C”). Both Asl and γ-tubulin were distributed on basal body composed of triplet microtubules in both centrioles of a pair (Fig. S2B). Asl was distributed along the whole basal body region, while the anti-γ-tubulin signal appeared on basal body as a dotted pattern (Fig. S2B, B’, C, C’). These results were consistent with those of previous reports (Lattao et al., 2017; Moritz et al., 1995). However, these two proteins are not suitable for specifically measuring centriole length, as they localise both on centrioles and the PCM region. The
β-tubulin distribution represents doublet and triplet microtubules in centriole (Fig. S2A, A’). The average length of the β-tubulin region on the centrioles was 1.08 ± 0.03 µm (n = 20). Although the tubulin was distributed along the entire centriole, it also constitutes the subcellular microtubules, which renders specific observation of centrioles difficult. In contrast, the C-terminal domain of Dplp (a PCM protein), called the pericentrin-AKAP450 centrosomal targeting domain (PACT), is known to be localises along the centrioles (Gillingham and Munro, 2000; Jana et al., 2018; Richens et al., 2015). PACT distribution almost overlapped with the β-tubulin region on centrioles, although it was shifted slightly toward the proximal end of the centrioles compared to the β-tubulin region (>50 premeiotic spermatocytes from 10 males were examined) (Fig. S2A, A’’). The PACT region was 1.10 ± 0.02 µm in length (n = 54), which was as long as that of the β-tubulin region. Therefore, we used mRFP-PACT as a marker to visualise basal body of centrioles and measure their length.

**Orbit, a microtubule polymerisation factor, was localised along the centriole basal body, and predominantly accumulated at the distal tip of centrioles**

Orbit/CLASP is well known as an essential factor for microtubule polymerisation that acts by adding α- and β-tubulin heterodimers at the plus-end of the microtubules. To determine whether Orbit was also involved in elongation of the triplet microtubules, we first performed simultaneous immunostaining of wild type spermatocytes with anti-Orbit and anti-Asl antibodies. Asl was localised on the basal body structure composed of triplet microtubules in the paired centrioles (Fig. S2C’, Fig. 2A, A’’). In contrast,
Orbit was observed along the centrioles, while the inside of the basal body structure was visualised using anti-Asl immunostaining (Fig. 2A, A’). Importantly, Orbit was especially enriched at the distal end of centrioles, projecting from the Asl-localisation region (arrows in Fig. 2A, 2A’). Line analysis quantitating the fluorescence confirmed the protein distribution (Fig. 2B, C). Next, the predominant localization at the distal end encouraged us to investigated whether the protein was accumulated to the transition zone (TZ) of centriole (Fig. S1B, Fig. S3). We induced expression of GFP-Orbit in the cells expressing a TZ protein with a fluorescence tag, Cby-Tomato. Cby was localized only with the most distal part of the Orbit-localizing region (Fig. S3C, C’, C”). These observations suggest that Orbit over-expression did not result in excess elongation of the TZ region. These localization data, together with the known microtubule polymerization role of Orbit, suggested that it was possibly involved in elongation of the centriole at the distal end in spermatocytes before meiosis.

**Orbit was essential for centriole elongation in the spermatocytes before male meiosis**

To investigate this hypothesis, we performed spermatocyte-specific overexpression of Orbit using the *bam-Gal4* driver (*bam>orbit*) and measured centriole length. We defined the PACT-localising region in the centrosome as the centriole region and measured the length in premeiotic spermatocytes at the end of the growth phase before meiosis. We selected premeiotic spermatocytes in the mature stage based on the organisation of DAPI-stained chromatin and cell size. The average length (1.28 μm on
average, n = 51 centrioles) of the centrioles in bam>orbit was significantly longer (25%) than that (1.02 µm, n = 53) in normal control premeiotic spermatocytes (bam>GFP) in the mature stage (Fig. 3A-D, and I). Moreover, we induced overexpression of Orbit at three different levels using the Gal4/UAS system and compared the average centriole length (Fig. S4A, B). By quantitative fluorescence that a total GFP-Orbit fluorescence were measured in each spermatocyte, we confirmed that spermatocytes harbouring a single copy of UAS-GFP-Orbit with bam-Gal4 from males raised at 25°C can induce overexpression of Orbit at relatively lower level, whereas spermatocytes carrying bam-Gal4 and two copies of UAS-GFP-Orbit from males raised at 25°C can induce moderate overexpression, and those carrying bam-Gal4 and two copies of UAS-GFP-Orbit from males raised at 28°C can induce overexpression at relatively higher level (Fig. S4C). The length of the centrioles labelled by GFP-Orbit increased with the extent of overexpression. We also confirmed that Orbit overexpression even at a lower level resulted in a significant extension of centrioles labelled by anti-Asl immunostaining (Fig. S4B). Therefore, we conclude that Orbit over-expression can stimulate excessive centriole extension. In the cells over-expressing GFP-Orbit at the moderate level (w; Chy-Tom/+;bam-Gal4/UAS-GFP-Orbit), we sometimes observed centriole pairs with longer Orbit-localizing regions protruding from basal bodies (Fig. S6C, C”). In the cells, Cby was localized only with the most distal part, not the whole Orbit region. These observations suggest that Orbit over-expression did not result in excessive elongation of the TZ region. Moreover, we also observed the axonemal microtubules extended from basal bodies by anti-acetylated tubulin
immunostaining (Fig. S5G). Consequently, overexpression of Orbit resulted in excessive elongation of axonemal microtubules extending from the basal bodies.

To verify the involvement of Orbit in regulation of centriole elongation, we measured the length of centrioles in premeiotic spermatocytes homozygous for hypomorphic allele, orbit⁷. Spermatocytes failed to be observed in orbit mutants homozygous for the null allele because of their earlier stage death. The average length of the centrioles (0.70 µm, n = 62 centrioles) in the hypomorphic mutant was significantly lesser (63% of the control) than that in the control cells (w¹¹¹⁸) (1.10 µm on average, n = 54 centrioles) (Fig. 3E-I). These genetic results suggest that Orbit plays an essential role in both microtubule polymerisation and centriole elongation in spermatocytes.

**Klp10A acted as a negative regulator of centriole elongation in premeiotic spermatocytes**

A previous study has shown that mutation in Klp10A leads to production of longer centrioles in *Drosophila* primary spermatocytes. We confirmed that Klp10A localised on centrioles in a premeiotic spermatocyte (Fig. 4E-E′′, also see inset in the panels). Next, we induced spermatocyte-specific depletion of Klp10A using dsRNA against its mRNA, together with dcr2 to raise the RNAi efficiency. Results of qRT-PCR confirmed that UAS-Klp10ARNAi efficiently depleted the Klp10A mRNA (< 10% of the control (bam>dcr2, GFP) level) (Fig. 4C). In Klp10A-depleted spermatocytes (bam>dcr2, Klp10ARNAi), the average length (1.48 µm, n = 52 centrioles) of centrioles
was 137% of the control length (1.09 μm, n = 58 centrioles). Klp10A depletion in the spermatocytes resulted in the production of significantly longer centrioles. Conversely, the average length of centrioles in premeiotic spermatocytes overexpressing Klp10A (bam>Klp10A) was 0.79 μm (n = 74 centrioles), which was 73% of the average length (1.08 μm, n = 67 centrioles) in normal spermatocytes (bam>GFP) (Fig. 4F-J). These results were consistent with the previous finding that Klp10A acts a negative regulator of centriole length in premeiotic spermatocytes (Delgehyr et al., 2012).

