

## GENERAL ARTICLE

# RNA kinase CLP1/Cbc regulates meiosis initiation in spermatogenesis

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## Abstract

CLP1, TSEN complex, and VCP are evolutionarily conserved proteins whose mutations are associated with neurodegenerative diseases. In this study, we have found that they are also involved in germline differentiation. To optimize both quantity and quality in gametes production, germ cells expand themselves through limited mitotic cycles prior to meiosis. Stemming from our previous findings on the correlation between mRNA 3'-processing and meiosis entry, here we identify that the RNA kinase Cbc, the *Drosophila* member of the highly conserved CLP1 family, is a component of the program regulating the transition from mitosis to meiosis. Using genetic manipulations in *Drosophila* testis, we demonstrate that nuclear Cbc is required to promote meiosis entry. Combining biochemical and genetic methods, we reveal that Cbc physically and/or genetically intersects with Tsen54 and TER94 (VCP ortholog) in this process. The C-terminal half of Tsen54 is both necessary and sufficient for its binding with Cbc. Further, we illustrate the functional conservation between Cbc and mammalian CLP1 in the assays of subcellular localization and *Drosophila* fertility. As CLP1, TSEN complex, and VCP have also been identified in neurodegenerations of animal models, a mechanism involving these factors seems to be shared in gametogenesis and neurogenesis.

## Introduction

Meiosis, the germline-specific process to reduce the genetic materials in half, is essential for sexual reproduction. To optimize both quantity and quality, germ cells generally expand themselves through limited mitotic cycles prior to meiosis. Although certain external signals such as nutritional status or retinoic acid (RA) have been identified (1–3), the regulatory mechanism that switches germ cells from mitosis to meiosis has not been clarified. Firstly, it is puzzling that RA receptors are dispensable in germ cells to enter meiosis (4). Most recently, MEIOSIN has been found to act at the transcriptional level with STRA8 in response to RA, though co-expression of both was

not sufficient to induce meiosis *in vitro* (5). The defined factors adequate to initiate meiosis remain to be uncovered.

Spermatogenesis of *Drosophila melanogaster* has been a superior model to study cell proliferation and differentiation due to the morphology of germ cells visibly trackable at different stages and the precise number of mitotic amplification before entering meiosis (6). Bam and Bgcn are the initial factors identified as a part of the meiosis 'gate-keeper', and the molecular functions of both are associated with RNA regulation (7). The germ cells of *bam* or *bgcn* mutant are stuck in the amplifying mitotic cycle and thus displaying a tumor-like phenotype. Manipulating the dosage of *bam* gene can alter the number of mitotic divisions before meiosis entry, further indicating its critical role

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at the transition (8). Bam and Bgcn act in the same molecular complex in this process (8,9). Interestingly, parallel factors and their mutant phenotypes have been discovered in mouse gametogenesis. Mutations in YTHDC2 or MEIOC caused meiotic arrest in mouse germ cells (10–12). YTHDC2 and Bgcn share the same ancestral gene and multiple protein domains, while MEIOC and Bam are diverged homologs (11). Additionally, YTHDC2 and MEIOC also form a protein complex to target a set of RNA transcripts and promote meiotic program (12). YTHDC2/Bgcn and MEIOC/Bam likely represent the major components of an evolutionarily conserved mechanism to control the transition from mitosis to meiosis.

In the past years, we have carried out genetic screens and transcriptome analyses to search for the factors involved at the mitosis-to-meiosis transition. In the *Drosophila* mutagenesis screen, we have found that Tut, an RNA binding protein, which recruits Bam and Bgcn to target RNAs, is required for promoting germ cells to meiosis (9). From the transcriptome analyses, we observed the distinct 3'UTR (UnTranslated Region of mRNA) profiles displayed by mitotic or meiotic cells, and that a set of genes showing 3'UTR changes in their transcripts are shared by *bam*, *bgcn*, and *tut* mutant germ cells (13). Furthermore, we demonstrated that Bam-Tut-Bgcn complex could repress 3'-processing factors post-transcriptionally by recruiting CCR4/Twin of deadenylation machinery (13).

To examine the causal relationship between 3'UTR and germline differentiation, we also changed the levels of 3'-processing factors to see the effect. Decreasing the levels of Cbc, a component of the 3'-processing machinery, resulted in germline over-proliferation. Cbc is an RNA kinase and a homolog of the mammalian CLP1 (cleavage factor polyribonucleotide kinase subunit 1). Cases from multiple families carrying the same mutation in human CLP1 are diagnosed of pontocerebellar hypoplasia (PCH), a neurodegenerative autosomal recessive disorder (14–16). In PCH and *Clp1* mouse model, neurodegeneration and abnormal tRNA splicing were consistently observed (14,15). In this study, our goal is to reveal how CLP1/Cbc engages in germ cell development, especially in the transition to meiosis.

## Results

### Cbc restricts germline mitotic amplification in *Drosophila* testis

In a normal testis of fruit fly, germ cells mitotically amplify four times before entering meiosis. Thus, a cyst containing more than 16 germ cells is rarely present before meiosis in a wild-type testis (Fig. 1A and B). To search for the factors involved in the transition from mitosis to meiosis, in the germline of fly testis, we carried out RNA interference (RNAi) and over-expression screens of candidate genes based on previous RNAseq analyses (13), using an early germline driver *nos-GAL4*. Cbc, a *Drosophila* homolog of mammalian RNA kinase CLP1, displayed germline over-amplification when its level was knocked down (Fig. 1C–G). Three different RNAi lines were tested to confirm this phenotype.

