Tissue-Specific DNA Replication Defects in *Drosophila melanogaster* Caused by a Meier-Gorlin Syndrome Mutation in Orc4

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ABSTRACT Meier-Gorlin syndrome is a rare recessive disorder characterized by a number of distinct tissue-specific developmental defects. Genes encoding members of the origin recognition complex (ORC) and additional proteins essential for DNA replication (CDC6, CDT1, GMNN, CDC45, MCM5, and DONSON) are mutated in individuals diagnosed with MGS. The essential role of ORC is to license origins during the G1 phase of the cell cycle, but ORC has also been implicated in several nonreplicative functions. Because of its essential role in DNA replication, ORC is required for every cell division during development. Thus, it is unclear how the Meier-Gorlin syndrome mutations in genes encoding ORC lead to the tissue-specific defects associated with the disease. To begin to address these issues, we used Cas9-mediated genome engineering to generate a *Drosophila melanogaster* model of individuals carrying a specific Meier-Gorlin syndrome mutation in *ORC4* along with control strains. Together these strains provide the first metazoan model for an MGS mutation in which the mutation was engineered at the endogenous locus along with precisely defined control strains. Flies homozygous for the engineered MGS allele reach adulthood, but with several tissue-specific defects. Genetic analysis revealed that this *Orc4* allele was a hypomorph. Mutant females were sterile, and phenotypic analyses suggested that defects in DNA replication was an underlying cause. By leveraging the well-studied *Drosophila* system, we provide evidence that a disease-causing mutation in *Orc4* disrupts DNA replication, and we propose that in individuals with MGS defects arise preferentially in tissues with a high-replication demand.

KEYWORDS *Drosophila*; Meier-Gorlin syndrome; Disease Model; DNA Replication

MEIER-GORLIN syndrome (MGS) is a rare developmental disorder. Individuals with MGS present with several tissue-specific developmental defects, including primordial dwarfism, small or missing patella, and small ears (Bicknell *et al.* 2011b; Guernsey *et al.* 2011; de Munnik *et al.* 2012). A significant number of patients also present with microcephaly, although typically have normal cognitive function (de Munnik *et al.* 2012). The first case of MGS was reported in 1959 (Meier *et al.* 1959), with a second case following in 1975 (Gorlin *et al.* 1975), but the underlying genetic cause of the disease was unknown. Advances in next-generation sequencing enabled the identification of mutations causing MGS. The identified mutations are in a set of genes essential for the function of DNA replication origins, the chromosomal positions required for the initiation of DNA replication, (*ORC1*, *ORC4*, *ORC6*, *CDT1*, *CDC6*, *GMNN*, *CDC45*, and *MCM5*) or replication fork progression (*CDC45*, *MCM5*, and *DONSON*) (Bicknell *et al.* 2011b; Guernsey *et al.* 2011; de Munnik *et al.* 2012; Burrage *et al.* 2015; Fenwick *et al.* 2016; Evrony *et al.* 2017; Reynolds *et al.* 2017; Vetro *et al.* 2017). The surprising discovery that defects in essential DNA replication proteins underlie a disease characterized by highly specific tissue defects raises important questions about how a fundamental process essential for all cells can differentially affect the development of particular tissues. Establishing

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controlled models in experimentally facile metazoans is an important step toward addressing these questions.

The average human undergoes 10^{16} cell divisions in a lifetime, and every cell division requires faithful duplication of the genome. Genome duplication begins at multiple individual DNA replication origins that are formed in a cell-cycle regulated process requiring many proteins that are conserved throughout eukaryotic organisms (Remus and Diffley 2009). In G1 phase, origins are selected by the binding of the origin recognition complex (ORC) comprised of six subunits (Orc1, Orc2, Orc3, Orc4, Orc5, and Orc6). ORC recruits Cdc6 and together this complex recruits the Cdt1 chaperone bound to the MCM hexamer, the core component of the replicative helicase. In an ATP-dependent process, an MCM complex, comprised of two head-to-head hexamers, assembles onto the double-stranded origin DNA, "licensing" the origin (Stillman 2005; Sclafani and Holzen 2007; Remus *et al.* 2009). In S phase, multiple proteins, including S phase kinases and the MCM helicase accessory factors Cdc45 and GINS, convert the MCM complex into two active replicative helicases, culminating in the initiation of DNA replication (origin function) (Moyer *et al.* 2006; Ilves *et al.* 2010). In most cell divisions, the genome must be replicated exactly once, and the cell-cycle separation of origin licensing (G1) and origin activation (S) ensures that only one complete round of genome duplication occurs per cell division (Diffley 2011). In addition to this standard form of cell division, as part of their normal differentiation some cell types undergo multiple rounds of genome duplication to generate polyploid cells (Lee *et al.* 2009). Both types of cell divisions depend on the same proteins for origin function.

