APC/C<sup>FZR-1</sup> Controls SAS-5 Levels To Regulate Centrosome Duplication in Caenorhabditis elegans

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ABSTRACT As the primary microtubule-organizing center, centrosomes play a key role in establishing mitotic bipolar spindles that secure correct transmission of genomic content. For the fidelity of cell division, centrosome number must be strictly controlled by duplicating only once per cell cycle. Proper levels of centrosome proteins are shown to be critical for normal centrosome number and function. Overexpressing core centrosome factors leads to extra centrosomes, while depleting these factors results in centrosome duplication failure. In this regard, protein turnover by the ubiquitin-proteasome system provides a vital mechanism for the regulation of centrosome protein levels. Here, we report that FZR-1, the Caenorhabditis elegans homolog of Cdhl/Hct1/Fzr, a coactivator of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, functions as a negative regulator of centrosome duplication in the C. elegans embryo. During mitotic cell division in the early embryo, FZR-1 is associated with centrosomes and enriched at nuclei. Loss of <i>fzr-1</i> function restores centrosome duplication and embryonic viability to the hypomorphic <i>zyg-1(it25)</i> mutant, in part, through elevated levels of SAS-5 at centrosomes. Our data suggest that the APC/C<sup>FZR-1</sup> regulates SAS-5 levels by directly recognizing the conserved KEN-box motif, contributing to proper centrosome duplication. Together, our work shows that FZR-1 plays a conserved role in regulating centrosome duplication in C. elegans.

The centrosome is a small, nonmembranous organelle that serves as the primary microtubule-organizing center in animal cells. Each centrosome consists of a pair of barrel-shaped centrioles that are surrounded by a network of proteins called pericentriolar material (PCM). During mitosis, two centrosomes organize bipolar spindles that segregate genomic content equally into two daughter cells. Thus, tight control of centrosome number is vital for the maintenance of genomic integrity during cell division, by restricting centrosome number to once, and only once, per cell cycle. Proper levels of centrosome proteins are shown to be critical for normal centrosome number and function. Overexpressing core centrosome factors leads to extra centrosomes, while depleting these factors results in centrosome duplication failure. In this regard, protein turnover by the ubiquitin-proteasome system provides a vital mechanism for the regulation of centrosome protein levels. Here, we report that FZR-1, the Caenorhabditis elegans homolog of Cdhl/Hct1/Fzr, a coactivator of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, functions as a negative regulator of centrosome duplication in the C. elegans embryo. During mitotic cell division in the early embryo, FZR-1 is associated with centrosomes and enriched at nuclei. Loss of <i>fzr-1</i> function restores centrosome duplication and embryonic viability to the hypomorphic <i>zyg-1(it25)</i> mutant, in part, through elevated levels of SAS-5 at centrosomes. Our data suggest that the APC/C<sup>FZR-1</sup> regulates SAS-5 levels by directly recognizing the conserved KEN-box motif, contributing to proper centrosome duplication. Together, our work shows that FZR-1 plays a conserved role in regulating centrosome duplication in C. elegans.

In the nematode <i>Caenorhabditis elegans</i>, extensive studies identified a set of core centrosome factors that are absolutely essential for centrosome duplication: the protein kinase ZYG-1 and the coiled-coil proteins SPD2, SAS-4, SAS-5, and SAS-6 (O’Connell et al. 2001; Kirkham et al. 2003; Leidel and Gőnçzy 2003; Dammermann et al. 2004; Delattre et al. 2004; Kemp et al. 2004; Pelletier et al. 2004; Leidel et al. 2005). SPD-2 and ZYG-1 localize early to the site of centriole formation and are required for the recruitment of the SAS-5/SAS-6 complex that sequentially recruits SAS-4 to the centriole (Delattre et al. 2006; Pelletier et al. 2006). These key factors are also present in other animal systems, suggesting an evolutionary conservation in centrosome duplication. For instance, the human genome contains homologs of the five centrosome factors found in <i>C. elegans</i>, Cep192/SPD-2 (Zhu et al. 2008), Plk4/ZYG-1 (Habeledanck et al. 2005), STIL/SAS-5 (Arquint et al. 2012), HsSAS-6/SAS-6 (Leidel et al. 2005) and CPAP/SAS-4 (Kleylein-Sohn et al. 2007; Tang et al. 2009), and all these factors are shown to play a critical role in centrosome biogenesis (Fu et al. 2015; Gőnçzy 2015). However, the recently identified core centriole factor, SAS-7, that acts upstream of SPD-2 during <i>C. elegans</i> centriole duplication has not been found outside of nematodes (Sugioka et al. 2017).
Maintaining the proper levels of centrosome proteins is critical for normal centrosome number and function (Kleylein-Sohn et al. 2007; Strnad et al. 2007; Rogers et al. 2009; Tang et al. 2009, 2011; Holland et al. 2010; Brownlee et al. 2011; Pukadowski et al. 2011; Song et al. 2011; Meghini et al. 2016; Levine et al. 2017). In light of this, protein turnover by proteolysis provides a key mechanism for regulating the abundance of centrosome factors. A mechanism regulating protein levels is their degradation by the 26S proteasome that catalyzes the proteolysis of polyubiquitinated substrates (Livneh et al. 2016). The anaphase promoting complex/cyclosome (APC/C) is a multi-subunit E3 ubiquitin ligase that targets substrates for degradation (Acquaviva and Pines 2006; Peters 2006; Chang and Barford 2014). The substrate specificity of the APC/C is directed through the sequential, cell-cycle-dependent activity of two coactivators, Cdc20/Fzy/FZY-1 (Hartwell and Smith 1985; Dawson et al. 1995; Kitagawa et al. 2002) and Cdh1/Fzr/Hct1/FZR-1 (Schwab et al. 1997; Sigrist and Lehner 1997; Visinonti et al. 1997; Fay et al. 2002). During early mitosis, Cdc20 acts as coactivator of the APC/C, and Cdh1 functions as coactivator to modulate the APC/C-dependent events at late mitosis and in G1 (Irniger and Nasmyth 1997; Visinonti et al. 1997; Fang et al. 1998; Prinz et al. 1998; Shirayama et al. 1998). Upregulated targets in Cdh1-deficient cells are shown to be associated with the genomic instability signature of human cancers, and show a high correlation with poor prognosis (Carter et al. 2006; García-Higuera et al. 2008). Furthermore, a mutation in SIL/STIL (a human homolog of SAS-5) linked to primary microcephaly (MCPH; Kumar et al. 2009) results in deletion of the Cdh1-dependent destruction motif (KEN-box), leading to deregulated accumulation of STIL protein and centrosome amplification (Arquint and Nigg 2014). In Drosophila, the APC/C/Fzy/Cdh1 directly interacts with Spd2 through KEN-box recognition and targets Spd2 for degradation (Meghini et al. 2016). Therefore, the APC/C/Cdh1/Fzy/Fce1 plays a critical role in regulating the levels of key centrosome duplication factors in mammalian cells and flies.