Simultaneous overexpression of Orbit and depletion of Klp10A resulted in additive enhancement of centriole elongation

Next, we investigated whether these two factors interacted and antagonised each other. Toward this purpose, we overexpressed Orbit and depleted Klp10A simultaneously in spermatocytes (bam>dcr2, Klp10ARNAi, orbit) and measured the centriole length (Fig. 5A). Interestingly, spermatocytes harbouring Orbit overexpression and Klp10A depletion possessed an overly elongated GFP-Orbit signal protruding from the distal end of basal body. Orbit was distributed along centrioles overly extended from basal body and predominantly accumulated at the distal end of the centrioles (Fig. 2B, 5B). In spermatocytes, the effects of the modified expression of these two genes on centriole elongation were additive (1.62 μm average length, n = 54 centrioles) compared to those in bam>orbit (1.27 μm average length, n = 51 centrioles) and bam>dcr2, Klp10ARNAi (1.48 μm average length, n = 52 centrioles) (Fig. 5A). These results suggest that these two factors act antagonistically for the production of centrioles of specific length.
Loss of centriole integrity was frequently observed in the premeiotic spermatocytes with both Orbit overexpression and Klp10A depletion

In addition to elongation of centrioles in spermatocytes showing both Orbit overexpression and Klp10A depletion, we observed many centrioles with abnormal structure in these spermatocytes. Control spermatocytes (bam>dcr2,LacZ) contained a pair of properly engaged centrioles (100%, n = 11). Surprisingly, in spermatocytes harbouring both Klp10A depletion and Orbit overexpression (bam>dcr2, Klp10ARNAi, orbit), loss of centriole integrity was frequently observed in 77% cells (Fig. 5G). We classified the abnormal centrioles to three classes, disengagement, breakage, and overduplication. When we observed abnormal cell with two or four unpaired centrioles of full-length (approximately 1µm or longer), we considered that disengagement of either or both pairs of centrioles occurred precociously before meiosis. Thus, we categorized the cell as disengagement. If we found a cell with unpaired centriole pieces shorter than 1µm, we classified it as breakage, rather than disengagement. A cell carrying extra pairs or single full-length centrioles were classified as overduplication. Shorter centrioles in orbit7 mutant cells and bam>Klp10A cells were not included in the loss of integrity phenotypes, as far as they were engaged. In addition, we have observed spermatocytes showing two types of phenotypes at the same time. For example, we found the cells showing both disengagement and breakage phenotypes (Fig. 5E). To avoid counting these cells twice, we categorized them as breakage. When we have categorized the cell showing both breakage and overduplication, we categorized it as
overduplication. The cells showing disengagement and overduplication phenotypes were classified into overduplication. In total, 20% of bam>dcr2, Klp10ARNAi, orbit spermatocytes contained abnormal sets of centrioles that were precociously separated (disengagement, Fig. 5C). Half of the cells at mature stage harboured abnormally shorter pieces of the centrioles (breakage, Fig. 5D). In addition, 7% cells contained excessively duplicated centrioles (overduplication, Fig. 5E). Only 23% of the cells possessed properly engaged centrioles (Fig. 5B). These findings suggested that excessively elongated centrioles may easily lose their integrity.

A reduced or loss of Cp110 resulted in production of slightly longer centrioles and enhanced the centriole excessive elongation phenotype by Orbit overexpression

Cp110 is a member of distal tip complex involved in regulation of centriole elongation. First, we confirmed that Cp110 was localised on centrioles at the distal end in the earlier stages of premeiotic spermatocytes, but not at later stages (Fig. S6A-D). Orbit co-localized with Cp110 on distal tips of centriole basal body at early stage (Fig. S6A). Until mid-stage, Cp110 was localized on the most distal area of the Orbit-localizing region (Fig. S6B). The GFP-Orbit signal protruding from the distal end of centrioles overlapped with anti-acetylated tubulin immunostaining signal (Fig. S5G). Therefore, the GFP-Orbit was possibly distributed to axonemal microtubules emanating from the distal tips of basal body (Fig. S5B-D). Cp110 disappeared from centrioles at late spermatocyte stage, while Orbit continued to be localized on centrioles (Fig. S6C, D). A previous study reported that Cp110 co-localised with Klp10A at the distal ends in
Drosophila cultured cells (Delgehyr et al., 2012). We performed proximity ligation assay (PLA) and showed that Orbit and Cp110 are closely associated with each other at the end of the centrioles in earlier, but not later stage spermatocytes. (Fig. S7).

Thus, we next investigated whether these proteins are interdependent on each other in centriole localization. We first performed anti-Cp110 immunostaining of control (Fig. S8A-C) and orbit

mutant spermatocytes (Fig. S8D-F) during spermatocyte development. The Cp110 was localized at the distal ends of centrioles in control spermatocytes at the early and mid-stages (75/84, 89.3% of the cells, no signals in 10.7% (9/84)). In the hypomorphic mutant cells at the same stages, the protein was comparably observed in 82.7% of the centrioles (91/110) (no signals in 17.3% (19/110)). On the contrary, we investigated whether Orbit is localized at the distal centriole ends in the spermatocytes from Cp110 null mutant males. Among 66 centrioles in control spermatocytes expressing GFP-Orbit, the signal was present at distal ends on 97.0% (64/66) of centrioles (no signal in 3 % (2/66)). Similarly, among 85 centrioles in spermatocytes at mid and late stages from Cp110 null mutant with GFP-Orbit expression, the signal was still localized at distal ends on 98.8% (84/85) of the centrioles (1.1 % (1/85) possessed no signal at either end of two pairs) (Fig. S8G). Therefore, we speculate that Orbit and Cp110 are not required for each other in mutual localization on centrioles.

Next, we investigated whether the distal end complex protein is required for centriole elongation using a Cp110 null mutant, Cp110

A previous study reported that most of centrioles of the Cp110

mutant spermatocytes and embryos behaved normally
(Franz et al., 2013). By contrast, we showed that spermatocytes from the $Cp110^{Δ1}$ mutant possessed slightly longer centrioles (1.20 µm on average, $n = 66$ centrioles in 19 spermatocytes) than the control (1.10 µm) (Fig. 6A, B, J). Among 66 centrioles, the longest centriole was 1.93 µm in length and the shortest was 0.67 µm. In total, nine centrioles were longer than 1.5 µm (Fig. 6J).

If the distal tip protein would act as a cap that restricts the access of the factors dynamically regulating centriole length, we expected that the loss of $Cp110$ enhanced the phenotype of overly longer centrioles in the cells overexpressing Orbit. In the $Cp110$ null mutant, average length of centrioles increased (1.20 µm on average, $n = 66$ centrioles examined), compared to that in control spermatocytes (1.02 µm, $n = 53$). Comparing to the length in the cells overexpressing Orbit ($bam>orbit$) (1.33 µm, $n = 49$) or that in the $Cp110^{Δ1}$ mutant cells, the length significantly increased in $Cp110^{Δ1}/Y; bam>orbit$ cells (Fig. 6A-D) (1.46 µm, $n = 21$) (Fig. 6J, $p < 0.0001$ in both cases, Student’s $t$-test). Consistently, the centrioles in $Cp110^{Δ1}/Y; bam>Klp10ARNAi$ cells also significantly increased in length (Fig. 6G-I) (1.67 µm, $n = 45$, Fig. 6K), compared to that in the cells harbouring Klp10A depletion ($bam>Klp10ARNAi$) (1.48 µm, $n = 52$) (Fig. 6E-G) (Fig. 6K, $p < 0.0001$ in both cases). Contrary to the conditions that stimulate centriole elongation, the length did not change in the $Cp110^{Δ1}$ cells overexpressing Klp10A ($Cp110^{Δ1}/Y; bam>Klp10A$ cells (0.94 µm, $n = 55$), compared to that in $bam>Klp10A$ cells (Fig. 6, H, I) (0.95 µm, $n = 51$, Fig. 6K), even though centrioles decreased in length in both cases (Fig. 6K). These results suggested that
Cp110 is also involved in determination of centriole length by restricting the access of these dynamic factors at the distal ends.