As expected, based on the requirement for origin licensing for every cell division, null mutations in genes encoding origin-regulatory proteins, including ORC, are lethal (Bell *et al.* 1993; Micklem *et al.* 1993; Landis *et al.* 1997; Pinto *et al.* 1999; Pflumm and Botchan 2001; Park and Asano 2008; Shu *et al.* 2008; Baldinger and Gossen 2009; Balasov *et al.* 2009; Guernsey *et al.* 2011; Okano-Uchida *et al.* 2018). Thus, mutations in the genes that underlie MGS must either be hypomorphic for their DNA-replication functions or affect undefined, nonessential roles. Because origin function is essential in every cell division, it is unclear how MGS mutations that affect origin licensing result in tissue-specific defects. Although ORC is essential for origin licensing, individual ORC subunits also function in other biological processes, such as heterochromatin formation (Prasanth *et al.* 2010) and cilia development (Hossain and Stillman 2012; Stiff *et al.* 2013). Thus, it is possible that replication-independent defects in ORC function drive some or all of the MGS developmental phenotypes. However, the continuing identification of point mutations in many different genes encoding replication proteins that cause MGS, including *DONSON*, which affects replication fork progression, supports an underlying role for DNA-replication defects in the etiology of MGS (Bandura *et al.* 2005; Evrony *et al.* 2017; Reynolds *et al.* 2017).

To probe the molecular mechanism underlying MGS mutations, MGS models have been generated in several organisms. In particular, the Orc4 MGS mutation has been generated in *Saccharomyces cerevisiae* (Sanchez *et al.* 2017). Yeast with this Orc4 (Y232C) substitution grow slowly and show substantial defects in replicating and retaining the high-copy number of ribosomal DNA (rDNA) repeats. This observation is consistent with $orc4^{Y232C}$ functioning as a hypomorphic replication allele because the yeast rDNA array, which normally contains 100 of copies of the 9 kb rDNA locus each with its own origin, is particularly sensitive to defects in origin-regulatory proteins (Ide *et al.* 2007; Kwan *et al.* 2013; Salim *et al.* 2017). Nonetheless, additional, replication-independent effects may result in the slow-growth phenotype (Sanchez *et al.* 2017). In *Drosophila*, a transgenic system has been used to model MGS mutations in Orc6 (Bleichert *et al.* 2013; Balasov *et al.* 2015). These flies have several tissue-specific defects, and biochemical analysis provides evidence that these phenotypes result from a destabilization of ORC, which in turn reduces MCM loading (Bleichert *et al.* 2013). Replication defects are also evident in cultured cells derived from MGS patients with multiple different *ORC1* alleles (Hossain and Stillman 2012). However, in contrast to the replication defects identified in yeast and flies, cell culture and zebrafish models for Orc1 mutants show defects in cilia development, and these defects in turn may generate the various morphological phenotypes observed in transgenic fish models. (Bicknell *et al.* 2011b; Hossain and Stillman 2012; Yao *et al.* 2017; Maerz *et al.* 2019). Although these models provide insights into this pleotropic disease, they reveal the challenges of discerning the molecular causes of tissue-specific defects even when the relevant proteins have been studied deeply at the biochemical level. Thus, it remains unclear whether the different cellular defects identified reflect differences in the underlying causes of MGS-associated phenotypes or whether they reflect differences in how the mutations were modeled. Finally, it is important to note that the metazoan models to date rely on exogenous expression of the mutant protein and therefore do not precisely mimic the conditions observed in MGS individuals.

To reveal insights into how the MGS mutation in *ORC4* results in tissue-specific defects, we used Cas9 genome-editing to generate a *Drosophila* model of MGS. The existence of a single identified MGS mutation in a highly conserved region of human Orc4 (Y174C), facilitated the generation of a *Drosophila* model for this mutation ($Orc4^{Y162C}$). Similar to MGS individuals, flies homozygous for this mutation were viable and reached adulthood with a number of tissue-specific developmental defects. Our creation of wild-type control and a null-mutant strains enabled us to demonstrate that the MGS mutation was a hypomorphic allele, and to identify specific tissues that were particularly sensitive to this mutation in *Orc4*. We showed that females homozygous for $Orc4^{Y162C}$ were sterile, with decreased numbers of germline nurse cells as well as defects in chorion gene amplification in somatic follicle cells, a feature shared with other hypomorphic alleles in genes essential for DNA replication. We also identified

bristle phenotypes that may be distinctive to MGS alleles, as we did not identify a similar phenotype in flies homozygous for hypomorphic alleles in other replication components. Together our data suggest that the tissue-specific defects identified in patients with MGS may result from cells within these tissues having a high-replication demand that cannot be met by the MGS mutant replication factors. Further, this work demonstrates the power of genome-editing in the fly to model human disease.

Materials and Methods

Fly lines and husbandry

Flies were grown on standard molasses food at 25°. Fly lines used in this study were as follows: *Orc4^{WT}* (this study); *Orc4^{Y162C}* (this study); *Orc^{null}* (this study); *Mcm6^{K1214} v[24]/FM3* [Bloomington *Drosophila* Stock Center (BDSC) #4322]; *w^{1118};Df(2R)BSC856/CyO* (BDSC #27927); *hsFlp122;Sp/SM6-TM6b*, *FRTG13 Orc4^{Y162C}/CyO* (this study); and *P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=ovo^{D1-18}}2R/T(1;2)OR64/CyO* (BDSC #4434).