In C. elegans, FZR-1 has been shown to be required for fertility, cell cycle progression and cell proliferation during embryonic and post-embryonic development via synthetic interaction with lin-35/Rb (Fay et al. 2002; The et al. 2015). However, the role of FZR-1 in centrosome assembly has not been described. In this study, we molecularly identified fr-1 as a genetic suppressor of zyg-1. Our results suggest that APC/C-FZR-1 negatively regulates centrosome duplication, in part, through proteasomal degradation of SAS-5 in a KEN-box dependent fashion. Therefore, FZR-1, the C. elegans homolog of Cdh1/Hct1/Fzr, plays a conserved role in centrosome duplication.

MATERIALS AND METHODS

C. elegans strains and genetics

A full list of C. elegans strains used in this study is listed in Supplemental Material, Table S1 in File S1. All strains were derived from the wild-type Bristol N2 strain using standard genetic methods (Brenner 1974; Church et al. 1995).

Strains were maintained on MYOB plates seeded with Escherichia coli OP50 and grown at 19°C unless otherwise indicated. The fr-1::gfp-3xflag construct containing 21.6 Kbp of the fr-1 5′ UTR and 6 Kbp of the fr-1 3′ UTR was acquired from TransgenOne (construct number: 2127141463160758 F11, Sarov et al. 2012), which was used to generate the transgenic line, MTU10, expressing C-terminal GFP-tagged FZR-1. For the generation of N-terminal GFP-tagged FZR-1 (OC190), we used Gateway cloning (Invitrogen, Carlsbad, CA) to generate the construct. Coding sequence of fr-1 was PCR amplified from the cDNA clone yk1338f2, and cloned into pDONR221 (Invitrogen) and then the resulting entry clone was recombined into pID3.01 (pMS9.3), which is driven by the pie-1 promoter. The transgenes were introduced into worms by standard particle bombardment (Praitt et al. 2001). For embryonic viability and brood size assays, individual L4 animals were transferred to clean plates, and allowed to self-fertilize for 24 hr at the temperatures indicated. For brood size assays, this was repeated until animals no longer produced embryos. Progeny were allowed at least 24 hr to complete embryogenesis before counting the number of progeny. The fr-1(RNAi) experiments were performed by RNAi soaking (Song et al. 2008). To produce dsRNA for RNAi soaking, we amplified a DNA template from the cDNA clone yk1338f2 using the primers 5′-ATGGATGAGCAACCCCGCC-3′ and 5′-GCACTGTACGTAAAGGTAC-3′ that contained a T7 promoter sequence at their 5′ ends. In vitro transcription was performed using the T7-MEGAscript kit (Thermo-Fisher, Hanover park, IL). L4 animals were soaked overnight in M9 buffer containing either 0.1–0.4 μg dsRNA/ml or no dsRNA (control).

Mapping and molecular identification of szy-14

Both szy-14(bs31) and szy-14(bs38) suppressors were previously mapped between dpy-10 and unc-4 on chromosome II as described in Kemp et al. (2007). As szy-14(bs31) appears to be a stronger suppressor (Figure 1B and Table 1, Kemp et al. 2007), we chose to use szy-14(bs31) for further mapping. Because szy-14 mutants show no embryonic lethality, we decided to use the suppression of the zyg-1(it25) embryonic lethality by the szy-14(bs31) mutation for phenotyping. For single-nucleotide polymorphism (SNP) mapping, we mated zyg-1(it25) dpy-10(e128) szy-14(bs31) unc-4(e120) hermaphrodites with Hawaiian CB4856 males as described in Song et al. (2008), and isolated a total of 104 independent Dpy-nonUnc recombinants from the F2 generation. After establishing homozygous recombinant lines, we screened for the fr-1(bs31) presence using the suppression of the zyg-1(it25) lethality at 24°C supplemented by reduced brood size phenotype (Fay et al. 2002). For each phenotyping, we used the following control strains in parallel to accurately score the suppression: zyg-1(it25), zyg-1(it25) dpy-10(e128), zyg-1(it25) dpy-10(e128) szy-14(bs31) unc-4(e120), zyg-1(it25) szy-14(bs31), and zyg-1(it25) szy-14(bs31) unc-4(e120). After careful phenotype examination, we determined 27 (out of 104) of the Dpy-nonUnc recombinants contain the fr-1(it25) mutation, which restores the zyg-1(it25) embryonic lethality, and that 77 (out of 104) of the Dpy-nonUnc recombinants do not contain the fr-1(it25) mutation, causing no suppression of the zyg-1 lethality. Through fine mapping, we narrowed down the szy-14 locus to a region of 57 kb between 9621265 and 9678204 on chromosome II. Then, we continued to molecularly screen for the szy-14 gene by sequencing several candidate genes (nos-3, kin-15, kin-16, wee-1.1, wee-1.3, and fr-1) located within a 57-kb interval on chromosome II. For sequencing the fr-1(it25) gene, we used the following primers: forward 5′-TCTTGTCTTCTGTTGAGGTT-3′ and reverse 5′-ACAGTACTGTAGCCCAA-3′ for the bs31 suppressor, and forward 5′-ATGGATGAGCAAGCACGCC-3′ and reverse 5′-CAAGCTTGACGCTGTG-3′ for the bs38 suppressor. Purified PCR amplicons were sequenced and aligned to the ORF, ZK13076.7 to identify the nucleotide substitution.