The Cp110 null mutation substantially enhanced the loss of centriole integrity phenotype in spermatocytes harbouring Orbit overexpression or Klp10A depletion

In addition to moderately excessive elongation of centrioles, we observed the loss of centriole integrity in 24% of the Cp110 null mutant spermatocytes (n = 21, Fig. 7I). These centriole phenotypes are similar to those seen in the cells harbouring Orbit overexpression and Klp10A depletion simultaneously. They are classified into centriole breakage inferred from a presence of shorter pieces labelled by mRFP-PACT (arrow in Fig. 7B) (14%, 3 of 21 cells, Fig. 7I) and centriole over-duplication (arrows in inset of Fig. 7B) (10%, 2/21, Fig. 7I). Surprisingly, we observed loss of centriole integrity in Cp110Δ1 cells harbouring Orbit overexpression at significantly higher frequency (78% of the centrioles in the 32 spermatocytes observed), compared to the loss of integrity phenotypes in either bam>orbit cells (Fig. 7C) or Cp110Δ1 cells (Fig. 7I). We observed several types of abnormal cells containing the following centriole abnormalities: premature separation of the centriole pair (disengagement) (arrows in Fig. 7D, 34%, Fig. 7I), putative centriole fragments (breakage) (arrowhead in Fig. 7D, 38%, Fig. 7I), and presence of extra pairs of centrioles (overduplication) (6%, Fig. 7I). These centriole phenotypes were also observed in the bam>dcr2, Klp10ARNAi, orbit spermatocytes (Fig. 5C-F). Consistently, these centriole integrity phenotypes can be seen in Cp110Δ1; bam>dcr2, Klp10ARNAi spermatocytes (n = 23); 35% cells harboured prematurely
separated centrioles (arrowheads in Fig. 7F), 22% cells contained shorter centrioles (arrow in Fig. 7F), and 4% cells had extra pairs of centrioles, while 39% spermatocytes contained normally engaged centrioles (Fig. 7D, I). We observed the centriole integrity phenotypes in the *Cp110*<sup>Δ1</sup> cells with Klp10A depletion at much higher frequencies (61% of the centrioles), compared to those in either *bam>orbit* cells or *Cp110*<sup>Δ1</sup> cells (Fig. 7I). Conversely, abnormal centrioles were not observed in *Cp110* mutant cells overexpressing Klp10A (*n* = 15) (Fig. 7H, I).

Moreover, we investigated whether post-translational modifications of tubulins were indeed affected by depletion of the *Cp110* gene, or the null mutation. In the absence of another component of the distal end complex, Cep97, tubulin acetylation was inhibited (Dobbelaere *et al.* 2020). Acetylated tubulin foci appeared on the distal ends of centrioles in control spermatocytes at mid stage (Fig. S5A) and became elongated at late stage (Fig. S5B). The foci failed to be observed at the distal ends in the *Cp110* null mutant cells (Fig. S5C, D). These observations were consistent with the published results in the *Cep97* null mutants (Dobbelaere *et al.* 2020). Furthermore, we examined whether Orbit overexpression or Klp10A depletion influences tubulin acetylation in the absence of Cp110. Interestingly, we found that acetylated microtubules excessively grew from the distal ends in both cases, indicating that modification and elongation of the stabilized microtubules was not perturbed in altered expression of Orbit or Klp10A without Cp110. Rather, these genetic alterations resulted in enhanced elongation of axonemal microtubules.
Abnormal centrioles associated with the multipolar spindles in meiotic cells derived from the \textit{Cp110} null mutant spermatocytes overexpressing Orbit

Furthermore, we investigated whether the loss of centriolar integrity in the \textit{Cp110} mutant spermatocytes overexpressing Orbit led to the formation of multipolar spindle microtubules in male meiosis. The control spermatocytes (\textit{w/Y; Ub-mRFP-PACT/+;Ub-GFP-\(\beta\)tubulin/bam-Gal4}) possessed bipolar spindle microtubules in metaphase I (175 cells/175 cells examined from 12 males) (Fig. 8A). Similarly, the cells with spermatocyte-specific overexpression of Orbit alone (\textit{w/Y; UAS-GFP-Orbit/+;Ub-mRFP-PACT, bam-Gal4/Ub-GFP-\(\beta\)tubulin}) did (153 cells/153 cells examined from 16 males) (Fig. 8C). Most of \textit{Cp110} null mutant spermatocytes (\textit{Cp110}\textsuperscript{\textDelta1}/\textit{Y};\textit{Ub-mRFP-PACT, bam-Gal4/Ub-GFP-\(\beta\)tubulin}) (156 cells/159 cells examined from 12 males) showed normal bipolar spindle microtubules in meiosis I (Fig. 8B), while we found a few spermatocytes carrying multipolar spindle microtubules in which pieces of centrioles were associated with each spindle pole at a lower frequency (3/159) (inset in Fig. 8B). In contrast, we observed multipolar spindle structure in the \textit{Cp110}\textsuperscript{\textDelta1} mutant cells with spermatocyte-specific overexpression of Orbit at a considerable frequency (10.4%) (22 cells with tri-polar (Fig. 8D) and 2 cells with tetra-polar spindles among 231 meiosis I cells from 20 males) (Fig. 8E). Among 36 spindle poles in 12 tripolar cells, 12 poles harboured paired centrioles, as visualised by mRFP-PACT. By contrast, 24 poles harboured unpaired single centrioles, which corresponded to a category classified as disengagement in Fig. 7I (arrow in Fig. 8D”, magnified view in inset D”). The remaining 6 multipolar cells possessed two sets of
paired centrioles, and single shorter centrioles, which corresponded to a category classified as breakage in Fig. 7I (arrow in Fig. 8E). Additionally, we found two tripolar cells, in which every spindle pole (6 poles) contained a set of paired centrioles, corresponding to a category of overduplication in Fig. 7I. In total, all unpaired centrioles (34/34 examined in 22 multipolar cells) were associated with spindle poles in the abnormal cells. Conversely, we did not observe any free centrioles, which are not associated with spindle poles, in any of 50 bipolar cells or 24 multipolar cells from \textit{Cp110}^{\Delta1} mutant males overexpressing Orbit. Based on these observations, we concluded that spermatocytes with altered expression of factors regulating centriole dynamics showed excess centriole elongation in the absence of the distal end complex. This is involved in the production of unpaired and/or fragmented centrioles, subsequently giving rise to abnormal meiotic cells harbouring multipolar spindle microtubules.

**DISCUSSION**

**Antagonistic roles of microtubule polymerising factor, Orbit and depolymerising factor, Klp10A in determination of centriole length in spermatocytes**