Generation of Orc4^{WT} and Orc4^{Y162C} fly lines

Single-stranded donor oligonucleotides (ssODNs) were generated to target the region of *Orc4* encoding Y162. Each ssODN had silent mutations to mutate the protospacer adjacent motif site and generate a novel *Nde*I cut site for molecular screening. The *Orc4^{Y162C}* ssODN also contained the necessary alterations to create the Y162C mutation. The *Orc4^{WT}* ssODN did not contain this mutation. Guide RNA plasmids and ssODNs were mixed, injected, and screened as in Hamm *et al.* (2017). Injections were done by Best Gene Inc.

Orc4^{WT} ssODN:

AAGAGAAAGACCTGCCGGTGCGAGAAACGCGACTTGACC
CGCTTCTCCAGCAGCTCGATCACGTCGAGGCGACAGGTA
ACGCCAAGTACACATATGGGCGCCTGGGCTACTGGGAG
ACGTCGAAGAGGTTGTAAAGCAGGGTCTGGTTGTGGTG
AGCACAGAAGAGGTCGAACTCCTCGAGAAT

Orc4^{Y162C} ssODN:

AAGAGAAAGACCTGCCGGTGCGAGAAACGCGACTTGACC
CGCTTCTCCAGCAGCTCGATCACGTCGAGGCGACAGGT
AACGCCAAGTACACATATGGGCGCCTGGGCTACTGGGA
GACGTCGAAGAGGTTGCAAAGCAGGGTCTGGTTGTGG
TGAGCACAGAAGAGGTCGAACTCCTCGAGAAT

Forward screening primer: GAAGTCCATCACTGTGCAGAT

Reverse screening primer: TGGTTGCGGGAGAAGTAAAG

Viability assays

Three to five heterozygous males and five to 10 heterozygous females of the indicated genotypes were mated in standard molasses vials with dry yeast and flipped twice at 2-day intervals. Two days after the final flip, the adult flies were cleared from the vials and their progeny were allowed to reach adulthood. Over 800 adults were counted for each cross. The ratio of CyO and non-CyO adults was determined and the χ^2 value was calculated for each cross, correcting for the observed ratio from the *Orc4^{WT}/CyO* self-cross.

Adult phenotyping

Adult flies from the indicated genotypes were imaged on a Nikon SMZ745 dissection microscope (back bristles) or frozen at −20° and then imaged on a Zeiss Axioplan2 epifluorescence microscope (wing bristles).

Ovary 4′,6-diamidino-2-phenylindole staining

Females of the indicated genotypes were mixed with males in molasses vials with a small amount of yeast paste and grown for 2 days. Flies were flipped into a fresh vial after 24 hr. Ovaries were dissected into Grace's medium. The media was removed, and the ovaries were resuspended in 0.5 ml of fix solution (4% formaldehyde in 1 × PBS) and incubated for 15 min at room temperature, while rocking. The ovaries were washed twice with 1 ml of PBST (1 × PBS + 0.2% Triton X-100) and then washed for 5 min with 1 × PBS to remove the detergent. Ovaries were then incubated with 1 × PBS + 4′,6-diamidino-2-phenylindole (DAPI; 1:1000) for 15 min and then mounted on a slide, covered with a coverslip, and sealed with clear nail polish. Ovaries were imaged on Zeiss Axioplan2 epifluorescence microscope.

Nurse cell counts

DAPI-stained ovaries from the indicated genotypes were imaged on a Zeiss Axioplan2 epifluorescence microscope, and the nurse cells were counted. Nurse cells from 50 to 100 stage 10 egg chambers were counted for each genotype.

5-Ethynyl-2′-deoxyuridine assay

Ovaries from 10 females were dissected as described above and resuspended in 100 μl of Grace's media. Then, 100 μl of 2 × 5-ethynyl-2′-deoxyuridine (EdU) in Grace's media (15 μM) stock solution was added to each sample and incubated for 1.25 hr at room temperature. The ovaries were washed twice with 200 μl of 3% BSA in 1 × PBS for 5 min each time. The ovaries were then fixed for 15 min in 200 μl of 4% formaldehyde in 1 × PBS. After fixation, the ovaries were washed twice with 200 μl of PBST (1 × PBS + 0.5% Triton X-100) for 5 min and 20 min. Ovaries were then washed twice for 5 min each with 1 × PBS + 3% BSA and then carried through the Click-iT Plus EdU Imaging Kit protocol (Thermo Fisher Scientific). Ovaries were imaged on a Nikon A1R-SI+ confocal microscope and >50 stage 10 chambers were examined for EdU foci in each genotype.

Generation of germline mitotic clones and egg counting

FRTG13 Orc4^{Y162C}/CyO females were mated with *hsFLP122*; *P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=ovoD1-18} 2R/T(1;2)OR64/CyO* males. Their progeny were incubated

for 30 min at 37° in a circulating water bath either 24–48 hr after egg laying (1 × heat shock) or 24–48 and 48–72 hr after egg laying (2 × heat shock). These embryos were reared to adulthood. Non-CyO females were isolated and mated to males in standard molasses vials. Homozygous *Orc4^{WT}* and *Orc4^{Y162C}* females were also mated to males as controls. The crosses were flipped every 24 hr for 4 days, and the number of eggs laid each day were counted.