We then examined the germline clones of *cbc* mutants including two null alleles ('Q5STOP' and a frameshift '8 fs5') and 1 point mutation (A37T) illustrated in Figure 2A. We hardly recovered any clones of the *cbc* null alleles, which are likely cell lethal. Germline over-proliferation was only observed in *cbc*<sup>A37T</sup> mutant clones at 30°C (Fig. 2C and D,

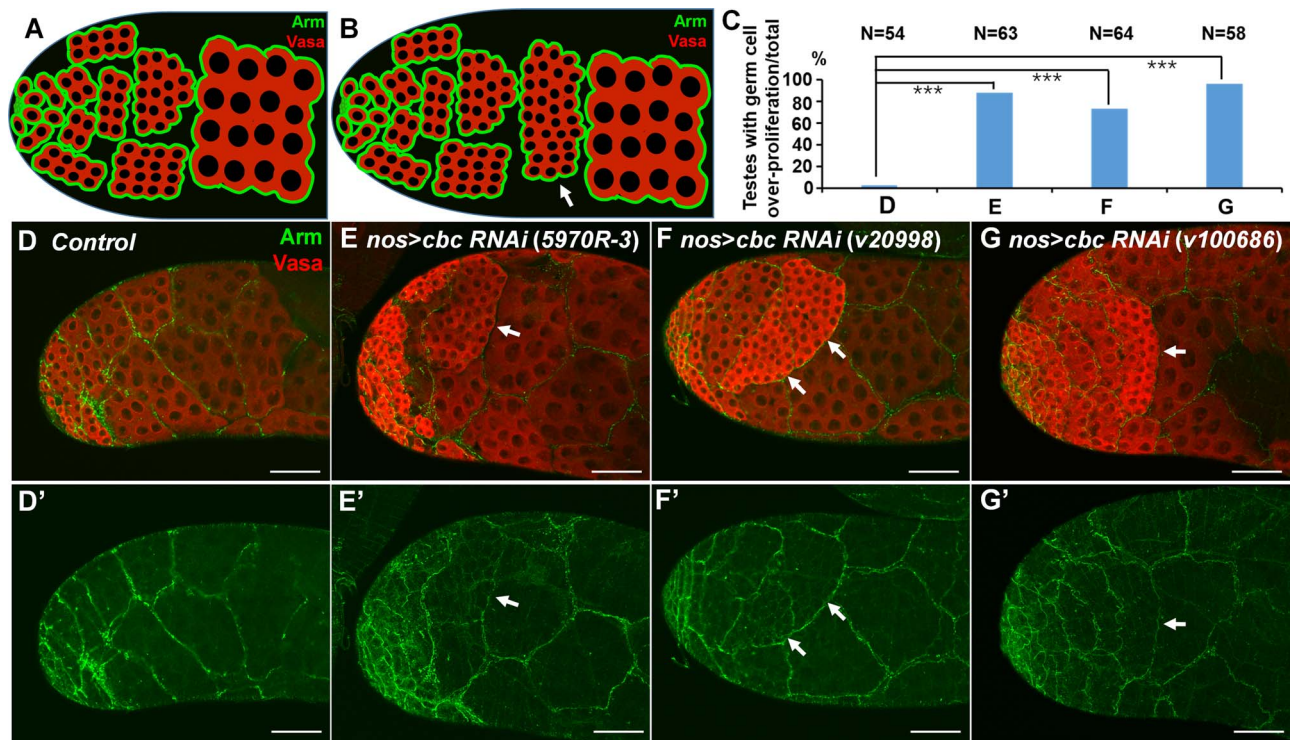
every over-amplified germline clone was GFP-negative). Both germline over-amplification and lethality were reversed by Cbc expression (Fig. 2E–H, *da-GAL4* is active in both somatic and germ cells to produce Cbc). Thus, we confirmed by RNAi and mutant clonal analyses that a proper level of Cbc is necessary to prevent germline from mitotic over-amplification.

### Meiosis initiation is tightly associated with Cbc nuclear localization

To determine the subcellular localization of Cbc during germline development, we genomically tagged the encoded product at the C-terminus and examined the temporal and spatial patterns of Cbc-HA fusion protein (Fig. 3A). We found that Cbc-HA was predominantly nuclear but devoid from nucleolus in the germ cells until the late meiotic growth phase (Fig. 3B, part of the nucleolus was marked by Fibrillarlin). As the germ cells grew bigger and approached 1<sup>st</sup> meiotic division, Cbc-HA shifted from nuclear plasma or DNA-occupied space to the nucleolar region complementary to Fibrillarlin, then disappeared once 1<sup>st</sup> meiotic division was completed (indicated by 32-nuclei in one cyst). In all somatic tissues we examined, Cbc-HA was always nuclear but absent in the nucleolar region (Fig. 3B5). The same spatio-temporal pattern was also observed by another genomically tagged product (Cbc-GFP, Supplementary Material, Fig. S1).

How is the dynamic localization of Cbc related to germline development? The point mutation *cbc*<sup>A37T</sup> provided a clue to connect Cbc nuclear localization and the transition to meiosis. *cbc*<sup>A37T</sup> is homozygous lethal and germline over-amplification was only observed in *cbc*<sup>A37T</sup> clones at 30°C (Fig. 2C and D). Both wild-type Cbc and Cbc<sup>A37T</sup> could restore the viability of *cbc*<sup>A37T/8fs5</sup>, but Cbc<sup>A37T</sup> failed to restore fertility at 30°C (Fig. 4, no big growth-phase meiotic cells observed, as shown in D; and Fig. 5D for fertility test at 25°C). Detailed analysis showed that the majority of Cbc<sup>A37T</sup> was nuclear at 25°C but cytoplasmic at 30°C in *cbc* mutant background (Fig. 4C, nuclear Flag signal complementary with cytoplasmic Vasa; and in 4D, Flag signal colocalized with Vasa). Interestingly, Cbc<sup>A37T</sup> was mostly excluded from the nucleus at 25°C in the presence of wild-type Cbc (Supplementary Material, Fig. S2A, Cbc<sup>A37T</sup> expressed in wild-type background). The proper transition to meiosis and thus normal fertility were coupled with nuclear Cbc (Fig. 4A–C), and the failure to enter meiosis and thus germline mitotic over-amplification were coupled with cytoplasmic Cbc (Fig. 4D, and all such testes contained >16-cell germline cysts after the temperature shift to 30°C).