Centrioles in \textit{Drosophila} spermatocytes consist of nine-fold triplets of microtubules (Lattao \textit{et al.}, 2017). We showed that the centrioles elongated to a certain length as the cells grew before male meiosis. Klp10A, which is a \textit{Drosophila} kinesin-13 orthologue essential for shortening the microtubules, plays an indispensable role in regulation of centriole length (Delgehyr \textit{et al.}, 2012, this study). Microtubule length can be
determined by the balance between polymerisation and depolymerisation of a tubulin heterodimer into protofilament. Thus, dynamic factors that promote microtubule elongation might also play critical roles in the determination of centriole length. In this study, we presented evidences suggesting that overexpression of Orbit results in excessively long centrioles in premeiotic spermatocytes. Conversely, we observed shorter centrioles in hypomorphic \textit{orbit} mutants. These results are consistent with the idea that Orbit is essential for promotion of centriole elongation in spermatocytes. Orbit was initially identified as a microtubule-associated protein (Inoue \textit{et al}., 2000) that adds tubulin heterodimers at the plus end of microtubules (Maiato \textit{et al}., 2005, Al-Bassam \textit{et al}., 2008). Hence, the possibility that Orbit might also elongate the triplet microtubules of centrioles by adding tubulin dimers, similar to its function in microtubule elongation, is high. In contrast, Klp10A/kinesin-13 acts as a microtubule depolymerising factor at the plus ends of microtubules in the interphase (Sharp \textit{et al}., 2005) and is an important regulator of centriole elongation (Delgehyr \textit{et al}., 2012). In \textit{Drosophila} S2 cells, Klp10A antagonises Orbit in bipolar spindle formation and its maintenance (Laycock \textit{et al}., 2006). Here, we showed that simultaneous overexpression of Orbit and depletion of \textit{Klp10A} further enhanced centriole elongation. Based on these findings, we proposed that these two factors act antagonistically to produce centrioles of specific length. We observed very long GFP-Orbit signal that extended from basal bodies in spermatocyte overexpressing the protein. Accordingly, we hypothesise that these are overly long axoneme microtubules produced as a consequence of excessively stimulated polymerization of tubulins by the overexpression. Orbit/CLASP has a microtubule
binding activity to stimulate tubulin polymerization at the plus end (Inoue et al., 2000, Al-Bassam et al., 2010). Alternatively, we cannot exclude a possibility that Orbit polymerizes itself to construct microtubule-like structure on the distal tip of the basal body. It is interesting to investigate whether basal body and axonemal microtubules overly elongate in cells overexpressing Orbit without fluorescence tag.

In addition to these two factors regulating centriole microtubules, we hypothesised that Cp110 plays a role as a cap to restrict these factors acting on the distal ends of the microtubules at earlier stage. After the Cp110 releases from the ends at mid stage, Orbit becomes possible to access the distal ends more easily and stimulate the centriole microtubules to extend to a certain length. Although the loss of the cap protein resulted in only a subtle extension of centrioles, Orbit overexpression in the absence of Cp110 can change the centriole microtubule dynamics significantly. Consequently, remarkably long centriole microtubules would be produced in the spermatocytes. Consistent with the hypothesis, \textit{Cp110}^{Al} mutation also significantly enhanced the overly long centriole phenotype in Klp10A-depleted spermatocytes. By contrast, the null mutation did not enhance a shorter centriole phenotype by Klp10A overexpression. Further experiments need to be performed to verify the model. The fact that Cp110 influences centriole length depending on the cell type and cellular context as shown in previous studies (Delgehyr et al., 2012; Dobbelaere et al. 2020; Franz et al. 2013). It is likely that differential regulation of the conserved core components underlies ciliary basal diversity in different cell types. As argued previously (Jana et al., 2018), cellular-
specific and tissue specific regulation in centriole duplication may be indispensable to regenerate diverse centriole structure.

Previous reports have not investigated whether Orbit and Klp10A are localised on centrioles or around PCM in Drosophila mitotic cells. However, a considerable amount of Orbit accumulated in centrosomes in early embryos, Drosophila cultured cells, and germ line stem cells during Drosophila oogenesis and spermatogenesis (Inoue et al., 2000; Inoue et al., 2004; Lemos et al., 2000; Mathe et al., 2003). Whether the protein was localised in the PCM or in the centrioles was not clear. Similarly, studies on the cellular localisation of Orbit/CLASP orthologues in other species also showed centrosome localisation of these proteins. A previous anti-human CLASP1 immunostaining in Hela cells demonstrated that the protein was localised on centrosomes at the M phase (Maiato et al., 2003). A CLASP orthologue in Caenorhabditis elegans was also observed in the centrosomes of its embryonic cells (Espiritu et al., 2012). These reports did not mention whether the orthologues were associated with the centrioles. Recently, it has been reported that Klp10A is dominantly localized in the TZ of the ciliary structures in spermatocytes, spermatids and sensory neurons (Persico et al., 2019). Centrosomal localisation of the protein has also been reported in mitotic cells and germ line stem cells in both sexes (Goshima and Vale, 2005, Zou et al., 2008, Radford et al., 2012). Whether Klp10A localises on the cylindrical microtubules of the centrioles or in the PCM of mitotic cells has not been demonstrated. Therefore, whether these antagonistic regulators are localised on centrioles in mitotic cells should be determined at a higher resolution. Furthermore,
whether these two regulators are required for centriole length determination in somatic cells warrant further investigation.

**Improvised construction of overly long centriolar microtubules by altered expression of the dynamic factors and production of disintegrated centrioles, and thereby centriole disengagement**

In addition to excessively elongated centrioles, we observed several abnormalities in centriole organisation and structure in spermatocytes overexpressing Orbit and/or harbouring *Klp10A* depletion. In the absence of Cp110, the loss of centriole integrity phenotypes was also enhanced. Small pieces of centrioles observed may be broken pieces of over-elongated centrioles, as observed in cancer cells (Marteil et al., 2018). Alternatively, they may have been unpaired centrioles separated precociously from centriole pairs containing daughter procentrioles, which are smaller than normal centrioles (Karki et al., 2017). By contrast, the loss of centriole integrity phenotypes was not observed in the cells overexpressing a shortening factor, Klp10A. Hence, we consider that improvised construction of the basal body microtubules may be associated with the loss of integrity phenotype. And thereby, centriole engagement would be lost. We found centriole fragments with shorter diameter in the centriole microtubules. The presence of disintegrated centrioles supports this idea. Further investigations are necessary to test the current hypothesis.
Spermatocytes homozygous for a loss of function mutation of Sas6 and that of Ana2 commonly demonstrated premature centriole separation before meiosis (Stevens et al., 2010, Rodrigues-Martins et al., 2007, Lattao et al., 2017). Hence, Sas6 and Ana2 considered to be required for centriole engagement and/or maintenance of the pairs. Orbit overexpression and/or Klp10A depletion may affect centriole engagement through interfering the Sas6 (or Ana2) function. It is also possible to consider that the premature disengagement can occur independent on Sas-6 or Ana2. Besides, it was reported that APC/C activation, and activation of separate, thereby unexpected cleavage of Sec1 cohesin can take place in mammalian cultured cells (Karki et al., 2016). We cannot exclude a possibility that alteration of microtubule dynamics in centrioles by altered expression of Orbit and/or Klp10A led to unexpected APC/C activation. This hypothesis would be tested by several experiments in our next study.

**Production of centrioles with constant length is important for centriole integrity and proper meiotic division**

Previous studies have also mentioned that Cp110 null mutant spermatocytes or syncytial stage embryos do not show detectable defects in centrosome behaviour, spindle formation, or chromosome segregation (Franz, et al., 2013). By contrast, we showed in this study that disintegrated centrioles and multipolar spindle microtubules emanating from the centriolar fragments existed in Cp110 null mutant spermatocytes, cells overexpressing Orbit, and Klp10A-depleted cells less frequently. Cells
homozygous for the loss-of-function \textit{Klp10A} mutation also displayed multipolar spindle structure (Delgehyr \textit{et al}., 2012). However, we cannot exclude that the spindle phenotype would result from abnormal microtubule organization by \textit{Klp10A} mutation, rather than centriole disintegration. Surprisingly, in \textit{Cp110} mutants overexpressing Orbit and undergoing meiosis I, we observed that unpaired single centrioles, even a part of them could act as the MTOC. Centrosomes must be “licensed” to function as a MTOC that nucleates microtubules, although the mechanism whereby the “license” is granted remains unclear. Nevertheless, a recent study has reported that excessive elongation of centrioles in cancer cells is related to the generation of over-duplicated, fragmented, or hyper-active centrosomes that nucleate considerably more microtubules during cell division (Marteil \textit{et al}., 2018). Chromosome segregation was disturbed in cells harbouring these abnormal centrioles. Interestingly, generation of cells harbouring extra centrosomes has been suggested to be able to drive spontaneous tumorigenesis in mice (Levine \textit{et al}., 2017; Marteil \textit{et al}., 2018). Additional studies have reported that excessively elongated centrioles in \textit{Drosophila} spermatocytes affect spermatogenesis via the production of defective flagella (Bettencourt-Dias \textit{et al}., 2005; Nigg and Raff, 2009). Consistently, we observed immotile sperm production and significant decrease in male fertility in the \textit{Cp110} mutant males with spermatocyte-specific \textit{Klp10A} depletion (Shoda and Inoue, manuscript in preparation). These observations suggested that production of excessively elongated centrioles can affect cell division and subsequently spermatogenesis. Once an abnormal spindle microtubule structure is constructed, the
extra MTOC results in chromosome mis-segregation and eventually chromosome instability, as observed in cancer cells (Ganem et al., 2009, Marteil et al., 2018).