Generation of orc4^{Y232C} yeast strains

Yeast strains were generated via CRISPR gene editing. The Cas9 coding cassette from pML104 (Laughery *et al.* 2015) was cloned into pDB18 (www.nieduszynski.org/methods/crispyCas9.php). Oligos containing a 20 mer guide sequence (forward: 5′-TGATCATACATTTGCTGGGCCTGTGG-3′, reverse: 5′-AAAACCACAGGCCCAGCAAATGTATG-3′) were cloned adjacent to the structural region of the single-guide RNA in the modified pDB18 plasmid. Along with the guide RNA-encoding plasmid, a double-stranded, rescue DNA template with sequence 5′-GATAGTGGTGAGGTTGACAGAGA GAGTATAACAAAGATAACAGTTGTTTTTATATTCGATGAAAT TGATACATTTGCTGGGCCTGTGAGACAAACTTTATTATGTAA TCTTTTTGACATGGTAGAACATTCTCGGGTACCTGTTTGC ATTTTTGGCTGCACAACGAAATTAAATATCTTGGAATATTTA GAAAAGAGGGTAAAGAGTAGATTTTCTCAAAGAGTGATT TATATGCCGCAAATACAGAATCTAGACGATATGGTTGAC GCCGTCAGAAATTTACTTACAGTTCGCTCTGAAATCTCC-3′ was transformed into *S. cerevisiae* (W303 background) using a one-step protocol (Laughery *et al.* 2015). Transformants were selected on URA dropout media for uptake of the URA3-containing, pDB18-derived vector, and individual colonies were screened and validated by PCR and sequencing.

Yeast whole-protein extraction

Whole-protein extraction from yeast was performed according to the standard procedure (Zhang *et al.* 2011). A_{600} cell equivalents of 0.12, 0.06, or 0.03 were boiled with 1 × Laemmli buffer for 5 min, loaded onto SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies against Orc1 and Orc4 (Bose *et al.* 2004).

Yeast chromatin fractionation

Yeast chromatin fractionation was performed as described in Liang and Stillman (1997), with the following modifications. Spheroplasting was performed with 0.4 μl of 1 mg/ml β-glucanase per A_{600} cell equivalent. A salt-wash step was added after cell lysis and sucrose cushion centrifugation: pellets were incubated with 50 μl of Extraction Buffer with 0.25% Triton X-100 (EBX) containing 100 mM or 750 mM NaCl for 10 min on ice with occasional mixing, then centrifuged at 10,000 × *g* for 2 min. MNase digestion steps were omitted. Samples were boiled with 1 × Laemmli buffer for 5 min, cell debris was pelleted at 10,000 × *g* for 1 min, and A_{600} cell equivalents of 0.25 were analyzed via Western blot as described above.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures and tables. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11303561.

Results

Generating endogenous point mutations in Drosophila to model MGS

The effect of an MGS mutant *ORC4* allele has not been examined in a metazoan model. The only reported mutation in *ORC4* known to cause MGS is the substitution of tyrosine 174 to cysteine (Guernsey *et al.* 2011). Tyrosine 174 is in the AAA + ATPase domain of human ORC4 (Figure 1A), a region highly conserved from yeast to humans, suggesting that it plays a critical role in ORC function. To model MGS caused by this tyrosine-to-cysteine mutation, we exploited the high conservation of this region to identify the homologous residue (tyrosine 162) in *Drosophila* and precisely engineer the *Orc4^{Y162C}* mutation in the endogenous locus by Cas9-mediated genome engineering (Figure 1B).

Introducing the mutation encoding a tyrosine-to-cysteine substitution (Y162C) at the endogenous *Orc4* locus assured that any phenotypic changes observed were due to the mutation and not to changes in transcription that might be caused by transgenic constructs. In addition to the Y162C substitution, our genome engineering strategy introduced a silent mutation in the protospacer adjacent motif to inhibit the guide RNA from directing cleavage of the engineered genome. We also engineered a silent mutation introducing an *Nde*I restriction site to allow for rapid molecular screening of the modified genomes (Figure 1B). To ensure that the two silent mutations did not affect Orc4 activity, we also generated a control strain, *Orc4^{WT}*, which lacked the Y162C substitution, but included both silent mutations. In addition, our editing generated a likely null allele, *Orc4^{null}*, which encoded a missense mutation (R183H) followed by a single base-pair deletion resulting in a frameshift after I187. This frameshift likely results in nonsense-mediated decay, but if a stable protein product is produced from this allele it would include 147 random amino acids following the frameshift before a stop codon. Thus, only the N-terminal 187 amino acids of the 458 amino acid Orc4 protein would be wild type, and the resulting protein would likely be nonfunctional. Although we have not analyzed protein product, this allele is likely a null allele because the phenotype of animals homozygous for this allele is as severe as the phenotype of this allele *in trans* to a deficiency (Figure 1C). Similar to null alleles in other replication subunits, homozygous Orc4^{null} animals are not viable with the majority of these animals dying as L3 larvae or during metamorphosis (Landis *et al.* 1997; Pinto *et al.* 1999; Pflumm and Botchan 2001; Bandura *et al.* 2005; Park and Asano 2008; Baldinger and Gossen 2009;