Since Cbc is an RNA kinase highly concentrated in the area of nucleolus during the meiotic growth phase of germ cells, what function does Cbc serve there? Nucleolus is a space for rRNAs production and Cbc contains a putative domain for rRNA biogenesis (17), it is sensible to examine rRNA processing in response to Cbc changes. 3' external transcribed spacers (3'-ETS) are present in the 47S rRNA precursor, which eventually gives rise to the smaller mature rRNAs including 18S and 28S ones. We examined the testes using the RNA probes against 3'-ETS, 18S, and 28S rRNAs by the fluorescence in situ hybridization (FISH) assay (18). Consistent with the predicted location of the RNA species that the probes detect, 3'-ETS signal was mainly nucleolar, whereas 18S or 28S mostly cytoplasmic in germ cells (Supplementary Material, Fig. S3A and C). We did not detect any obvious change in these rRNA species when we reduced Cbc levels and caused the accumulation of germ cells stuck in mitosis (Supplementary Material, Fig. S3B and D). To validate the efficiency of this method, we repeated a previously reported experiment and



**Figure 1.** Decreasing Cbc level caused germ cell over-amplification. (A) A schematic illustration of the cellular architecture at the apical part of a normal *Drosophila* testis. Armadillo (Arm), a somatic marker, which outlines each unit of the germline lineage; Vasa, a germline marker. (B) A schematic illustration of the apical part of a testis containing a cyst of more than 16 mitotic germ cells (arrow). (C) Scoring the testes containing germ cell over-amplification. N, number of testes scored. The data were analyzed by Wilcoxon Rank Sum,  $***P < e^{-12}$ . (D) genotype: *UAS-Dicer2/+;nos-GAL4/+*. *nos-GAL4*, a germline-specific GAL4 driver. (E-G) Knock-down of Cbc (*UAS-Dicer2/+;nos-GAL4 > cbc RNAi*) led to germ cell over-proliferation (arrows). Note: crosses were raised at 25°C for 4 days, and then transferred to 30°C. All flies were dissected within 0–3 days after eclosion. Scale bars: 50  $\mu$ m.

obtained the same results as those documented (Supplementary Material, Fig. S3E–H) (18). Additionally, we noticed that *Cbc*<sup>A37T</sup>-Flag at 25°C ensured normal progress through meiosis without concentrating in nucleolus (Fig. 4C, arrowhead). Taken together, we demonstrated that the nuclear but not nucleolar Cbc is required for germ cells' switch to meiosis.

### Cbc and its mammalian homolog CLP1 are functionally conserved

To investigate the conservation between Cbc and its mammalian homologs, we checked the conserved amino acids regarding their effect on subcellular distribution. A37T of Cbc corresponds to A42T in human CLP1; R140H of CLP1, the genetic mutation associated with PCH, corresponds to R135H in Cbc. In their respective host cells in culture, more proteins of CLP1<sup>A42T</sup> or Cbc<sup>R135H</sup> shifted from nucleus to cytoplasm in comparison with their wild-type versions (Supplementary Material, Fig. S4). It appears that the A-to-T change exhibited a stronger effect on Cbc/CLP1 localization than R-to-H (Supplementary Material, Fig. S4). Nevertheless, these two conserved residues were favored for the nuclear localization of both Cbc and human CLP1.

To determine the functional conservation of Cbc and mammalian CLP1 in spermatogenesis, we tested mouse CLP1 activity in the *cbc* mutant background. *cbc*<sup>Q5stop</sup> is a null allele and its mutant clones hardly survived. We occasionally recovered a few *cbc*<sup>Q5stop</sup> cells which are shown in Figure 5A' (GFP-negative cells). In contrast, *cbc*<sup>Q5stop</sup> cells were very abundant and advanced to meiotic growth phase when mouse CLP1 (mCLP1) was supplied in germ cells (Fig. 5B). *cbc*<sup>A37T/8fs5</sup> mutants were lethal but became

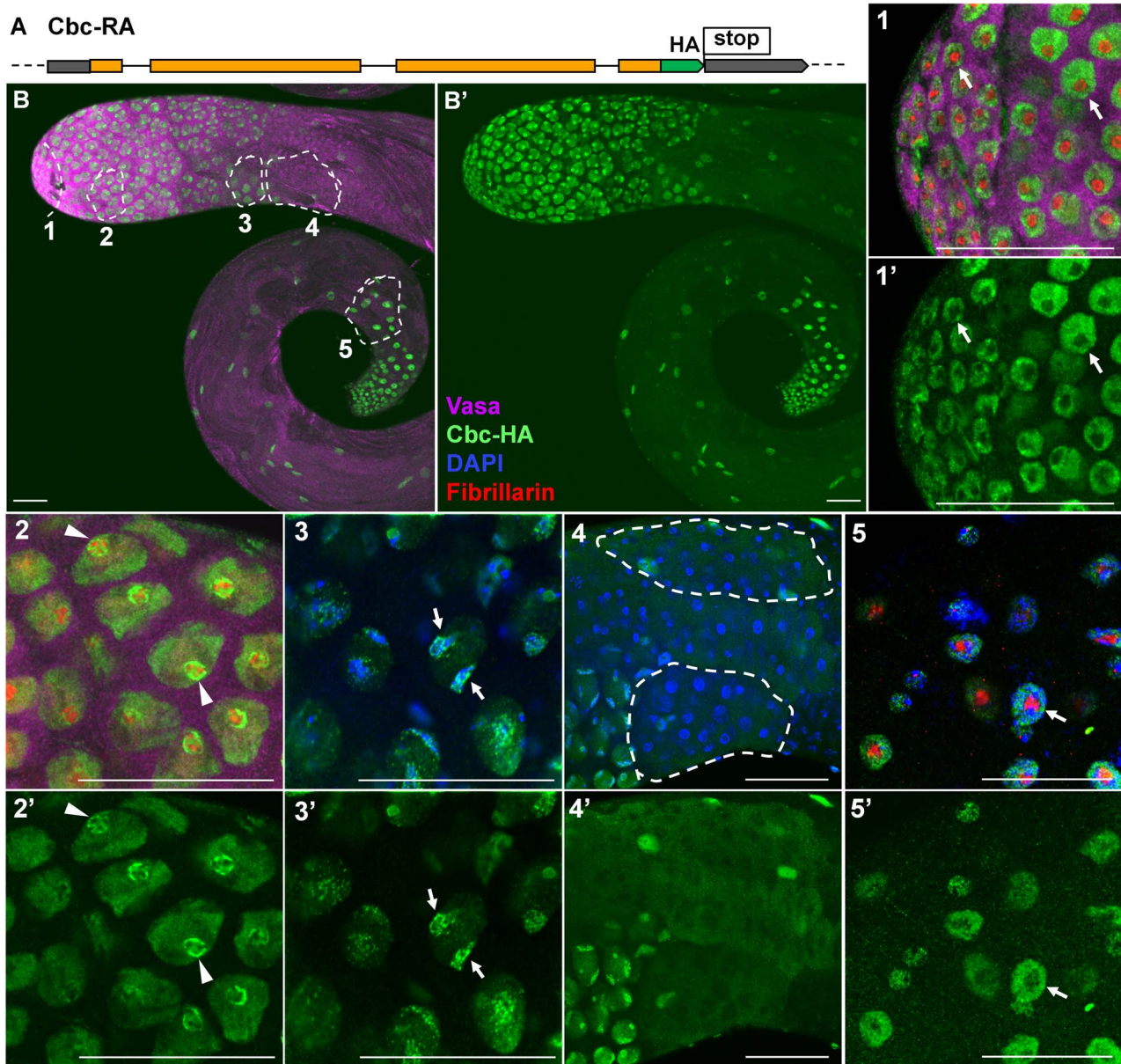
viable and even partially fertile when mouse CLP1 was expressed in both soma and germline (Fig. 5C and D). Activities of A37T, K122A (putatively kinase-dead), or R135H mutant proteins at 25°C were similar against lethality but varied in restoring male fertility (Fig. 5D), which was unlikely related to meiosis entry but post-meiotic differentiation upon examination of fixed testes (data not shown). Intriguingly, human CLP1 is different from mouse CLP1 in only five amino acids, and it could restore the viability of *cbc*<sup>A37T</sup> flies but not their fertility. Nonetheless, the structural and functional conservation between Cbc and mammalian CLP1 was illustrated by the assays in subcellular distribution, fertility and viability.