Certain abnormalities such as missegregation of chromosomes and the resulting aneuploidy might appear in the subsequent division of cells containing abnormal centrioles. Conversely, excessively short centrioles may be inadequate as templates for the duplication of centrioles in the S phase. Hence, regulation of centrosome length via the antagonistic activities of elongation and shrinking factors, such as Orbit and Klp10A, is important. However, the loss of centriole integrity and the resulting aneuploidy may occur in meiotic cells, but not in mitotic cells, which have stricter microtubule assembly checkpoints. Further investigations are required to understand how centrosome length is regulated in mitotic cells.

Current findings suggest a presence of mutually antagonistic regulation to determine centriole length and the significance of the production of centrioles with a certain length for centriole integrity, and for assurance of proper chromosome segregation. We believe that these findings may enable the identification of a mechanism whereby the loss of centriole integrity causes chromosome mis-segregation in cancer cells.
MATERIALS AND METHODS

Drosophila stocks

For normal control, w^118 was used. We also used P{UAS-GFP^S65T}T2 (DGRC, #106363) and P{UAS-LacZ} (DGRC, #107532) as normal controls. To visualise microtubules in spermatocytes, P{Ubi-GFP-βTubulin} was used (Inoue et al., 2004). We used the orbit^7 hypomorphic allele for orbit gene (Inoue et al., 2004). To overexpress the Orbit protein, P{UASp-GFP-Orbit^924,^47} was used (Miyauchi et al., 2013, Kitazawa et al., 2014). To visualise the centrioles in premeiotic spermatocytes, P{Ub-mRFP-PACT} (Dix and Raff, 2007), P{Asl-YFP} (Rebollo, et al., 2007), P{GFP-Ana1} (Blachon, et al., 2007), and P{Cby-Tomato} (Enjolras et al., 2012) were used. These stocks were gifts from J. Raff (University of Oxford, Oxford, UK), C. Gonzalez (IRB Barcelona, Barcelona, Spain), T. Avidor-Reiss (Harvard Medical School), and B. Durand (University of Lyon, Lyon, France), respectively. To overexpress Klp10A, P{EPgy2}EY09320 (BDSC#17557) and P{UAS-GFP-Klp10A} were used. P{EPgy2}EY09320 carries P{EPgy2} inserted at 5’ of the Klp10A gene in the forward direction of the UAS sequences in the P-element so as to enable to induce Gal4-dependent transcription of the gene (Venken and Bellen, 2007). P{UAS-GFP-Klp10A} was used to visualise the localisation of GFP-tagged Klp10A in spermatocytes. The stock was kindly distributed by Y. Yamashita (University of Michigan, Ann Arbor, MI, USA). For depletion of Klp10A, UAS-Klp10A RNAi^HMS00920 (BDSC #33963) was used. These stocks were obtained from Bloomington Stock Centre.
For depletion of centriolar distal tip factors, Cp110 was used; \textit{UAS-Cp110 RNAi}^{KK105525} (VDRC \#v101161) from Vienna \textit{Drosophila} Resource Centre (VDRC) (Vienna, Austria) was used. When we induced dsRNA against each mRNA, we also induced co-expression of the \textit{dsr2} mRNA encoding a Dicer2 double-stranded RNA-specific endonuclease in every RNAi experiment to raise the RNAi efficiency in testis cells, where the \textit{dcr2} expresses at a lower level (flybase; https://flybase.org/reports/FBgn0034246) in testis cells. We used \textit{UAS-dcr2}; \textit{bam-GAL4::VP16} stock (Hayashi \textit{et al.}, 2016) as spermatocyte-specific Gal4 driver for overexpression various cDNAs and dsRNAs against endogenous mRNAs. We also used \textit{Cp110}^{\Delta1} as a null allele for the \textit{Cp110} gene (Franz \textit{et al.}, 2013). \textit{Cp110}^{\Delta1} stock was kindly distributed by J. Raff.

\textit{All Drosophila} stocks were maintained on standard cornmeal food at 25 °C, as previously described (Oka \textit{et al.}, 2015). Food: 7.2 g of agar, 100 g glucose, 40 g dried yeast, and 40 g of cornmeal was added into 1L water, mixed and boiled while stirring constantly. After the food medium had cooled down, 5 mL of 10% parahydroxybenzonate dissolved in ethanol and 5 mL of propionic acid were added as antiseptics. Gal4-dependent expression was done at 28 °C.

\textbf{Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis}

qRT-PCR analysis was performed to determine mRNA level of various target genes in adult testes. Total RNA was extracted from adult testes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis from the total RNA was
carried out using the PrimerScript II High Fidelity RT-PCR kit (Takara, Shiga, Japan) with random primers. qRT-PCR was performed using FastStart Essential DNA Green Master (Roche, Mannheim Germany) and a LightCycler Nano (Roche, Basel, Switzerland). We used RP49 as a normalisation reference (Oka et al., 2015). Relative mRNA levels were quantified using LightCycler Nano software version 1.0 (Roche, Basel, Switzerland). The primer sets used were as follows: Rp49 (Fw: 5’-TTCCTGGTGCAACAGTG-3’, Rv: 5’-TCTCCTGGCTTTCTTG-3’), Klp10A (Fw: 5’-GAATCTAGTCGTCTCGGCCAG-3’, Rv: 5’-GCTTGTCGGACAGAAGATCGA-3’), Cp110 (Fw: 5’-GCTTGTCGGACAGAAGATCGA-3’, Rv: 5’-GCTTGTCGGACAGAAGATCGA-3’).

Each sample was duplicated on the PCR plate, and the final results average three biological replicates. For the quantification, the ΔΔCt method was used to determine the differences between the target gene expression relative to the reference RP49 gene expression.