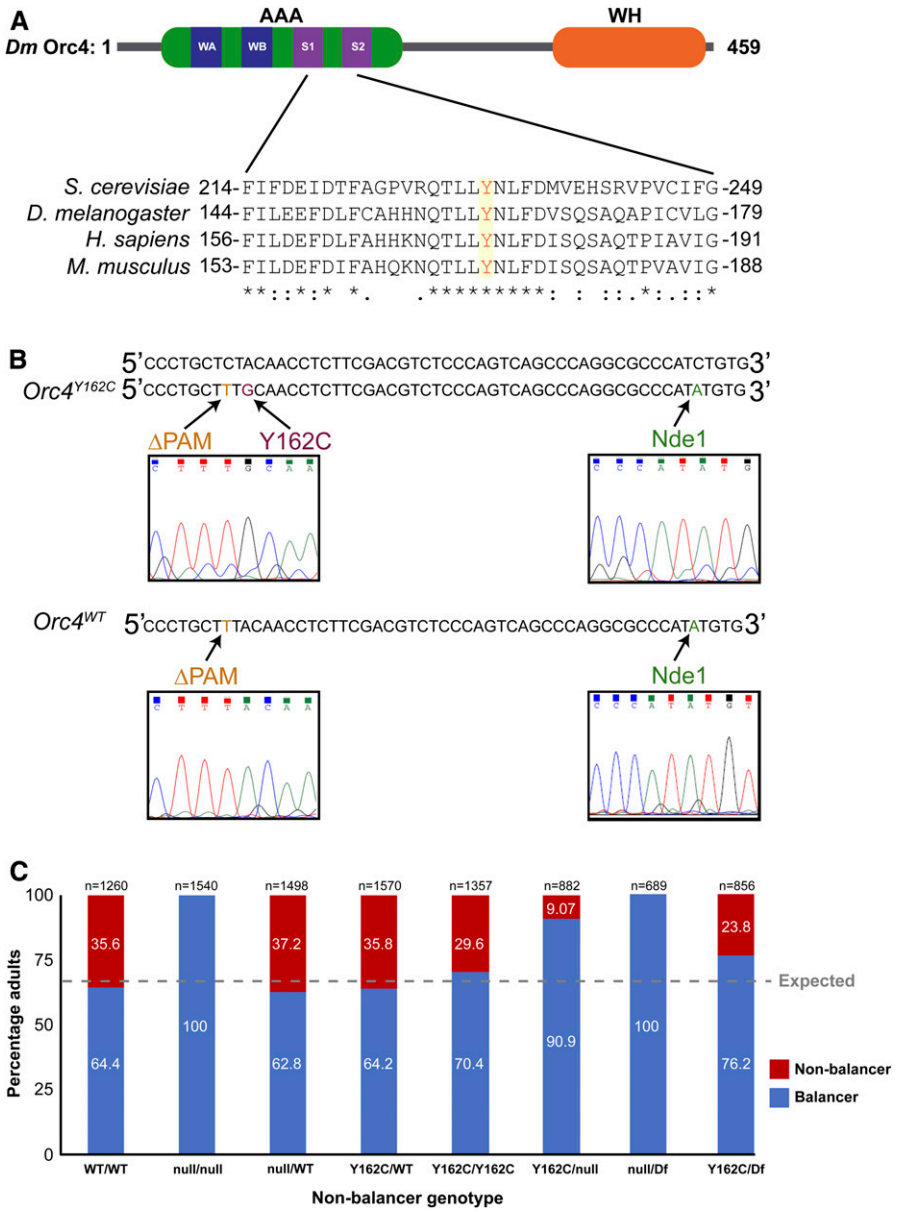

Figure 1 Similar to MGS individuals, *Orc4*^{Y162C} animals are viable. (A) Schematic of *Drosophila* Orc4 protein domains with the AAA + ATPase domain (AAA) and the winged helix (WH) domain indicated. The Walker A (WA), Walker B (WB), Sensor 1 (S1), and Sensor 2 (S2) motifs are also indicated in the AAA + ATPase domain (top). Clustal Omega alignment of multiple eukaryotes for the region surrounding the conserved tyrosine residue mutated in MGS (highlighted) (bottom). (B) Sequence of the genomic region encoding Y162 of Orc4 from a wild-type, unedited *Drosophila* strain (top). Below the sequence of the Cas9-edited genomes of the *Orc*^{Y162C} and *Orc*^{WT} strains are shown. The silent mutation removing the PAM site (gold), the Y162C mutation (maroon), and silent *Nde*I cut site (green) are noted along with the sequencing traces confirming the edited alleles. (C) The percent of balancer (CyO) to nonbalancer adults is shown for the crosses resulting in the indicated nonbalancer progeny. Heterozygote males and females were mated, and their progeny were scored for the presence of the CyO balancer. The dashed gray line represents the expected ratio from this cross (66% balancer: 33% nonbalancer). *n* = total number of flies assayed.