### Cbc interacts with Tsen54 and TER94 to regulate the transition to meiosis

To identify the protein partners of Cbc in spermatogenesis, we collected testes expressing Cbc-HA to perform co-immunoprecipitation (coIP) followed by mass-spectrometry. The reliability of this coIP experiment was indicated by the most abundant peptides from Pcf11, which is a well-known partner of CLP1 in yeast and mammalian 3'-processing machinery (Supplementary Material, Table S1) (19,20). Furthermore, Pcf11 has been shown as a post-transcriptional target of Tut, and Pcf11 genetically interacted with *tut* in the transition to meiosis (13). Thus we provided additional evidence confirming the relation between global 3'-processing and meiosis initiation.

Among the abundant interactors co-purified with Cbc, there were several TSEN complex subunits, especially Tsen54,





**Figure 3.** Cbc localization shifts from nucleus to nucleolus during germline development. (A) HA was genomically tagged to *cbc* by CRISPR-Cas9. (B) Cbc-HA localization. Vasa, a germ cell marker, which is mainly localized in cytoplasm. Fibrillarlin, a nucleolus marker. (1–5) Higher magnification of the outlined regions of the testis in B. (1 and 1') Early germ cells (arrows). (2 and 2') Relatively mature spermatocytes (arrowheads). (3 and 3') Mature spermatocytes, and two arrows point to the spots in the same nucleus. (4 and 4') Two outlined cysts after meiotic division. (5 and 5') somatic cells. At least 40 testes were examined. Scale bars: 50  $\mu$ m.

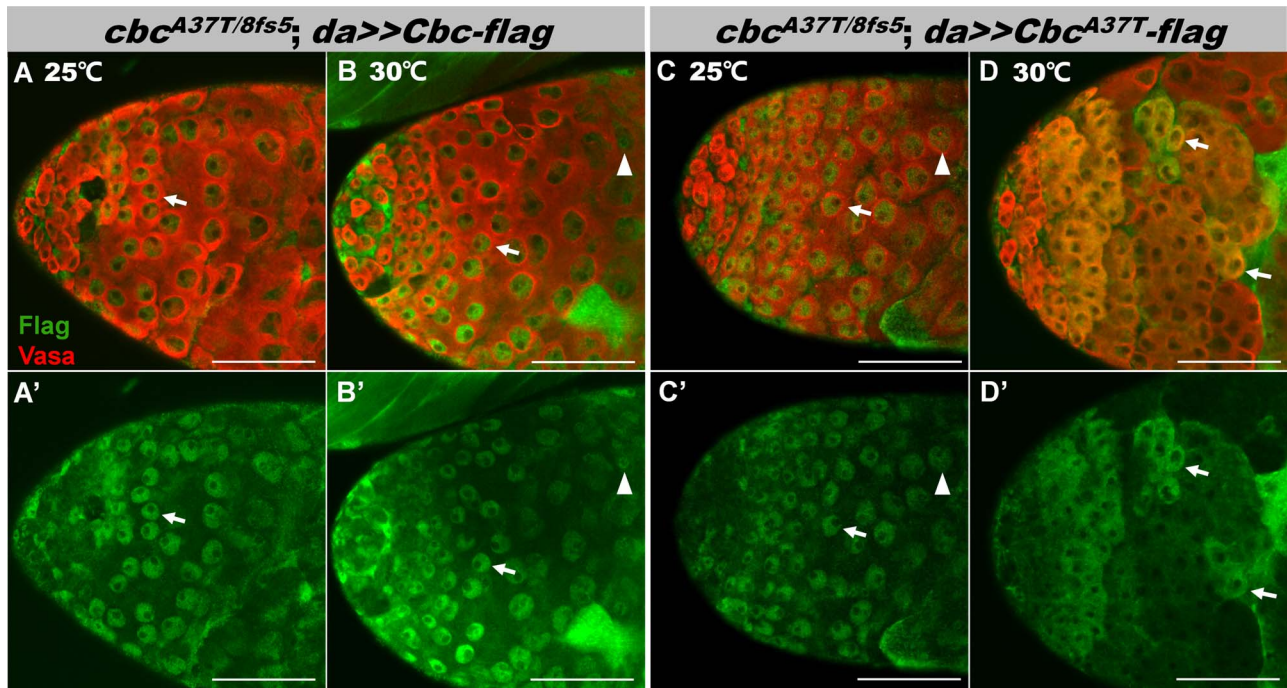
and the specificity of this RNAi using 3'UTR sequences was shown by the rescue with one of the TER94 isoforms (Fig. 7C). However, we did not detect any physical binding between Cbc and TER94 in either Y2H or coIP of tagged proteins in cultured cells (Supplementary Material, Fig. S5).