CRISPR/CAS-9 mediated genome editing

For genome editing, we used the co-CRISPR technique as previously described in C. elegans (Arribere et al. 2014; Paix et al. 2015). In brief, we microinjected N2 and zyg-1(it25) animals using a mixture containing recombinant SpCas9 (Paix et al. 2015), crRNAs targeting ssr-5 and dpy-10 at 0.4–0.8 μg/μl, tracrRNA at 12 μg/μl, and single-stranded
a great majority of zyg-1(i25) mutant embryos form monopolar spindles (3.3 ± 4.4% bipolar spindles, n = 660). In contrast, bipolar spindle formation is restored in zyg-1(i25) fzr-1(bs31) (79.9 ± 22.0% bipolar spindles, n = 276, P < 0.001) and zyg-1(i25) far-1(bs38) (51.4 ± 24.4%, n = 404, P < 0.001) double mutants. Average values are presented. Error bars represent SD. n is the number of blastomeres. * * * P < 0.001 (two-tailed t-test). (C) Schematic of FZR-1 protein structure illustrates functional domains and the location of the missense mutations: R65C within the C-box in the far-1(bs38) mutant, and C612Y within WD40 domain in the far-1(bs31) mutant allele.

Immunoprecipitation (IP)

Embryos were collected from gravid worms using hypochlorite treatment (1:2:1 ratio of M9 buffer, 5.25% sodium hypochlorite, and 5 M NaCl), washed with M9 buffer five times and frozen in liquid nitrogen. Embryos were stored at −80°C until use. IP experiment using α-GFP were performed following the protocol described previously (Stubenvoll et al. 2016). 20 μl of Mouse-α-GFP magnetic beads (MBL, Naka-ku, Nagoya, Japan) were used per reaction. The α-GFP beads were prepared by washing with 15 min in PBST (PBS, 0.1% Triton-X), followed by a third wash in 1X lysis buffer [50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM MgCl2, 200 mM KCl, and 10% glycerol (v/v)] (Cheeseman et al. 2004). Embryos were suspended in 1X lysis buffer supplemented with complete protease inhibitor cocktail (Roche) and MG132 (Tocris, Avonmouth, Bristol, UK). The embryos were then milled for 3 min at 30 Hz using a Retsch MM 400 mixer-mill (Verder Scientific, Newtown, PA). Lysates were sonicated for 3 min in ice water using an ultrasonic bath (Thermo-Fisher). Samples were spun at 45,000 rpm for 45 min using a Sorvall RC M120EX ultracentrifuge (Thermo-Fisher). The supernatant was transferred to clean microcentrifuge tubes. Protein concentration was quantified using a NanoDrop spectrophotometer (Thermo-Fisher) and equivalent amount of total proteins was used for each reaction. Samples and α-GFP beads were incubated and rotated for 1 hr at 4°C, and then washed five times for 5 min using PBST (PBS + 0.1% Triton-X 100). Samples were resuspended in 20 μl of a solution containing 2× Laemmli Sample
For western blotting, samples were sonicated for 5 min and boiled in a least three times for all experiments and subsequently analyzed for 2.2 zyg-1(or409) fzr-1(RNAi) zyg-1(it25) 22.5 100 4.0 Buffer (Sigma) and 10% β-mercaptoethanol (v/v), then boiled for 5 min before fractionating on a 4–12% NuPAGE Bis-Tris gel (Invitrogen).

Western blotting
For western blotting, samples were sonicated for 5 min and boiled in a solution of 2× Laemmli Sample Buffer and 10% β-mercaptoethanol (v/v), and boiled for 5 min before fractionating on a 4–12% NuPAGE Bis-Tris gel (Invitrogen). The iBlot Gel Transfer system (Invitrogen) was then used to transfer samples to a nitrocellulose membrane. The following antibodies were used at 1:3000–10,000 dilutions: α-Tubulin: α-Tubulin (DM11a; Sigma), α-GFP: IgG1κ (Roche), α-SAS-5 (Song et al. 2011), and α-TBG-1 (Stubenville et al. 2016). IRDye secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used at a 1:10,000 dilution. Blots were imaged using the Odyssey infrared scanner (LI-COR Biosciences), and analyzed using Image Studio software (LI-COR Biosciences).

Statistical analysis
All P-values were calculated using two-tailed t-tests assuming equal variance among sample groups. Statistics are presented as Average ± SD unless otherwise specified. Data were independently replicated at least three times for all experiments and subsequently analyzed for statistical significance.

Data availability
All strains used in this study are available upon request. File S1 contains the following: Figure S1, Centrosome-associated TBG-1 levels are unaffected in fzr-1(bs31) and sas-5KEN-to-3A mutant embryos; Figure S2, Brood size in sas-5KEN-to-3A and fzr-1(bs31) mutants; Figure S3, SAS-5 levels are increased in sas-5KEN-to-3A mutants; Table S1, List of strains used in this study; Table S2, List of oligonucleotides used for CRISPR/Cas9 genome editing.