Balasov *et al.* 2009). Together, these strains provide the first metazoan model for an MGS mutation in which the mutation was engineered at the endogenous locus along with precisely defined control strains.

Orc4^{Y162C} *is a hypomorph and animals are homozygous viable*

MGS individuals with *ORC4*^{Y174C} mutations are either homozygous for this mutation or carry it over a null allele (Bicknell *et al.* 2011a; Guernsey *et al.* 2011; de Munnik *et al.* 2012). Although these individuals possess a number of distinctive phenotypes, they survive and have a normal expected lifespan. Therefore, we initially tested the viability of homozygous *Orc4*^{Y162C} animals. We quantitatively assessed the number of nonbalancer (straight-winged) and balancer (curly-winged) progeny produced from crosses of balanced heterozygotes for either *Orc4*^{Y162C}, *Orc4*^{WT}, or *Orc4*^{null} heterozygotes. Because animals homozygous for the CyO balancer die as larvae, Mendelian ratios would predict that 66% of the adults would carry the balancer while the remaining 33% would not. As expected, a single wild-type copy of *Orc4* resulted in ratios close to those expected (Figure 1C). Furthermore, *Orc4*^{null} animals are inviable both as homozygotes and when *in trans* to a deficiency, similar to previously reported data for null alleles of other ORC members (Landis *et al.* 1997; Pinto *et al.* 1999; Pflumm and Botchan 2001; Park and Asano 2008; Baldinger and Gossen 2009; Balasov *et al.* 2009) (Figure 1C). Like individuals carrying the *ORC4*^{Y174C} mutation, *Orc4*^{Y162C} animals are homozygous viable. Unlike some prior models of MGS mutations (Bicknell *et al.* 2011b; Yao *et al.* 2017; Maerz *et al.* 2019), we did not observe any obvious reduction in size of the homozygous *Orc4*^{Y162C}

animals, suggesting either a difference between *Drosophila* and other organisms or that some of these phenotypes may have been caused by misexpression of the disease allele, which was avoided by our genome-editing strategy. When we corrected our expected ratio of *CyO* to non-*CyO* flies based on the observed ratio from the $Orc4^{WT}/CyO$ cross, we identified a statistically significant decrease in the number of $Orc4^{Y162C}/Orc4^{Y162C}$ adults (χ^2, $P < 1.0 \times 10^{-4}$). This corresponds to a 24% decrease in viability compared to wild type, which would have a significant effect on fitness.

The observed effect on viability of the homozygous $Orc4^{Y162C}$ mutation suggested it might be a loss-of-function allele. To directly test this, we scored the viability of $Orc4^{Y162C}/Orc4^{null}$ and $Orc4^{Y162C}/Df$ *trans*-heterozygotes. These flies reach adulthood at reduced levels as compared to $Orc4^{Y162C}$ homozygotes (χ^2, $P < 1.0 \times 10^{-60}$, $P < 1.0 \times 10^{-9}$), demonstrating that $Orc4^{Y162C}$ is a hypomorphic allele. The viability of the $Orc4^{Y162C}$ combined with data showing that a Y162C substitution did not inherently destabilize the protein in transfected S2 cells (Supplemental Material, Figure S1) suggests that the tyrosine-to-cysteine substitution in Orc4 results in a protein with reduced functionality.

Animals homozygous for $Orc4^{Y162C}$ have tissue-specific defects

Having demonstrated that $Orc4^{Y162C}$ animals reach adulthood, we were able to assay for tissue-specific phenotypes. When compared to a wild-type strain (w^{1118}), $Orc4^{WT}$ animals show no obvious phenotypic differences. Thus, we used these strains as our wild-type controls. By comparison, $Orc4^{Y162C}$ homozygous animals had several phenotypic abnormalities. We identified several missing bristles on the thorax (Figure 2A). In addition, we observed severe bristle defects on the wing. The bristles along the wing are normally uniform in length and evenly spaced. By contrast, the wing-margin bristles in the $Orc4^{Y162C}$ animals are disorganized and vary in length (Figure 2A). We did not observe either of these bristle phenotypes in two additional hypomorphic alleles of replication components, MCM6 and Chiffon (Figure 2B) (Komitopoulou *et al.* 1983; Landis and Tower 1999). However, the scutellar bristle defect was reported for a previously characterized MGS-associated mutation in *Orc6*, which was modeled using a ubiquitously expressed transgene (Balasov *et al.* 2015). Together, these data demonstrate that $Orc4^{Y162C}$ homozygous animals, while relatively healthy, possess tissue-specific defects, providing support for the relevance of our *Drosophila* system in modeling MGS.