## Discussion

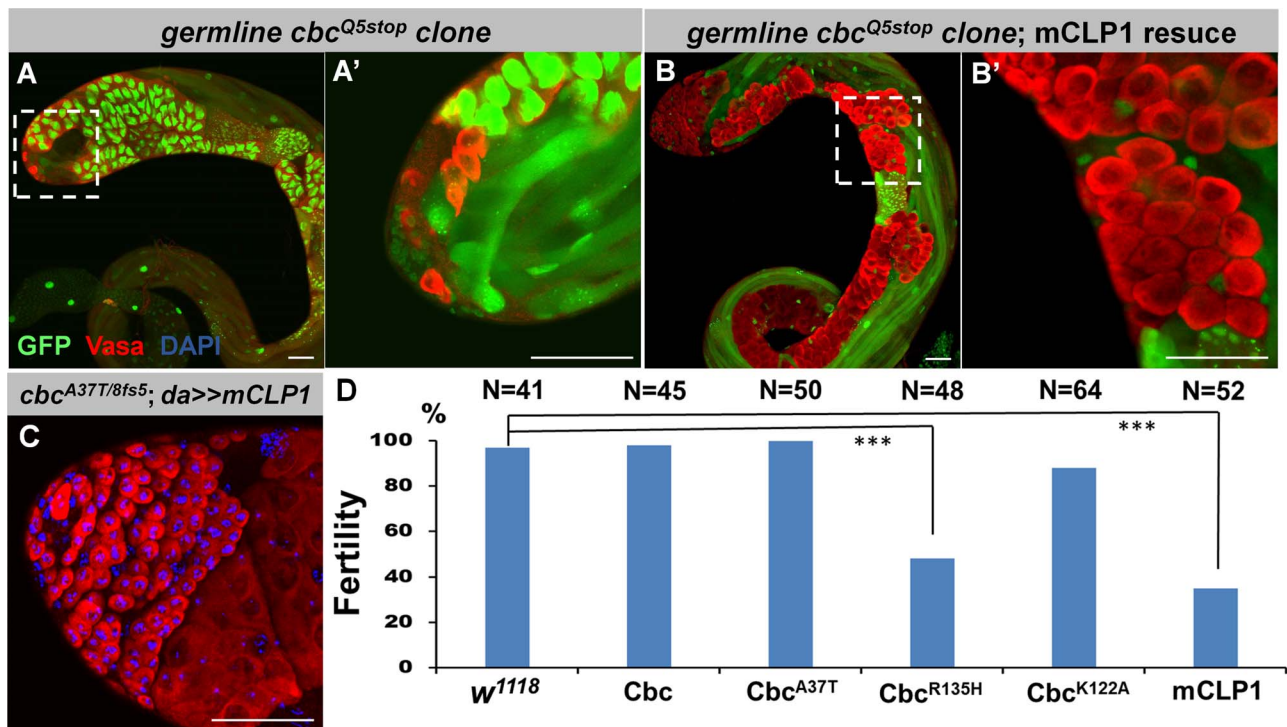
CLP1 polynucleotide kinase and TSEN tRNA splicing factors have been linked to neural development, and their mutations are closely associated with the congenital neurodegenerative disorder PCH (21). Here, we demonstrate that Cbc, the *Drosophila* homolog of CLP1, physically and genetically acts with Tsen54

at the transition to meiosis in spermatogenesis. Nuclear but not nucleolar Cbc is necessary in this process.

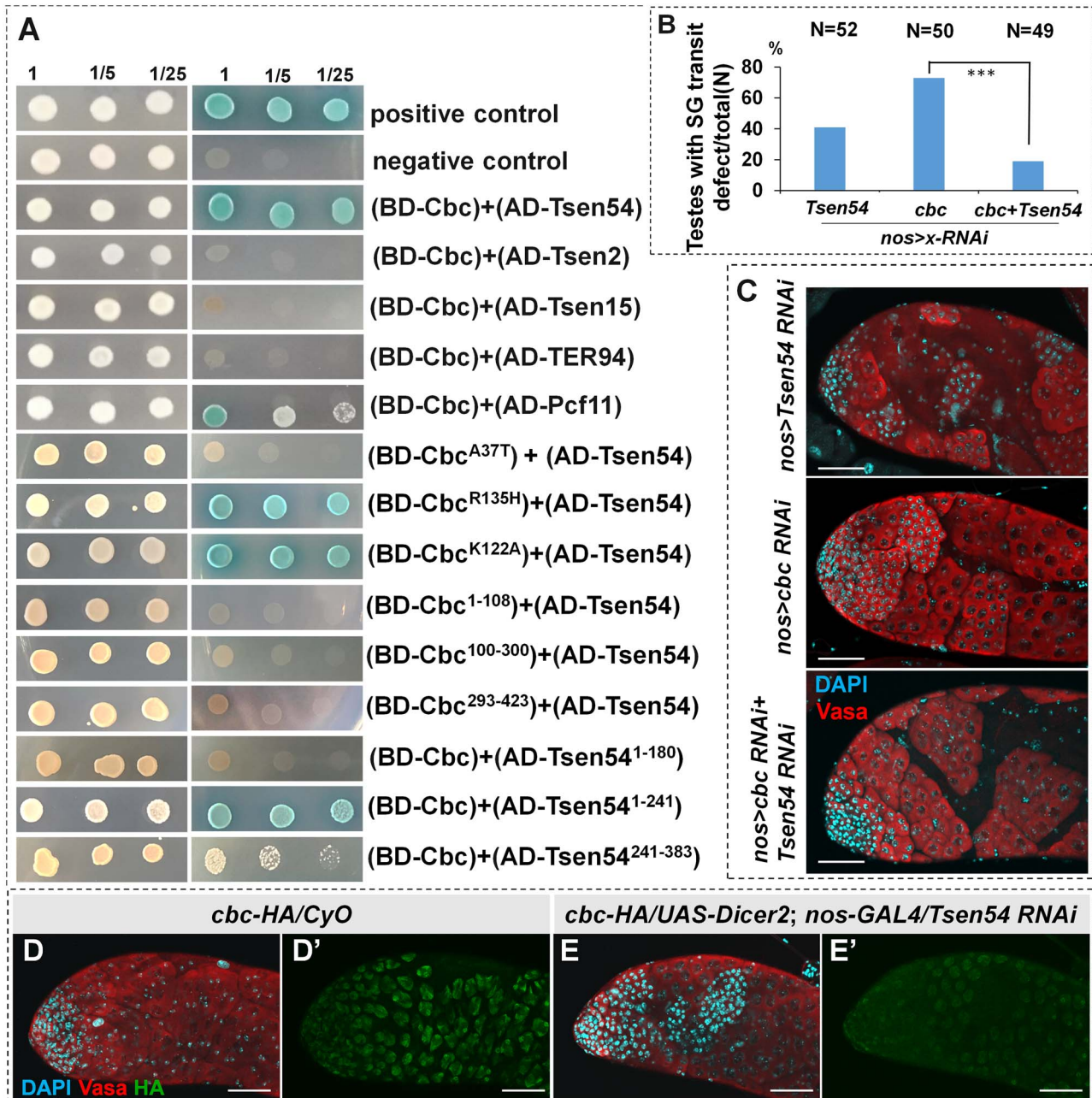
During spermatogenesis, Cbc's distribution shifts from nucleus, but devoid in nucleolus, to solely nucleolus (Fig. 3). Our current data do not support a nucleolar function of Cbc in meiosis initiation, but neither exclude the possibility that nucleolar localization is required for the post-meiotic differentiation. Regarding Cbc's nuclear import, the temperature-sensitive point mutation A37T showed some interesting behaviors. Cbc<sup>A37T</sup> was nuclear at 25°C but cytoplasmic at 30°C when wild-type Cbc was absent (Fig. 4C and D), and was mostly cytoplasmic when expressed in the wild-type background even at 25°C (Supplementary Material, Figs S2A, I and S4G). Cbc's primary structure does not possess a typical nuclear localization signal