RESULTS AND DISCUSSION
The szy-14 mutation restores centrosome duplication to zyg-1(it25) mutants
Through a genetic suppressor screen, the szy-14 (suppressor of zyg-1) gene was originally identified that restores embryonic viability of the partial loss-of-function zyg-1(it25) mutant (Kemp et al. 2007). The zyg-1(it25) mutant embryo grown at the restrictive temperature (24°C) fails to duplicate centrosomes during the first cell cycle, resulting in monopolar spindles at the second mitosis and 100% embryonic lethality (O’Connell et al. 2001). A complementation test identified two alleles, szy-14(bs31) and szy-14(bs38), of the szy-14 mutation that partially restore the embryonic viability of zyg-1(it25) but show slow growth phenotype without obvious cytological defects, indicating that the szy-14 gene is not essential for embryonic viability (Table 1, Kemp et al. 2007).

Given that ZYG-1 is essential for proper centrosome duplication (O’Connell et al. 2001), we speculated that the szy-14 mutation might suppress the embryonic lethality of zyg-1(it25) mutants via restoration of centrosome duplication. To examine centrosome duplication events, we quantified the percentage of bipolar spindles at the second mitosis, which indicates successful centrosome duplication during the first cell cycle (Figure 1A and B). At the restrictive temperature 24°C, both double mutant embryos, zyg-1(it25); szy-14(bs31) (79.9 ± 22.0%) and zyg-1(it25); szy-14(bs38) (51.4 ± 24.4%) produced bipolar spindles at a significantly higher rate, compared to zyg-1(it25) single mutant embryos (3.3 ± 4.4%) (Figure 1B). Our observation shows that the szy-14 mutation restores centrosome duplication in zyg-1(it25) embryos, thereby restoring embryonic viability to zyg-1(it25) mutants.

Molecular identification of szy-14
The szy-14 gene was initially mapped to the right arm of chromosome II between the morphological markers dpy-10 and unc-4 (Kemp et al. 2007). Using fine physical mapping, we located szy-14 to an interval of 57 kb (ChrlII: 9621265..9678204; Wormbase.org) that contains several known cell cycle regulators. Based on the genetic map position of the szy-14 suppressor, we sequenced candidate genes within this interval to detect any mutations in szy-14 mutants. Sequencing revealed that szy-14(bs38) mutants contain a single substitution (C-to-T) in exon 2, and szy-14(bs31) mutants carry a mutation (G-to-A) in exon 5 of the ORF of the szy-14 gene. Consistently, inhibiting FZR-1 by RNAi soaking partially restores embryonic viability in both zyg-1(it25) and zyg-1(or409) mutant alleles (Table 1), indicating that loss-of-function of fzf-1 leads to the restoration of embryonic viability to the zyg-1 mutants. Together, we determined that the bs31 and bs38 mutations are alleles of the fzf-1 gene. Hereafter, we refer to szy-14(bs31) and szy-14(bs38) mutants as fzf-1(bs31) and fzf-1(bs38) mutants, respectively.

fzf-1 encodes a conserved coactivator of the anaphase promoting complex/cyclosome (APC/C), the C. elegans homolog of Cdh1/Hct1/Fzr (Schwab et al. 1997; Sigrist and Lehner 1997; Visintin et al. 1997; Fay et al. 2002). The APC/C is an E3 ubiquitin ligase that orchestrates the sequential degradation of key cell cycle regulators during mitosis and early interphase (Song and Rape 2008). As part of this process, specific activators modulate the APC/C activity in different phases of mitosis. Specifically, FZR-1/Cdh1 modulates the APC/C at late mitosis and events in G1 during the time when centrosome duplication occurs.
In each of the *fzr-1* mutant alleles, the single substitution leads to a missense mutation (Figure 1C). The *fzr-1(bs31)* mutation results in a missense mutation (C612Y) within the conserved WD40 repeat domain that is known to be involved in protein–protein interactions and is important for substrate recognition (Kraft et al. 2005; He et al. 2013). The *fzr-1(bs38)* mutation produces a missense mutation (R65C) at the conserved C-box of FZR-1. The C-box is known to be crucial for the physical interaction between FZR-1 and other APC/C subunits (Schwab et al. 2001; Thornton et al. 2006; Chang et al. 2015; Zhang et al. 2016). Thus, both *fzr-1(bs31)* and *fzr-1(bs38)* mutations appear to affect conserved domains that are critical for the function of the APC/C complex, suggesting that FZR-1 might regulate centrosome duplication through the APC/C complex.

**FZR-1 localizes to nuclei and centrosomes during early cell division**

To determine where FZR-1 might function during the early cell cycle, we produced two independent transgenic strains that express FZR-1 tagged with GFP at the N- or C-terminus (see Materials and Methods). To label microtubules, we mated GFP-tagged FZR-1 transgenic animals with the mCherry::β-tubulin expressing line, and performed 4D time-lapse movies to observe subcellular localization of GFP::FZR-1 throughout the first cell cycle (Figure 2A). Confocal imaging illustrates that during interphase and early mitosis, GFP::FZR-1 is highly enriched at the nuclei. After the nuclear envelope breaks down (NEBD), GFP::FZR-1 diffuses to the cytoplasm and reappears to the nuclei at late mitosis when the nuclear envelop reforms. After NEBD, GFP::FZR-1 becomes apparent at spindle microtubules, and centrosomes that colocalize with SPD-2, a centrosome protein (Figure 2B). Both GFP-tagged FZR-1 transgenic embryos exhibit similar subcellular distributions, except a slight difference in fluorescent intensity (data not shown). While we do not exclude the possibility that FZR-1 functions in the cytoplasm to regulate cellular levels of centrosome factors, our observations suggest that *C. elegans* FZR-1 might direct APC/C activity at centrosomes during late mitosis in early embryos, which is consistent with the role of FZR-1 as the coactivator of the APC/C at late mitosis in other organisms (Raff et al. 2002; Zhou et al. 2003; Meghini et al. 2016).