**Immunofluorescence**

We performed immunostaining experiments for testis cells prepared using the following protocol described previously (Tanabe et al., 2017). A pair of testes was collected from newly eclosed adult flies (within 2 days from eclosion). The fly placed in a drop of the Testis buffer (183 mM KCl, 47 mM NaCl, 10 mM EDTA, pH 6.8) was dissected under a stereo microscope. Using a pair of the forceps, the fly abdomen was clamped and gently pulled the external genitalia outward. After carefully removed
accessory tissues away from a pair of testes, the testes were collected on a slide glass. Using a pair of fine tungsten needles, a sheath covering the testis was torn from the apical tip and covered with 18 x 18 mm-coverslip (Matsunami, Osaka, Japan). After freezing the slides in liquid nitrogen, the coverslip was removed with a razor blade. The slides were transferred into 100% ethanol for 10 minutes to dehydrate and fix the sample. Subsequently, testis cells were fixed again with 3.7% formaldehyde for 7 minutes. The slides were permeabilised in PBST (PBS containing 0.01% Triton-X) for 10 minutes and blocking with 10% normal goat serum in PBS (Wako, Osaka, Japan) for 30 minutes at room temperature. For immunostaining experiments, the following primary antibodies were used at dilution; mouse anti-γ Tubulin (GTU88\textsuperscript{★}) antibody (#T6557 Sigma-Aldrich, St. Louis, MO, USA): 1:200, rabbit anti-Orbit antibody (Inoue et al., 2004): 1:200, guinea-pig anti-Asl antibody (a gift of J. Raff, University of Oxford, Oxford, UK): 1:800, anti-Cp110 antibody (Franz et al., 2013, a gift of J. Raff, University of Oxford, Oxford, UK): 1:200, and anti-acetylated tubulin antibody (#6-11B-1 Sigma-Aldrich, St. Louis, MO, USA): 1:100. After incubating with the primary antibody overnight at 4˚C, the samples were washed in PBS for 10 minutes and labelled with Goat Anti-Mouse or Rabbit IgG (H+L) Alexa Fluor 488, 594 or 647 (Invitrogen, Carlsbad, CA, USA). The secondary antibodies were used at dilution of 1:400. After incubation for 2 hours at room temperature, the slides were washed in PBS for 10 minutes and pure water for 1 minute. For DNA staining, we used VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (#H-1200 Vector Laboratories, Burlingame, CA, USA) or Prolong Gold with DAPI (Invitrogen,
Carlsbad, CA, USA). Imaging was performed on Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan) outfitted with excitation, emission filter wheels (Olympus, Tokyo, Japan). Cells were imaged with 40x or 100x lens. GFP and RFP fluorescence images were captured with a CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Image acquisition was controlled through the Metamorph (Molecular Device, Sunnyvale, CA, USA) software package running on a PC. We also used Nikon N-SIM super resolution microscopy (Nikon, Tokyo, Japan) to acquire the super-resolution microscope images of centrioles in premeiotic spermatocytes using an oil immersion objective lens (CFI SR Apochromat 100x, 1.49 NA, Nikon, Tokyo, Japan). Near-simultaneous GFP and/or RFP fluorescence images were captured with an EM-CCD camera (Hamamatsu, Photonics, Shizuoka, Japan). Image acquisition and quantification of the centriole length were performed through NIS-Elements (Nikon, Tokyo, Japan) software running on PC.

**Proximity Ligation Assay (PLA)**

PLA that enables detection of protein interaction within a cell was performed according to the Duolink kit method (Nacalai Inc., Kyoto, Japan) as described (Okazaki et al., 2020). We applied the PLA method to examine a close association between Cp110 and GFP-Orbit. For detection of the complexes, we used anti-Cp110 antibody (a gift from J. Raff) and anti-GFP antibody (A-6455, Thermo Fischer Scientific, Waltham, USA) to detect complexes containing Cp110 and Orbit. Samples were observed with a fluorescent microscope (Olympus, Tokyo, Japan, model: IX81). Image acquisition was
controlled through the Metamorph software version 7.6 (Molecular Devices) and processed with ImageJ version 1.51 or Adobe Photoshop CS4.
Abbreviations

MTOC, Major microtubule organising centre; Asl, Asterless

Author Contributions: T.S., Y.S., and Y.T. carried out immunostaining experiments, and qRT-PCR experiments. Y.H.I. planned, organised the project, and interpreted the data. T.S. and Y.H.I. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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REFERENCES


Figures

Fig.1 Centriole elongation as a progression of spermatocyte growth during the growth stages of premeiotic spermatocytes before male meiosis.

(A) Fluorescence observation of a pair of centrioles in the premeiotic spermatocytes expressing Asl-YFP under a confocal microscope. The centrioles in the spermatocytes at S1 to Prophase I during the growth phase were observed. (B) Average length of the centriole structures visualised by immunostaining with anti-Asl antibody, or anti-γ-tubulin antibody, or by expression of Ana1-GFP, or mRFP-PACT during the later stages of the growth phrase, in which centrioles elongate distinctively. Bar; 1μm.
**Fig. 2 Centriolar localisation of Orbit protein in premeiotic spermatocytes.**

(A and B) Observation of premeiotic spermatocyte immunostained with anti-Orbit antibody by structured illumination microscopy (SIM). (A) Two pairs of centrioles in a premeiotic spermatocyte. Anti-Orbit immunostaining of the spermatocyte (Red in A, white in A’). Anti-Asl immunostaining (Green in A, white in A’’). DNA staining by DAPI (Blue in A, white in A’’’). Inset represents a pair of the engaged centrioles. Arrows indicate the distal ends of centrioles. Among 60 centrioles in 15 premeiotic spermatocytes, fluorescence images showing the typical distribution of proteins were selected. (B) A magnified view of a pair of centrioles stained with anti-Orbit antibody in the wild type spermatocyte. (C) A line analysis for the anti-Orbit immuno-fluorescence along one of the pair of centrioles from the proximal end (distance = 0 μm) to the distal end. An arrow indicates the centriolar region used for line analysis. The graph indicates enrichment of Orbit around the distal tip of centriole. Bars: 5 μm in (A), 1 μm in (B).
Fig. 3 Orbit plays an essential role in elongating centrioles to a certain length in premeiotic centrioles.

(A-H) Observation of centrioles in premeiotic spermatocytes harbouring spermatocyte-specific overexpression of Orbit by SIM. Centrioles were visualised by mRFP-PACT expression. (A) Centrioles in control spermatocyte (bam>GFP). (C) Centrioles in premeiotic spermatocyte (bam>orbit). (B and D) Magnified views of a pair of centrioles in (A) and (C), respectively. (E-H) Visualisation of centrioles labelled by mRFP-PACT in the premeiotic spermatocyte homozygous for orbit<sup>7</sup>. (E) Centrioles in a control spermatocyte (w<sup>1118</sup>). (G) Centrioles in orbit<sup>7</sup> premeiotic spermatocyte. (F and H) Magnified views of a pair of centrioles in (E) and (G), respectively. Outer cell boundary of each cell is encircled by a white dotted line. Bars: 5 μm in (A), 1 μm in (B).

(I) The graph indicates the length of centrioles in premeiotic spermatocytes overexpressing Orbit and in homozygous cells that have the orbit hypomorphic allele. In this graph, the error bars represent 95% Confidence Interval and significance was tested using the Mann-Whitney test. ****, P <0.0001.
Fig. 4. Centriolar localisation of microtubule-severing factor Klp10A, and the effects of overexpression and depletion of it on centriole elongation in premeiotic spermatocytes.

(A, B) Observation of two pairs of centrioles in premeiotic spermatocytes by mRFP-PACT expression by SIM. (A) Control spermatocyte (bam>dcr2, GFP), (B) spermatocyte depleted of klp10A (bam>dcr2, Klp10ARNAi). Bar: 1 μm. (C) Relative mRNA levels of Klp10A, in testis expressing Klp10A dsRNA (bam>dcr2, Klp10ARNAi) using qRT-PCR analysis. (D) Average length of centrioles in premeiotic spermatocytes with spermatocyte-specific depletion of Klp10A. 58 centrioles from 18 control premeiotic spermatocytes at mature stage, 52 centrioles from 15 bam>Klp10ARNAi cells of the same stage were examined. (E-I) Observation of two pairs of centrioles by SIM. (E) Centriolar localization of GFP-tagged Klp10A in spermatocyte (bam>Klp10A)
(green in E, white in E’) immunostained with the anti-Asl antibody (red in E, white in E”). DNA staining by DAPI (blue in E, white in E”). Arrows indicate the distribution of Klp10A on the centrioles. Inset in E indicates a magnified view of one of the two pairs of centrioles in the cell. (F, H) Two paired centrioles in premeiotic spermatocytes, visualised by mRFP-PACT expression. (F) Control (bam>GFP). (H) Observation of the centrioles by Klp10A overexpression (bam>Klp10A). Bars: 5 μm. Outer cell boundary of each cell is encircled by a white dotted line. (G and I) Magnified images of a pair of centrioles in (F) and (H), respectively. Bars: 1 μm. (J) Average length of centrioles in premeiotic spermatocytes analysed. Error bars represent 95% CI and significance was determined using the Mann-Whitney test. ****; P <0.0001.
Fig. 5 Overexpression of Orbit in spermatocytes depleted of Klp10A resulted in both additive effect of the centriole elongation and a loss of integrity of the centrioles.