**Figure 4.** A point mutation hindered Cbc nuclear localization and inhibited meiosis entry. (A and B) genotype: *cbc<sup>A37T/8fs5</sup>; da-GAL4/UAS-Cbc-flag*. Cbc-flag was localized in the nucleus when overexpressed in *cbc<sup>A37T/8fs5</sup>* mutant at 25°C (A, arrow) or transferred to 30°C for 3 days (B, arrow), and germ cells developed normally. (C and D) genotype: *cbc<sup>A37T/8fs5</sup>; da-GAL4/UAS-Cbc<sup>A37T</sup>-flag*. (C and C') Cbc<sup>A37T</sup>-flag was localized in the nucleus when overexpressed in *cbc<sup>A37T/8fs5</sup>* mutant at 25°C and germ cells developed normally. (D and D') When the temperature was shifted to 30°C for 3 days, Cbc<sup>A37T</sup>-flag became predominantly cytoplasmic and germ cells failed to transit to meiosis. At least 40 testes were examined for each condition. Scale bars: 50 µm.



**Figure 5.** Overexpressing CLP1, the mammalian homolog of Cbc, rescued the lethality and sterility of *cbc* mutant. (A and B) Clones were specifically induced in germ cells. GFP negative cells are *cbc<sup>Q5stop</sup>* mutant clones. (A) Genotype: *FRT42B, cbc<sup>Q5stop</sup>/FRT42B, ubiGFP; nos-Gal4,UAS-FLP/+*. (B) Genotype: *FRT42B, cbc<sup>Q5stop</sup>/FRT42B, ubiGFP; nos-Gal4,UAS-FLP/UAS-mCLP1*. (A'&B') Higher magnification of the outlined region. (C) Genotype: *cbc<sup>A37T/8fs5</sup>; da-GAL4/UAS-mCLP1*. Overexpression of mCLP1 in *cbc* transheterozygous mutant rescued its lethality and promoted germ cell differentiation. (D) Scoring of male fertility at 25°C (details in materials and methods). Genotype: *cbc<sup>A37T/8fs5</sup>; da-GAL4/UAS-X* (X = different forms of Cbc or mCLP1). The data were analyzed by Wilcoxon Rank Sum. \*\*\*P < e<sup>-5</sup>. Scale bars: 50 µm.