**FZR-1 might function as a part of the APC/C complex to regulate centrosome duplication**

Given that FZR-1 is a conserved coactivator of the APC/C, an E3 ubiquitin ligase, we hypothesized that FZR-1 functions as a part of the APC/C complex in centrosome assembly. If so, depleting other APC/C subunits should have a similar effect that loss of FZR-1 had on the *zyg-1(it25)* mutant. To examine how other core subunits of the APC/C complex might affect *zyg-1(it25)* mutants, we mated the *zyg-1(it25)* strain with *mat-3(or180)* mutants for the core APC8/CDC23 subunit (Golden et al. 2000), and *emb-1(hc57)* mutants for the conserved subunit APC16 in the *C. elegans* APC/C complex (Kops et al. 2010; Green et al. 2011; Shakes et al. 2011). By generating double homozygote mutants, we assayed for bipolar spindle formation and embryonic viability in *zyg-1(it25); mat-3(or180)* and *zyg-1(it25); emb-1(hc57)* double homozygous mutants (Figure 3 and Table 1). At the restrictive temperature 24°C, *zyg-1(it25); mat-3(or180)* double-mutant embryos exhibit a ninefold increase in bipolar spindle formation (81.8 ± 14.3%), compared to *zyg-1(it25)* single mutant embryos (9.1 ± 8.8%) during the second mitosis (Figure 3A). Consistently, 5% of *zyg-1(it25)* single mutant embryos die at 24°C (Table 1). In support of our results, the *mat-3(bs29)* allele has been reported as a genetic suppressor of *zyg-1* (Miller et al. 2016). This result also indicates that the *zyg-1(it25)* mutation partially restores embryonic viability of *mat-3(or180)* mutants, suggesting a mutual suppression between *zyg-1* and *mat-3*. Furthermore, we observed that the *emb-1* mutation suppresses the centrosome duplication phenotype of *zyg-1(it25)* mutants at the semi-restrictive temperature 22.5°C. While 45.5 ± 11.9% of *zyg-1(it25)* embryos form bipolar spindles, 79.1 ± 12.4% of *zyg-1(it25); emb-1(hc57)* double-mutant embryos produce bipolar spindles (Figure 3A). We, however, observed no significant restoration of embryonic viability in *zyg-1(it25); emb-1(hc57)* double mutants (P = 0.691) compared to *zyg-1(it25)* single mutants (Table 1), presumably due to the strong embryonic lethality by the *emb-1(hc57)* mutation itself (Kops et al. 2010; Shakes et al. 2011). Our results indicate that loss of function mutations affecting the APC/C complex suppress the phenotype of *zyg-1(it25)* mutants. Therefore, FZR-1 might function as a component of the APC/C complex to regulate centrosome duplication in early *C. elegans* embryos.

**Loss of FZR-1 results in elevated SAS-5 levels**

Next, we wanted to understand how FZR-1 contributes to centrosome duplication. Since FZR-1 appears to function through the APC/C complex in centrosome assembly, we hypothesized that the APC/C/FZR-1 specifically targets one or more centrosome regulators for ubiquitin-mediated degradation. If that is the case, depleting FZR-1 should protect substrates from degradation leading to accumulation of target proteins. To identify a direct substrate of APC/C/FZR-1 that regulates...
to zyg-1(it25) single mutants (45.5 ± 11.9%, n = 238). n is the number of blastomeres. * P < 0.05, ** P < 0.001 (two-tailed t-test). (B) Still images of embryos expressing GFP::β-tubulin, mCherry::γ-tubulin (centrosome marker) and mCherry::histone raised at 24° illustrate monopolar spindle formation in the zyg-1(it25) embryo, and bipolar spindle formation in the zyg-1(it25); mat-3(or180) double-mutant embryo. Bar, 5 μm.

Protein stabilization by the fcr-1 mutation might lead to increased levels of a centrosome-associated substrate, which may compensate for impaired ZYG-1 function at the centrosome. In C. elegans, SAS-5 is the only core centrosome duplication factor containing a KEN-box, which suggests SAS-5 as a potential target of the APC/C^{FZR-1}. If the APC/C^{FZR-1} targets SAS-5 directly through KEN-box-mediated proteolysis, inhibiting FZR-1 should protect SAS-5 from degradation leading to SAS-5 accumulation. To examine how the fcr-1 mutation affects SAS-5 stability, we immunostained embryos with anti-SAS-5, and quantified the fluorescence intensity of centrosome-associated SAS-5 (Figure 4, A and B). As ZYG-1 is required for SAS-5 localization to centrosomes, hyper-accumulation of SAS-5 might compensate for partial loss-of-function of ZYG-1, thereby restoring centrosome duplication to zyg-1(it25) mutants. In fact, our quantitative immunofluorescence revealed that fcr-1(bs31) embryos exhibit a significant increase (1.41 ± 0.42 fold; P < 0.001) in centrosomal SAS-5 levels at the first anaphase, compared to wild-type (Figure 4B). Consistently, compared to zyg-1(it25) single mutants, zyg-1(it25); fcr-1(bs31) double mutant embryos exhibit a 1.48-fold increase (P < 0.001) in centrosome-associated SAS-5 levels (Figure 4B). Indeed, centrosomal SAS-5 are restored to near wild-type levels in zyg-1(it25); fcr-1(bs31) double mutants (0.95 ± 0.44 fold; P = 0.003). We, however, observed no significant changes in centrosomal TBG-1 (γ-tubulin) levels in fcr-1(bs31) mutants (Figure S1 in File S1).