Females homozygous for $Orc4^{Y162C}$ are sterile

While $Orc4^{Y162C}$ animals were homozygous viable, females were sterile, indicative of an additional tissue-specific defect. By contrast, homozygous $Orc4^{Y162C}$ males were fertile. Females produced eggs at very low frequencies compared to $Orc4^{WT}$ control animals. The few eggs that were produced did not have dorsal appendages and appeared watery and malformed, indicative of a thin eggshell. In contrast to the bristle phenotype, female sterility is shared among animals possessing loss-of-function mutations in a variety of replication factors, such as Orc2, MCM6, Cdt1, Chiffon, and Humpty dumpty (Orr-Weaver 1991; Landis *et al.* 1997; Landis and Tower 1999; Whittaker *et al.* 2000; Bandura *et al.* 2005).

Because the females are sterile, we examined the ovaries of $Orc4^{Y162C}$ homozygous animals to determine if there were specific defects in egg chamber development. We dissected ovaries from $Orc4^{WT}$ and $Orc4^{Y162C}$ homozygous females and stained them with DAPI to image the nuclei. The ovarioles appeared largely normal, and we could identify egg chambers through stage 14. We noted two distinct phenotypes in stage 10 chambers: disrupted follicle-cell patterning and decreased numbers of nurse cells (Figure 2C and Figure S2). Both cell types play critical roles in oocyte maturation. Thus, either or both of these deleterious phenotypes could be responsible for the sterility of $Orc4^{Y162C}$ females. The germline-derived nurse cells produce the maternal products that will be deposited into the egg. During stage 10B, the somatically derived follicle cells rapidly amplify the chorion genes required for eggshell production (Orr-Weaver 1991; Calvi *et al.* 1998). Both cell types are polyploid, and the disorganized follicle cell structure and reduced nurse cell numbers could be due to replication defects during oocyte maturation.

To more quantitatively assess these defects, we determined the number of nurse cells in $Orc4^{WT}$, $Orc4^{Y162C}$, and $Mcm6^{K1214}$ homozygous females. We included $Mcm6^{K1214}$ as a control because it is a female-sterile allele of an additional component of the replication machinery (Komitopoulou *et al.* 1983; Schwed *et al.* 2002). Using fixed, DAPI-stained ovaries, we counted the number of nurse cells in 50–100 stage 10 egg chambers. Wild-type stage 10 egg chambers possess 15 nurse cells. Ovaries from $Orc4^{Y162C}$ homozygous females have fewer nurse cells than $Orc4^{WT}$ females along with a wider distribution in the number of nurse cell per egg chamber (*t*-test, $P = 4.16 \times 10^{-12}$) (Figure 2D). By contrast, stage 10 chambers from the $Mcm6^{K1214}$ females showed only a minor decrease in nurse cell number as compared to ovaries from $Orc4^{WT}$ females (*t*-test, $P = 7.79 \times 10^{-3}$), and were significantly different in comparison to $Orc4^{Y162C}$ (*t*-test, $P = 1.03 \times 10^{10}$). Thus, while both the $Orc4^{Y162C}$ and $Mcm6^{K1214}$ alleles lead to female sterility, only $Orc4^{Y162C}$ females have a decreased number of nurse cells (Komitopoulou *et al.* 1983; Schwed *et al.* 2002).

Females homozygous for $Orc4^{Y162C}$ fail to amplify the chorion genes

Mutations in genes encoding replication factors are known to cause female sterility at least in part due to replication defects in the somatic follicle cells. At stage 10B, follicle cells in the egg chamber undergo selective amplification of a limited subset of loci including the chorion genes, which are essential for eggshell production later during oocyte maturation (Orr-Weaver 1991; Calvi *et al.* 1998). This results in a gene amplification of 16- to 20-fold for a region on the X chromosome and 60- to 80-fold for a region of the third