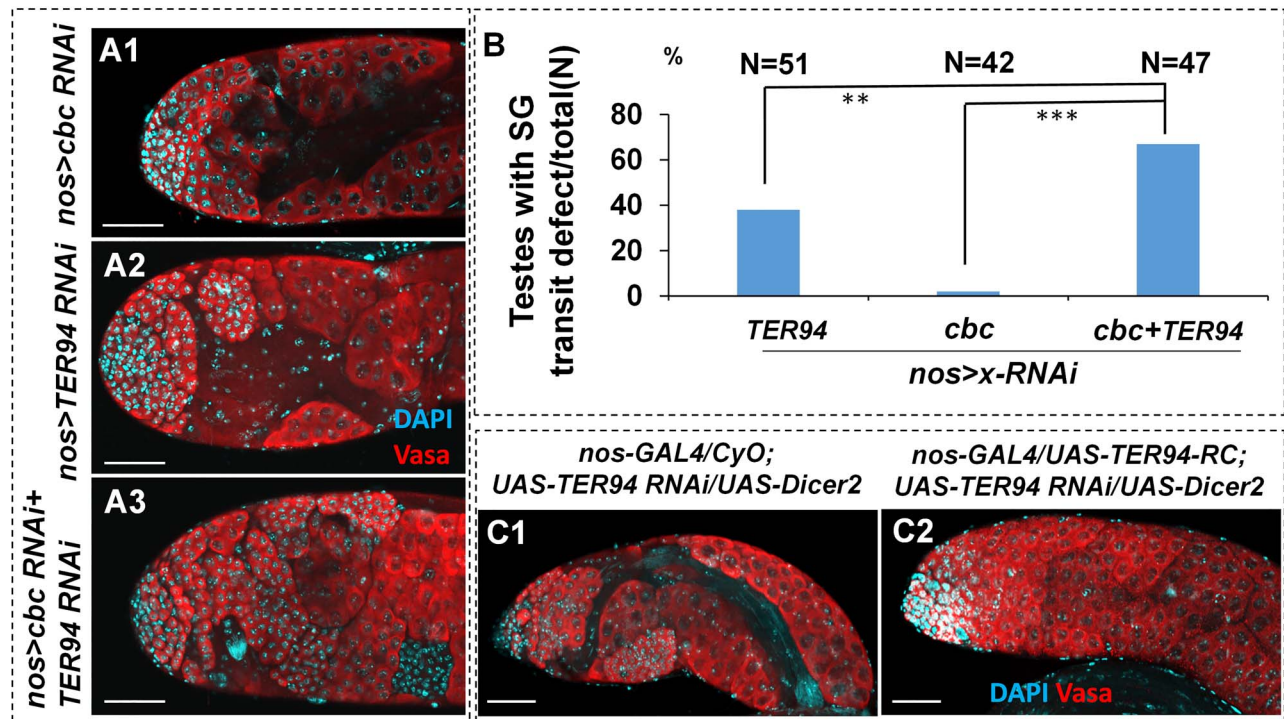


**Figure 6.** Cbc interacted with Tsen54 to regulate the transition to meiosis. (A) Yeast 2-Hybrid assay. Positive control: (BD-53) + (AD-T). Negative control: (BD-Lam) + (AD-T). (B) Phenotypes in panel C were scored. 'SG transition defect' meaning that the mitotic spermatogonia (SG) either over-amplified or arrested without entering meiotic division. 'nos>', germline-specific GAL4 driver. The data were analyzed by Wilcoxon Rank Sum,  $***P < e^{-5}$ . (C-E) Crosses were raised at 25°C for 4 days, and then transferred to 30°C. All flies were dissected 0–3 days after eclosion. (C) Simultaneous knock-down of Cbc and Tsen54 suppressed the phenotype of either *cbc* RNAi ( $v100686$ ) or *Tsen54* RNAi (BDSC35337). (D and E) Genomic Cbc-HA expression was decreased in *Tsen54* RNAi (BDSC35337) compared with that in wild-type. HA signals were collected with the same settings. Scale bars: 50  $\mu$ m.

that Tsen54 does. It appears that Cbc gets into the nucleus with the assistance of a factor which may have a higher affinity for the wild-type Cbc than for Cbc<sup>A37T</sup>. Since A37T compromised both Cbc's binding to Tsen54 (Fig. 6A) and nuclear localization (Supplementary Material, Fig. S2A), Tsen54 may be the candidate assisting Cbc to stay nuclear by simply trapping its binding partner. Notably, Cbc<sup>A37T</sup>-HA levels were apparently lower than Cbc-HA (Supplementary Material, Fig. S2A, B, and G), indicative of a stability difference. Additionally, Cbc protein levels were downregulated but remained nuclear when we knocked down

Tsen54 (Fig. 6D and E). Possibly, wild-type Cbc could be stabilized by its association with Tsen54 in the nucleus.

Is tRNA splicing involved in regulating meiosis entry? Only 6–7% of tRNA genes contain introns in the genome of fruit fly or human (21), and TSEN complex is responsible for tRNA processing. There are four subunits in TSEN complex: two structural ones, TSEN15 and TSEN54; and two catalytic ones, TSEN2 and TSEN34. CLP1 is frequently found in the same protein complex of TSEN subunits, but is not required for TSEN complex formation and pre-tRNA intron excision (22,23). Although three



**Figure 7.** Genetic interaction between *cbc* and *TER94*. (A1–3) Knock-down of *Cbc* and *TER94* enhanced the phenotype of either *cbc* RNAi (v100686) or *TER94* RNAi (THU1058). Crosses were raised at 25°C for 4 days, and then transferred to 28°C to optimize the phenotypic comparison. ‘*nos>*’, germline-specific GAL4 driver. (B) Phenotypes in A1–3 were scored. ‘SG transition defect’ means that the mitotic spermatogonia (SG) either over-amplified or arrested without entering meiotic division. The data were analyzed by Wilcoxon rank sum. \*\* $P = 0.014$ ; \*\*\* $P < e^{-6}$ . (C1–2) Over-expression of *TER94* isoform C rescued the defect caused by *TER94* RNAi (THU1058, containing *TER94* 3’UTR sequence). Crosses were raised at 25°C for 4 days, and then transferred to 30°C. All flies were dissected within 0–3 days after eclosion. Scale bars: 50  $\mu$ m.

Tsen subunits co-purified with *Cbc*, only *Tsen54* displayed any function in spermatogenesis upon knockdown, in accordance with its strong interaction with *Cbc* (Figs 6A and Supplementary Material, Fig. S5A–B). A recent study illustrated that *CLP1* or *Cbc* actually inhibited tRNA biogenesis *in vitro* or *in vivo*, respectively; and *CLP1/Cbc* may act downstream of TSEN complex (23). Accumulation of Tyr-tRNA products was also detected in kinase-dead *Clp1* mice (24). These results are consistent with our observation of the opposing effect between *cbc* and *Tsen54* in spermatogenesis (Fig. 6B and C). Whether tRNA biogenesis plays a role in this process requires further investigation since TSEN localization and function unrelated to tRNA processing have been reported (25,26).

*TER94*, the *Drosophila* ortholog of human valosin-containing protein (VCP), was present among the proteins co-purified with *Cbc*, and its adequate levels were needed to limit germ cell mitotic expansion (Supplementary Material, Table S1, Fig. 7). However, direct contacts between *TER94* and *Cbc* were not observed (Figs 6A and Supplementary Material, Fig. S5). *TER94* associates with different co-factors to process substrates marked by ubiquitin, and is necessary for restructuring Golgi stacks during mitotic cycle (27,28). We currently have limited clues to speculate how *TER94* acts in the switch from mitosis to meiosis. Regarding the pathogenesis of PCH in *CLP1*-mutant patients, VCP may also contribute to the abnormal neurodevelopment since VCP mutations have been associated with many neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).

How may *Cbc* function in spermatogenesis be linked to neurodegeneration phenotypes caused by *CLP1* mutation? A neuroblast-specific knockdown of *Cbc* results in less daughter

cells per neuroblast in the larval brain compared to the controls (29). This phenotype is consistent with the neurodegeneration found in PCH patients with *CLP1* mutation, and cell death was detected in *Clp1*-mutant vertebrate models (14,15). Whereas in spermatogenesis, *Cbc* is required for differentiation into the meiotic program, block of which leads to mitotic over-amplification. At the level of cellular differentiation/proliferation, a similar scenario of *Cbc* function in spermatogenesis and neurogenesis has not been observed.