Elevated protein levels might influence centrosome-associated SAS-5 levels in fcr-1(bs31) mutants. To determine how inhibition of the APC/C^{FZR-1} affected overall protein levels, we performed quantitative western blot analysis using embryonic protein lysates and antibodies against centrosome proteins (Figure 4C). Our data indicate that fcr-1(bs31) embryos possess increased SAS-5 levels (∼1.5-fold), relative to wild-type embryos, while the levels of SAS-6 and TBG-1 are not significantly affected in fcr-1(bs31) mutants (Figure 4C). This observation on the SAS-6 levels in fcr-1(bs31) mutants is consistent with previous work by Miller et al. (2016), showing no increase in SAS-6 levels by the mat-3(bs29)/APC8 mutation that inhibits the APC/C function. These results suggest that C. elegans utilizes a different mechanism to control SAS-6 levels, unlike Human SAS-6, which is regulated by the APC/C-mediated proteolysis (Strnad et al. 2007). Furthermore, our immunoprecipitation suggests a physical interaction between SAS-5 and FZR-1 in C. elegans embryos (Figure 4D), supporting that SAS-5 might be a direct substrate of the APC/C^{FZR-1}. Consistent with our results in this study, prior study has shown that inhibiting the 26S proteasome leads to increased levels of SAS-5 (Song et al. 2011). Thus, SAS-5 levels are likely to be controlled through the ubiquitin-proteasome system.

Collectively, our data show that the fcr-1 mutation leads to a significant increase in both cellular and centrosomal levels of SAS-5, suggesting that the APC/C^{FZR-1} might control SAS-5 levels via ubiquitin-mediated proteasomal degradation to regulate centrosome assembly in the C. elegans embryo.

**Mutation of the KEN-box stabilizes SAS-5**

If the APC/C^{FZR-1} directly targets substrates for destruction via the conserved KEN-box, mutating this motif should cause substantial resistance to ubiquitination-mediated degradation. To determine whether the APC/C^{FZR-1} targets SAS-5 through the KEN-box motif, we mutated the KEN-box at the endogenous sas-5 locus. By using CRISPR/CAS-9 mediated genome editing (Paix et al. 2015), we generated mutant lines (sas-5^KEN-to-3A) carrying alanine substitutions of the SAS-5 KEN-box (Figure 5A). The sas-5^KEN-to-3A mutant embryo exhibits no obvious cell cycle defects or embryonic lethality (Table 1), consistent with fcr-1 mutants (Kemp et al. 2007). sas-5^KEN-to-3A animals exhibit a slightly reduced (∼80%) and irregular distribution of brood size within the population (Figure S2 in File S1). Reduced brood size and slow growth phenotypes were previously reported in fcr-1 mutant alleles (Fay et al. 2002; Kemp et al. 2007).