(A) Average length of centrioles in the premeiotic spermatocytes having overexpression of Orbit and depletion of Klp10A (bam>dcr2, Klp10ARNAi, orbit). The centrioles of the cells with each genotype were visualized by expression of mRFP-PACT and measured the length. Error bars represent 95% CI and significance among was tested by Kruskal-Wails test and Mann-Whitney test. **; \( P < 0.01 \), ***; \( P < 0.001 \), ****; \( P < 0.0001 \) in Mann-Whitney test. >50 centrioles from 14 cells among the premeiotic spermatocytes at mature stage were examined. First column; spermatocytes overexpressing Orbit, second column; the cells having depletion of Klp10A, third column; the cells having overexpression of Orbit and depletion of Klp10A. Note that there is an additive effect on the centriole elongation. (B-F) Observation of centrioles in spermatocytes having orbit overexpression and Klp10A depletion (bam> dcr2, Klp10ARNAi, orbit) by SIM. Orbit
(Green), PACT (Red) and DNA (Blue). (B) Spermatocyte having the centrioles engaged properly, and overly longer cilium-like structure labelled by GFP-Orbit. (C-F) Several types of abnormal centriole structure seen in the premeiotic spermatocytes. Arrows indicate each example of abnormal centrioles. Insets in C-F indicate magnified images of the abnormal centriole pairs. (C) centrioles disengaged (arrows), (D, E) centrioles lost the centriolar integrity. Arrows indicate smaller pieces of basal body, associated with elongated cilia labeled by GFP-Orbit. (F) centrioles overly duplicated. Inset in D indicates magnified image of the left of abnormal centriole figures pointed by arrows. Inset in E indicates all of PACT-positive pieces indicating thinner basal body-like structure. (G) Frequencies of spermatocytes having properly engaged centrioles and abnormal centrioles. Control: bam>dcr2, LacZ. Grey: Properly engaged. Yellow: Disengagement. Red: Breakage. Green: Over-duplication. Bars: 5 μm.
Fig. 6. Effects of *Cp110* null mutation on centriole elongation in spermatocytes overexpressing either of Orbit or Klp10A, or those harbouring Klp10A depletion.

(A–I) Observation of centrioles labelled by mRFP-PACT using SIM. Two sets of centrioles in control spermatocyte (*bam-Gal4/+*, A), *Cp110* null mutant spermatocyte (*Cp110Δ1/Y*, B), spermatocyte overexpressing *orbit* (*bam>orbit*, C), *Cp110* null mutant spermatocyte overexpressing *orbit* (*Cp110Δ1/Y; bam>orbit*, D), control spermatocyte (*bam>dcr2*, E), spermatocyte depleted of Klp10A (*bam>dcr2, Klp10ARNAi*, F), *Cp110* null mutant spermatocyte depleted of Klp10A (*Cp110Δ1/Y; bam>Klp10ARNAi*, G), spermatocyte overexpressing Klp10A (*bam>Klp10A*, H), and *Cp110* null mutant spermatocyte overexpressing Klp10A (*Cp110Δ1/Y; bam>Klp10A*, I). Bars; 1µm. (J) Average length of centrioles in premeiotic spermatocytes with four different genotypes at mature stage. (K) Average length of centrioles in premeiotic spermatocytes with 7 different genotypes at mature stage. The error bars represent 95% CI and significance was tested using the Student’s *t*-test. ns; not significant, **; *P* < 0.01, ****; *P* < 0.0001.
Fig. 7 Abnormal centriole structure observed in the premeiotic spermatocytes of the *Cp110* null mutant, and the mutant cells overexpressing Orbit or having *Klp10A* depletion.

(A-H) Observation of premeiotic spermatocytes at mature stage carrying two pairs of centrioles labelled by mRFP-PACT (red) by SIM. DNA was stained with DAPI (blue).

(A) Control spermatocyte (w^{118}) containing two pairs of centrioles engaged, (B) spermatocyte hemizygous for a null mutation, *Cp110^{Δ1}* containing a centriole piece (arrow) with normal-looking two pairs of centrioles. Inset B indicates three pairs of centrioles engaged (arrows). (C) Spermatocyte overexpressing Orbit (*bam>*orbit) containing disengaged centrioles (arrow) and a pair of centrioles engaged. (D) *Cp110^{Δ1}* spermatocyte with Orbit overexpression (*Cp110^{Δ1}; bam>*orbit). Inset D indicates a
magnified view of unpaired centrioles (arrows) and small piece (arrowhead). (E)

Spermatocyte with Klp10A depletion with unpaired centrioles (arrowheads) (*bam>dcr2, Klp10ARNAi*). (F) *Cp110Δ1* spermatocyte with Klp10A depletion (*Cp110Δ1; bam>dcr2, Klp10ARNAi*) with unpaired centrioles (arrowheads) and a small centriole piece (arrow).

Inset F indicates a magnified view of unpaired centrioles and small piece. (G, H)

Fig. 8 Multipolar spindle microtubule structure in metaphase I spermatocytes of 

*Cp110* null mutant males overexpressing Orbit.

(A-E) Fluorescence micrographs of spermatocytes at metaphase I from control male 

(bam-Gal4/+) (A), *Cp110* mutant spermatocytes (*Cp110*Δ1/Y) (B), spermatocytes overexpressing Orbit (w/Y; UAS-GFP-Orbit/+; Ub-mRFP-PACT, bam-Gal4/Ub-βtubulin-GFP) (C), and *Cp110*Δ1 spermatocytes having Orbit overexpression (*Cp110*Δ1/Y; UAS-GFP-Orbit/+; Ub-mRFP-PACT, bam-Gal4/Ub-βtubulin-GFP) (D, E). The primary spermatocytes possess bipolar (A-C), tripolar (D), and tetrapolar (E) spindle
microtubules. Inset in B presents multipolar spindle microtubules observed at a lower frequency. The meiotic image is associated with centriole fragments (arrows in B”). Green in A-E (white in A’-E’) corresponds to microtubules. Red in A-E (white in A’’-E’’) indicates a fluorescence image of mRFP-PACT. Centrioles are visualised in yellow in A-E as merged images of Tubulin-GFP, mRFP-PACT and GFP-Orbit (C-E). Inset in panel D” represents magnified image of single unpaired centriole in a spindle pole pointed by an arrow. Note that four single centrioles that have already been separated were localized on each pole of the tetrapolar spermatocyte. Arrow in E” shows unpaired centriole, and arrowhead indicates a pair of centrioles in the neighbouring cell. DNA staining (blue in A-E, white in A’’-E’’). Bar; 10 µm.
Fig. S1. Overview of cell growth and division in *Drosophila* spermatogenesis, and centriole dynamics in accordance with the cell growth of primary spermatocyte.

(A) Illustration of asymmetric division of germ line stem cells, four rounds of mitosis to generate the 16-cell cysts, cell growth of spermatocyte, and two meiotic divisions, meiosis I and II in *Drosophila* testis. (B) Schematic presentation of a single centriole consisted of the basal body, transition zone (light blue) and the ciliary-like region (CLR). (C) Dynamic alteration of a pair of centrioles during the growth phase, which is classified into S1 to S6 stages, and male meiosis I. Plasma membrane (PM), centriole (cen) (red), and microtubules (MT) (green). Nucleus (N) and chromosome (chr) are shown in dark blue.
Fig. S2. Observation of two pairs of engaged centrioles by structured illumination super-resolution microscope (SIM), on which four types of centriolar proteins were distributed in premeiotic spermatocytes.