What are the new findings we can provide for the physiological and pathological functions of *CLP1* in mammals? Structurally, *Cbc* and *CLP1* are highly conserved at the amino acid levels of 70% similarity. Replacement of amino acids at positions conserved between *Cbc* and *CLP1* exhibited similar changes in subcellular distribution and fertility test (Figs 5D and Supplementary Material, Fig. S4). Functional conservation was further demonstrated by *CLP1*’s substitution of *Cbc* on viability and partial fertility in fruit flies (Fig. 5). Further, even the co-purified partner proteins, *Tsen54* and *Pcf11*, are shared by *Cbc* and *CLP1* (19,30–33). Though direct association of *CLP1* and *TSEN54* has not been reported in mammalian systems, it likely exists, judged by the functions and collaborators parallel in fly and mammal. Thus, we validate *Drosophila* as a suitable model to study *CLP1*-related organismal development and diseases.

## Materials and Methods

### Fly strains

For the genetic screens, flies were ordered from Tsinghua Fly Center, NIG-Fly, Vienna *Drosophila* RNAi Center, and FLYORF. The results of the screens are shown in Supplementary data S1. Tool



strains used in this study: *UAS-Dicer2* (a gift from T.Tabata); *nos-Cas9* (34); *vasa-GAL4* (35); balancers, *w<sup>1118</sup>*, *ZH-attP-86Fb*, *FRT42B*, *ubiGFP*, *FRT42B*, *FRT42D*, *ubiGFP*, *FRT42D*, *nos-GAL4*, *hs-FLP*, *nos-GAL4*, *UAS-FLP/TM6*, *Cre* and *da-Gal4* were ordered from Bloomington Drosophila Stock Center.

Cbc related flies: *UAS-cbcRNAi* (NIG#5970R-3) from NIG-Fly; *UAS-cbcRNAi* (v100686/v20998) from Vienna Drosophila RNAi Center. *cbc<sup>Q5stop</sup>* and *cbc<sup>A37T</sup>* from Bloomington Drosophila Stock Center; *cbc<sup>8fs5</sup>* was generated by CRISPR-Cas9 resulting in a 5 bp-deletion after the 8th amino acid. *Cbc-HA* was a genomically tagged fly, in which 3 × HA was fused to *cbc* C terminus before stop codon by CRISPR-Cas9. Transgenic flies of *UAS-Cbc*, *UAS-Cbc-flag*, *UAS-Cbc<sup>A37T</sup>-flag*, *UAS-mCLP1* and *UAS-hCLP1* were generated by inserting the pUAS-attB construct to attP landing site of *ZH-attP-86Fb* flies (36).

### Genomic editing by CRISPR-Cas9

*cbc<sup>8fs5</sup>* mutant was generated by CRISPR/Cas9 at Fungene Biotech (<http://www.fungene.tech>), which was validated by sequencing its PCR products.

*cbc-HA* was generated in our lab. The sgRNA sequence targeting *cbc* (5'-gcagagcattacaatgctagg-3') was selected around the stop codon (<http://targetfinder.flycrispr.neuro.brown.edu/>) (37). The selected sequence was cloned to pCFD4-U6:1\_U6:3tandemgRNAs (a gift from Simon Bullock, Addgene plasmid # 49411; <http://n2t.net/addgene:49411>; RRID: Addgene\_49411). Both homology arms sequences were cloned to pBSK-HA-LRL (a gift from Fungene Biotech) to generate the dsDNA donor. A mixture with 500 ng/μL donor and 100 ng/μL gRNA vector was injected into *nos-cas9* fly embryos as described previously (34,38). All P0 flies were crossed with balancers. The F1 flies with RFP marker in eyes were crossed with *Cre* flies to cross out RFP marker. The *cbc-HA* flies (F2) without RFP marker were validated by sequencing, Western blotting and immunostaining. Genomically tagged *cbc-GFP* and *HA-cbc* flies were also generated by the same method.

### Transgenic flies

cDNAs of *Cbc*, *mCLP1*, and *hCLP1* isoform1 were cloned respectively from Drosophila testes, mouse testes, and HeLa cells, and then inserted to pUAS-attB vector (39). The constructs were inserted to *ZH-attP-86Fb* flies to generate *UAS-cbc*, *UAS-mCLP1* and *UAS-hCLP1* transgenic flies. *Cbc-flag*, *Cbc<sup>A37T</sup>-flag* were generated by cloning flag sequence in C-terminus.

### Immunofluorescence

Fly testes were dissected in PBS, fixed in 4% FA/PBS, and immunostained as described (40), except that the primary antibodies were incubated for 2 days at 4°C. Primary antibodies used in this study: rabbit anti-Vasa (1:8000; against KLH-MSDDWDDEPIVDTRGARC-OH), guinea pig anti-Vasa (1:4000; against 6xHis-Vasa produced in *E. coli*), mouse anti-Armadillo (1:400; DSHB, N27A1), rabbit anti-Fibrillarin (1:400; HUABIO, ER1802-81), mouse anti-HA (1:300; MBL, M180-3), mouse anti-Flag (1:500; Sigma, F1804). Alexa-Fluor-conjugated secondary antibodies were used at 1:4000 (Molecular Probes, Invitrogen). Fluorescent images were collected by OLYMPUS FV1000 Confocal microimaging system.

### Fly fertility test

One male and three *w<sup>1118</sup>* female flies were cultured in one tube at 25°C. The male was considered fertile if empty pupal cases were present after 15 days.

### Yeast two-hybrid assay

*Cbc* wild-type or mutated sequence were cloned to pGBKT7 plasmid as BD. Coding sequences of *Tsen2*, *Tsen15*, *Tsen54*, *TER94*, and *Pcf11* were cloned to pGADT7 plasmid as AD. Yeasts with BD and AD plasmids were cultured on SD/−Ade/−His/−Leu/−Trp medium (QDO, Takara Bio, 630 412 and 630 428) supplemented with X-α-Gal (Takara Bio, 630 462) and aureobasidin A (QDO/X/A, Takara Bio, 630 466) to test protein–protein interactions or on SD/−Leu/−Trp medium (DDO, Takara Bio, 630 412 and 630 417) to confirm the transformation.

### Supplementary Material

Supplementary Material is available at HMG online.

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