Next, we asked how the sas-5^KEN-to-3A mutation affected zyg-1(it25) mutants. If the APC/C^{FZR-1}-mediated proteolysis of SAS-5 accounts for the suppression of zyg-1, sas-5^KEN-to-3A mutants should mimic the fcr-1 mutation that suppresses zyg-1 mutants. By mating the sas-5^KEN-to-3A mutant with zyg-1(it25) animals, we tested whether the sas-5^KEN-to-3A mutation could genetically suppress zyg-1 mutants, by assaying for embryonic viability and centrosome duplication (Figure 5B and Table 1).
For the zyg-1(it25) mutant control in this experiment, we used the strain MTU14 [zyg-1(it25); sas-5KEN-to-KEN, Table S1 in File S1] that contains the equivalent modifications, except the KEN-box, to the sas-5KEN-to-3A mutation (Figure 5A, see Materials and Methods). At the semirestrictive temperature 22.5°C, zyg-1(it25); sas-5KEN-to-3A animals lead to a 7.7-fold increase in the frequency of viable progeny (35.3 ± 9.2%; P < 0.0001, compared to zyg-1(it25); sas-5KEN-to-KEN mutant controls (4.6 ± 4.0%) (Table 1). Consistently, zyg-1(it25); sas-5KEN-to-3A embryos exhibit successful bipolar spindle assembly at a significantly higher rate (67.5 ± 16.3%; P = 0.02) than zyg-1(it25); sas-5KEN-to-KEN embryos (35.1 ± 10.7%) at the two-cell stage (Figure 5B). These results suggest that the sas-5KEN-to-3A mutation does partially restore embryonic viability and centrosome duplication to zyg-1(it25) mutants at 22.5°C. However, at the restrictive temperature (24°C), where the fzr-1 mutation shows a strong suppression (Figure 1B and Table 1), both zyg-1(it25); sas-5KEN-to-3A double mutants and zyg-1(it25); sas-5KEN-to-KEN mutant animals result in 100% embryonic lethality (Table 1). zyg-1(it25); sas-5KEN-to-3A embryos (14.7% bipolar, n = 68) grown at 24°C show only minor effect on centrosome duplication compared to zyg-1(it25); sas-5KEN-to-KEN control embryos (7.6% bipolar, n = 66). The data obtained at 24°C reveal that the sas-5KEN-to-3A mutation results in much weaker suppression to zyg-1(it25) mutants than the fzr-1 mutation, suggesting that the SAS-5 KEN-box mutation does not generate the equivalent impact that results from the fzr-1 mutation. If SAS-5 is the only APC/C^CZFR-1 substrate that contributes to the suppression of zyg-1 mutants, the fzr-1 or KEN-box mutation might influence SAS-5 stability differently. In this scenario, FZR-1 might target SAS-5 through KEN-box and additional recognition motifs (e.g., D-box), causing a greater effect on SAS-5 stability than the KEN-box mutation alone. To examine how the KEN-box mutation affected SAS-5 stability, we measured the fluorescence intensity of SAS-5 at centrosomes by quantitative immunofluorescence (Figure 5, C and D). At 22.5°C, where the sas-5KEN-to-3A mutation restores centrosome duplication and embryonic viability to zyg-1(it25), sas-5KEN-to-3A mutants exhibit a significant increase in centrosome-associated SAS-5 levels (~1.5-fold, P < 0.001), compared to wild-type (Figure 5, C and D). Consistently, zyg-1(it25); sas-5KEN-to-3A embryos display ~1.4-fold (P = 0.002) increased SAS-5 levels at centrosomes, compared to zyg-1(it25); sas-5KEN-to-KEN control embryos that contain reduced centrosomal SAS-5 levels (Figure 5D). Notably, zyg-1(it25); sas-5KEN-to-3A embryos exhibit centrosomal SAS-5 levels nearly equivalent (~0.97-fold) to those of wild-type embryos (Figure 5D). As a control, we also quantified centrosomal TBG-1 levels, but saw no changes between sas-5KEN-to-3A mutants and the wild type (Figure S1 in File S1). Furthermore, we examined overall SAS-5 levels by quantitative western blot, finding that relative to wild-type embryos, sas-5KEN-to-3A mutant embryos possess ~1.5-fold increased SAS-5 levels (Figure S3 in File S1). Together, our quantification data reveal that the sas-5KEN-to-3A or fzr-1 mutation leads to nearly equivalent fold change (~1.5-fold) in both cellular and centrosome-associated SAS-5 levels (Figure 4, B and C, Figure 5D, and Figure S3 in File S1). Together, these results suggest that APC/C^CZFR-1 directly targets SAS-5 in a KEN-box-dependent manner to control SAS-5 turnover, and that SAS-5 stabilization by blocking proteolysis results in elevated SAS-5 levels at the centrosome, partially contributing to the suppression of the zyg-1(it25) mutation. In human cells, APC/ C_Cdh1 recognizes a KEN-box to regulate the levels of STIL, the homolog of C. elegans SAS-5, and STIL deleted of the KEN-box leads to accumulation of STIL protein, and centrosome amplification (Arquint and Nigg 2014). While we do not observe extra centrosomes by the SAS-5 KEN-box mutation, our data show that that APC/C^CZFR-1 controls SAS-5 stability via the direct recognition of the conserved degron motif, KEN-box, to regulate centrosome duplication in C. elegans embryos, suggesting a conserved mechanism for regulating SAS-5 levels between humans and nematodes.
Interestingly, although either inhibiting FZR-1 or mutating KEN-box influences SAS-5 stability at a comparable level, we observe a notable difference in the suppression level of these two mutations. Weaker suppression by the sas-5KEN-to-3A mutation suggests that the APC/C\(^{\text{FZR-1}}\) might target additional substrates that cooperatively support the zyg-1 suppression. In this scenario, APC/C\(^{\text{FZR-1}}\) might target other centrosome proteins outside core duplication factors through the conserved degron motifs, such as destruction (D)-box and KEN-box (Glotzer et al. 1991; Pfleger and Kirschner 2000). Alternatively, APC/C\(^{\text{FZR-1}}\) might target additional core centrosome factors through other recognition motifs other than KEN-box, such as D-box (Glotzer et al. 1991) or unknown motif in the C. elegans system. In humans and flies, APC/C\(^{\text{Cdh1/Fzr}}\) has been shown to regulate the levels of STIL/SAS-5, Spd2, HisSAS-6, and CPAP/SAS-4 (Strnad et al. 2007; Tang et al. 2009; Arquint and Nigg 2014; Meghini et al. 2016). While C. elegans homologs of these factors, except SAS-5, lack a KEN-box, all five centrosome proteins contain at least one putative D-box. An intriguing possibility, given the strong genetic interaction observed between fzl-1 and zyg-1, is that ZYG-1 could be a novel substrate of APC/C\(^{\text{FZR-1}}\). Additional work will be required to understand the complete mechanism of APC/C\(^{\text{FZR-1}}\)-dependent regulation of centrosome duplication in C. elegans. In summary, our study shows the APC/C\(^{\text{FZR-1}}\)-dependent proteolysis of SAS-5 partially contributes to the suppression of the zyg-1 mutants, and we report that FZR-1 functions as a negative regulator of centrosome duplication in C. elegans.

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LITERATURE CITED

Acquaviva, C., and J. Pines, 2006 The anaphase-promoting complex/ cyclosome. APC/C. J. Cell Sci. 119: 2401–2404.

Arquint, C., and E. A. Nigg, 2014 STIL microcephaly mutations interfere with APC/C-mediated degradation and cause centriole amplification. Curr. Biol. 24: 351–360.

Arquint, C., K. F. Sonnen, Y. D. Stierhof, and E. A. Nigg, 2012 Cell-cycle-regulated expression of STIL controls centriole number in human cells. J. Cell Sci. 125: 1342–1352.

Arribere, J. A., R. T. Bell, B. X. Fu, K. L. Artiles, P. S. Hartman et al., 2014 Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. Genetics 198: 837–846.

Brenner, S., 1974 The genetics of Caenorhabditis elegans. Genetics 77: 71–94.

Brownlee, C. W., J. E. Klebb, D. W. Buster, and C. G. Rogers, 2011 The protein phosphatase 2A regulatory subunit twins stabilizes Plk4 to induce centriole amplification. J. Cell Biol. 195: 231–243.

Carter, S. L., A. C. Eklund, I. S. Kohane, L. N. Harris, and Z. Szallasi, 2006 A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat. Genet. 38: 1043–1048.

Chang, L., and D. Barford, 2014 Insights into the anaphase-promoting complex: a molecular machine that regulates mitosis. Curr. Opin. Struct. Biol. 29: 1–9.

Chang, Z., Z. Zhang, J. Yang, S. H. McLaughlin, and D. Barford, 2015 Atomic structure of the APC/C and its mechanism of protein ubiquitination. Nature 522: 450–454.

Cheeseman, I. M., S. Niessen, S. Anderson, F. Hyndman, J. R. Yates, III et al., 2004 A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. Genes Dev. 18: 2255–2268.

Church, D. L., K. L. Guan, and E. J. Lambie, 1995 Three genes of the MAP kinase cascade, mek-2, mpk-1/sur-1 and let-60 ras, are required for mitotic cell cycle progression in Caenorhabditis elegans. Development 121: 2525–2535.

Dammernann, A., T. Müller-Reichert, L. Pelletier, B. Habermann, A. Desai et al., 2004 Centriole assembly requires both centriolar and pericentriolar material proteins. Dev. Cell 7: 815–829.