(A-C) Immunostaining of premeiotic spermatocytes expressing GFP-β-Tubulin or mRFP-PACT with the antibodies against the centriole-localized proteins, γ-Tubulin, and Asl. (A) Two pairs of centrioles in the premeiotic spermatocyte expressing GFP-β-Tubulin and mRFP-PACT. (A’) GFP fluorescence of GFP-β-Tubulin on the centrioles. (A’’) RFP fluorescence of mRFP-PACT on the centrioles. (B and C) Immunostaining of two pairs of centrioles in the premeiotic spermatocyte expressing mRFP-γ-Tubulin and mRFP-PACT. (B’) Anti-γ-Tubulin immunostaining of the centrioles. (B’’) mRFP-PACT fluorescence of the centrioles. Note that γ-Tubulin and Asl are localized along outer region of the centriole cylinder. PACT and β-Tubulin are localized in lumen of the cylindrical structure of the centriole. The fluorescence images of the cells showing typical localization of the proteins were selected among more than 50 premeiotic spermatocytes for β-Tubulin and PACT fluorescence, 15 spermatocytes for γ-Tubulin and Asl immunostaining. bars: 1 µm.
**Fig. S3.** Co-localization of Orbit with a transition protein, Cby and effect of Orbit overexpression on elongation of axoneme microtubules consisted of acetylated tubulin.

(A-D) Anti-Asl immunostaining of two sets of centrioles in premeiotic spermatocyte expressing GFP-Orbit and Cby-Tomato. Green in A-D (white in A’-D’) represents a fluorescence of GFP-Orbit. Red in A-D (white in A”-D”) represents a fluorescence of Cby-Tomato. Blue in A-D (white in A’”-D’”) indicates anti-Asl immunostaining. GFP-Orbit is enriched at the distal region protruding from the distal end of basal body visualized by anti-Asl immunostaining. Cby-Tomato is localized on the most distal part of the Orbit-localizing region. Bar; 5µm. (E) Anti-acetylated tubulin immunostaining of two sets of centrioles in premeiotic spermatocyte expressing GFP-Orbit and mRFP-PACT. Green in E (white in E’) represents a fluorescence of GFP-Orbit. Red in E (white in E’”) represents axoneme microtubules recognized by anti-acetylated tubulin immunostaining. Blue in E (white in E’”) indicates mRFP-PACT fluorescence. Arrows indicate overly elongated axoneme microtubules from the distal ends of centrioles.
Fig. S4. Centrioles elongation is dependent on extent of Orbit overexpression.

(A, B) Average length of centrioles in control spermatocytes at S6 stage from control (bam-Gal4/) males (while column), spermatocytes harbouring a single copy of UAS-GFP-Orbit and bam-Gal4 (bam>orbit(X1)) from the males raised at 25°C as lower level overexpression of the Orbit (light grey column), spermatocytes harbouring bam-Gal4 and two copies of UAS-GFP-Orbit (bam>orbit(X2)) from the males raised at 25°C as moderate level over expression (grey column), spermatocytes carrying bam-Gal4 and two copies of UAS-GFP-Orbit (bam>orbit(X2)) from the males raised at 28°C as higher level over expression (black column). (A) Average length of centrioles in control cells and the cells at same stage having Orbit overexpression at three different levels. Centriole length was measured by GFP-Orbit fluorescence. The length in control cells was measured by anti-Asl immunostaining. (B) Average length of centrioles in control cells and the cells having Orbit overexpression at three different levels. The length was measured by anti-Asl immunostaining. (C) Relative levels of GFP fluorescence intensity in the spermatocytes expressing GFP-Orbit at lower, moderate, or higher level overexpression. Total fluorescence intensity in whole regions of each cells was measured, and a relative intensity value was calculated, compared to that in the bam>orbit(X1) males. Charts show mean±S.E.M. Statistical significance was tested by Student’s t-test, compared to average centriole length in control cells **; P < 0.01, ***; P < 0.001, and ****; P < 0.0001.
Fig. S5. Excess elongation of acetylated microtubules emanating from the distal ends of centrioles in \textit{Cp110} null mutant spermatocytes overexpressing Orbit. (A-F) Anti-acetylated tubulin and anti-Asl immunostaining of spermatocytes expressing mRFP-PACT. (A, B) Control (\textit{bam-Gal4}+/+) spermatocytes at mid (A), and late (B) stages. (C, D) \textit{Cp110} mutant (\textit{Cp110}^{D1}/Y;\textit{bam-Gal4}+/+) spermatocytes at mid (C), and late (D) stages. (E, F) \textit{Cp110} mutant spermatocytes harbouring \textit{Klp10A} depletion at mid (E), and late (F) stages. (G) \textit{Cp110} mutant spermatocytes overexpressing Orbit (\textit{Cp110}^{D1}/Y;\textit{bam>orbit}) at mid-late stage. Green in A-F (white in A'-F') and red in G (white in G') indicate anti-acetylated tubulin immunostaining. Red in A-F (white in A"-F") and blue in G (white in G") indicate fluorescence of mRFP-PACT. Green in G (white in G") indicates GFP-Orbit fluorescence. Note that microtubule caps composed of acetylated tubulin (arrowheads) exist at distal ends of centrioles visualised by PACT. The caps are absent at the distal ends in \textit{Cp110}^{D1} mutant cells (open arrowheads). \textit{Cp110}^{D1} spermatocytes having depletion of Klp10A or those having overexpression of \textit{orbit} carry elongated acetylated microtubules from centrioles in both cases (arrowheads). Bars: 5 µm.
Fig. S6. The CP110 cap protein co-localized with Orbit at the distal ends of the centrioles in the earlier spermatocytes and disappeared from the ends at later stages.

Anti-CP110 and anti-Asl immunostaining of premeiotic spermatocytes expressing Orbit at early (A), mid (B), late stages (C), and meiotic stage (anaphase I) (D). Anti-Cp110 immunostaining (red in A-D, white in A’-D’), GFP-Orbit fluorescence (green in A-D, white in A’’-D’’), and anti-Asl immunostaining (blue in A-D, white in A’’’-D’’’). Bar, 1 μm. Magnified views of a set of centrioles in inset in A, and B. Orbit is enriched on the region extending from the distal end of basal body recognized by anti-Asl immunostaining. The Cp110 protein is localized on the distal tip of the Orbit-localizing region. The cap protein is diminished at the mid stage and fails to be observed at late stage and thereafter. Bars; 2μm.
Fig. S7. Proximity Ligation Assay (PLA) to detect a complex containing Orbit and CP110. 
(A, B) PLA signal indicating that Orbit and CP110 were closely associated with each other within 40 nm in a pair of centrioles in early spermatocytes (A), but not in late spermatocytes (B). The PLA signals (Red in A, white in A’) are observed on the one end of the centriole, while any PLA signals was not observed in B and B’. The GFP signals (Green in A and B, white in A” and B”). Bar, 1 µm.
Fig. S8. A mutual dependence between Orbit and Cp110 on the localization on centrioles. (A-F) Immuno-localization of Cp110 on centrioles visualised by anti-Asl immunostaining in early, mid, and late stage spermatocytes from control (w1118) (A-C), and orbit7 mutant males (D-F). Green in A-F (white in A'-F’) corresponds to anti-Cp110 immunostaining. Red in A-F (white in A''-F'”) corresponds to anti-Asl immunostaining. Four Cp110 foci can be seen in every mutant spermatocyte among more than 50 earlier cells examined (filled arrowheads in A). The Cp110 foci become very faint at mid stage (arrowheads in B) or disappeared at late stage (open arrowheads in C). Consistently, the foci in orbit7 cells can be seen in early stage spermatid, thereafter they disappear as the growth phase proceeds (E, F), as shown in control cells (A-C). (G) A SIM observation of Orbit localization at the distal end of the basal body in a Cp1104/Y; bam>Orbit mutant spermatocyte. Green; GFP-Orbit, red; mRFP-PACT. Bars, 2 µm.