Dawson, I. A., S. Roth, and S. Artavanis-Tsakonas, 1995 The Drosofila cell cycle gene fzzy is required for normal degradation of cyclins A and B during mitosis and has homology to the CDC20 gene of Saccharomyces cerevisiae. J. Cell Biol. 129: 725–737.

Delattre, M., S. Leidel, K. Wani, K. Baumer, J. Bamat et al., 2004 Centriolar SAS-5 is required for centrosome duplication in C. elegans. Nat. Cell Biol. 6: 656–664.

Delattre, M., G. Canard, and P. Gόnczy, 2006 Sequential protein recruitment in C. elegans centriole formation. Curr. Biol. 16: 1844–1849.

Fang, G., H. Yu, and M. W. Kirschner, 1998 The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. Cell Res. 26: 869–885.

Green, R. A., H. L. Kao, A. Audhya, S. Arur, J. R. Mayers et al., 2011 A high-resolution C. elegans essential gene network based on phenotypic profiling of a complex tissue. Cell 145: 470–482.

Habedank, R., Y. D. Stierhof, C. J. Wilkinson, and E. A. Nigg, 2005 The Polo kinase Plk4 functions in centriole duplication. Nat. Cell Biol. 7: 1140–1146.

Hartwell, L. H., and D. Smith, 1985 Altered fidelity of mitotic chromosome transmission in cell cycle mutants of S. cerevisiae. Genetics 110: 381–395.

He, J., W. C. Chao, Z. Zhang, J. Yang, N. Cronin et al., 2013 Insights into degron recognition by APC/C coactivators from the structure of an Acm1–Cdh1 complex. Mol. Cell 50: 649–660.

Holland, A. J., W. Lan, S. Niessen, H. Hoover, and D. W. Cleveland, 2010 Polo-like kinase 4 kinase activity limits centrosome overduplication by autoregulating its own stability. J. Cell Biol. 188: 191–198.

Irimmer, S., and K. Nasmyth, 1997 The anaphase-promoting complex is required in G1 arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase. J. Cell Sci. 110: 1523–1531.

Kemp, C. A., K. R. Kopish, P. Zipperlen, J. Ahringer, and K. F. O’Connell, 2004 Centrosome maturation and duplication in C. elegans require the coiled-coil protein SPD-2. Dev. Cell 6: 511–523.

Kemp, C. A., M. H. Song, M. K. Addepalli, G. Hunter, and K. O’Connell, 2007 Suppressors of zyg-1 define regulators of centrosome duplication and nuclear association in Caenorhabditis elegans. Genetics 176: 95–113.

Kirkham, M., T. Müller-Reichert, K. Oegema, S. Grill, and A. A. Hyman, 2003 SAS-4 is a C. elegans centriolar protein that controls centrosome size. Cell 112: 575–587.

Kitagawa, R., E. Law, L. Tang, and A. M. Rose, 2002 The Cdc20 homolog, FYZ-1, and its interacting protein, IFY-1, are required for proper chromosome segregation in Caenorhabditis elegans. Curr. Biol. 12: 2118–2123.

Kleylein-Sohn, J., J. Westendorf, M. Le Clech, R. Habedank, Y. D. Stierhof et al., 2007 Plk4-induced centriole biogenesis in human cells. Dev. Cell 13: 190–202.

Kops, G. J., M. van der Voet, M. S. Manak, M. H. van Osch, S. M. Naini et al., 2010 APC16 is a conserved subunit of the anaphase-promoting complex/cyclosome. J. Cell Sci. 123: 1623–1633.

Kraft, C. H., C. Vodernaier, S. Maurer-Stroh, F. Eisenhaber, and J. M. Peters, 2005 The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. Mol. Cell 18: 543–553.

Kumar, A., S. C. Girimaji, M. R. Duvvari, and S. H. Blanton, 2009 Mutations in STIL, encoding a pericentriolar and centrosomal protein, cause primary microcephaly. Am. J. Hum. Genet. 84: 286–290.

Leidel, S., and P. Gόnczy, 2003 SAS-4 is essential for centrosome duplication in C. elegans and is recruited to daughter centrioles once per cell cycle. Dev. Cell 4: 431–439.

Leidel, S., M. Delattre, L. Cerutti, K. Baumer, and P. Gόnczy, 2005 SAS-6 defines a protein family required for centrosome duplication in C. elegans and in human cells. Nat. Cell Biol. 7: 115–125.

Levine, M. S., B. Bakker, B. Boecka, J. Moyett, J. Lu et al., 2017 Centrosome amplification is sufficient to promote spontaneous tumorigenesis in mammals. Dev. Cell 40: 313–322.e5.

Livneh, Y., I. Cohen-Kaplán, C. Cohen-Rosenzweig, N. Avni, and A. Ciechanover, 2016 The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. Cell Res. 26: 869–885.

Medley, J. C., M. M. Kabara, M. D. Stubvenvoll, L. E. DeMeyer, and M. H. Song, 2017 Casein kinase II is required for proper cell division and acts as a negative regulator of centrosome duplication in Caenorhabditis elegans embryos. Biol. Open 6: 17–28.

Meghini, F., T. Martins, X. Tait, K. Fujimoto, H. Yamano et al., 2016 Targeting of Fzr/Cdh1 for timely activation of the APC/C at the centrosome during mitotic exit. Nat. Commun. 7: 12607.

Miller, J. G., Y. Liu, C. W. Williams, H. E. Smith, and K. F. O’Connell, 2016 The EZF–DP1 transcription factor complex regulates centriole duplication in Caenorhabditis elegans. G3 6: 709–720.

Nigg, E. A., and T. Stourns, 2011 The centrosome cycle: centrosome duplication, and inheritance asymmetries. Nat. Cell Biol. 13: 1154–1160.