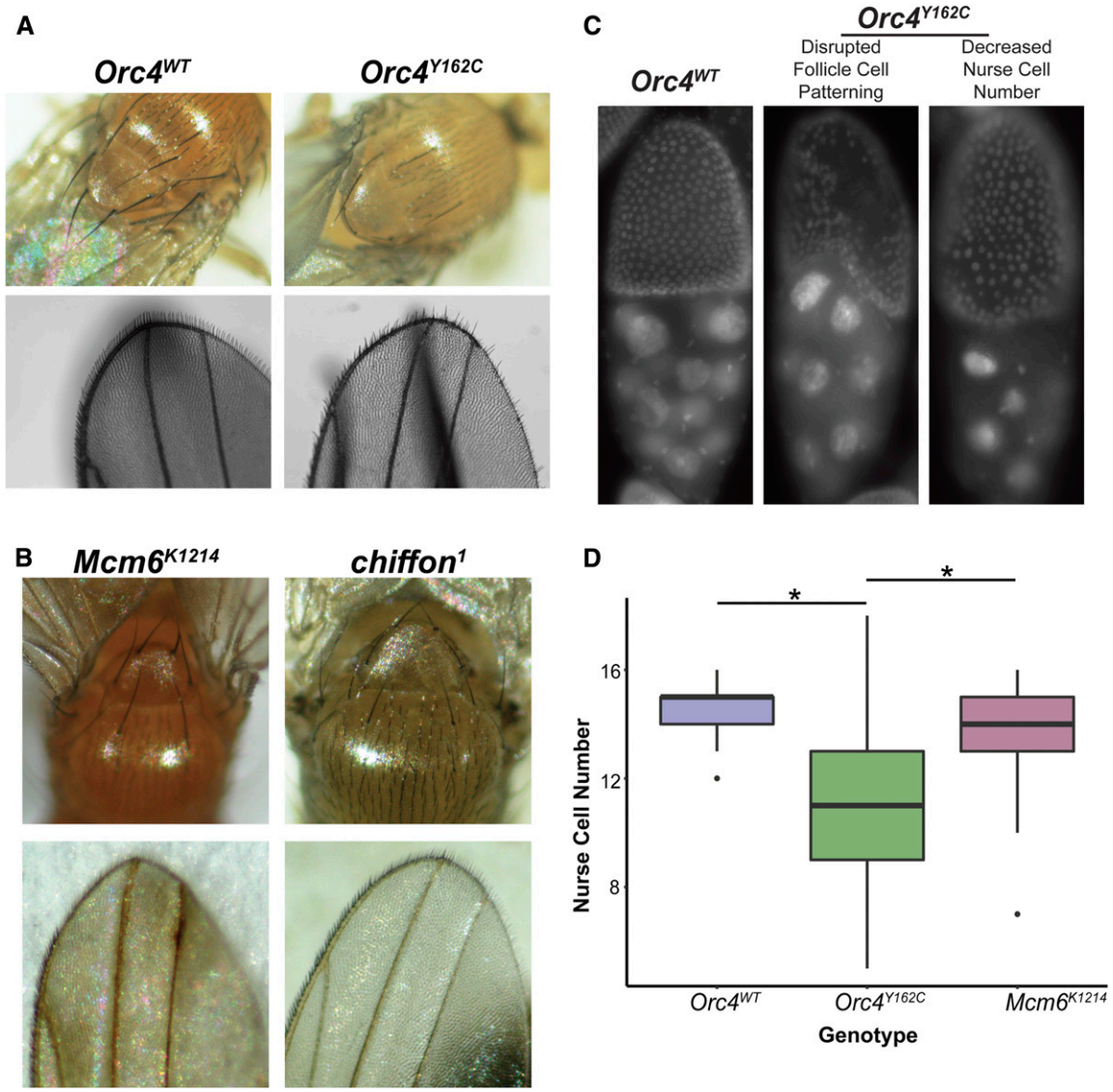

Figure 2 *Orc4*^{Y162C} animals have tissue-specific phenotypes. (A) Images of homozygous *Orc4*^{WT} and *Orc4*^{Y162C} animals. Mutant animals have missing scutellar bristles (top) and absent as well as disorganized wing-margin bristles (bottom). (B) Scutellar (top) and wing-margin (bottom) bristles are normal in animals homozygous for mutations in *Mcm6* and *chiffon*. (C) DAPI-stained images of stage 10 egg chambers from homozygous *Orc4*^{WT} and *Orc4*^{Y162C} animals. Examples of *Orc4*^{Y162C} animals with disorganized follicle cells and reduced nurse cells are shown. (D) Quantification of the number of nurse cells in *Orc4*^{WT}, *Orc4*^{Y162C}, and *Mcm6*^{K1214} ovaries. * *Orc4*^{Y162C} females have significantly fewer nurse cells than both *Orc*^{WT} ($P < 2.7 \times 10^{-17}$ *t*-test) and *Mcm6*^{K1214} females ($P < 1.0 \times 10^{-10}$, *t*-test). Fifty stage 10 chambers were counted in *Orc4*^{WT} and *Mcm6*^{K1214} animals; 100 stage 10 chambers were counted in *Orc4*^{Y162C} animals.

chromosome. Amplification occurs through repeated rapid and precise rounds of origin firing and replication fork elongation. Failure to adequately amplify these loci leads to thin, fragile eggshells, which results in female sterility. Thus, the female sterility of *Orc4*^{Y162C} may result from a failure to adequately amplify the chorion genes during oocyte maturation. Furthermore, well-established assays for chorion gene amplification provide a system by which to directly assay whether the MGS mutation in *Orc4* affects DNA replication (Calvi *et al.* 1998; Park and Asano 2012).

To test if *Orc4*^{Y162C} animals are replication deficient, we dissected ovaries from *Orc4*^{WT}, *Orc4*^{Y162C}, and *Mcm6*^{K1214} homozygous females and incubated them with the modified thymidine analog EdU for 1.25 hr, which allowed for the incorporation of EdU into replicating DNA that could subsequently be imaged using a small molecule–based fluorescence assay. Amplification of the chorion gene loci can be visualized as distinct EdU foci in the follicle cells of stage 10B egg chambers (Calvi *et al.* 1998). In the *Orc4*^{WT} females we observed large robust foci in the follicle cells (Figure 3A). As expected, no foci were detected in stage 10 egg chambers from *Mcm6*^{K1214} ovaries (Schwed *et al.* 2002) (Figure 3A). Despite clear incorporation of EdU in other stages of egg chamber development, no EdU foci were evident in the stage

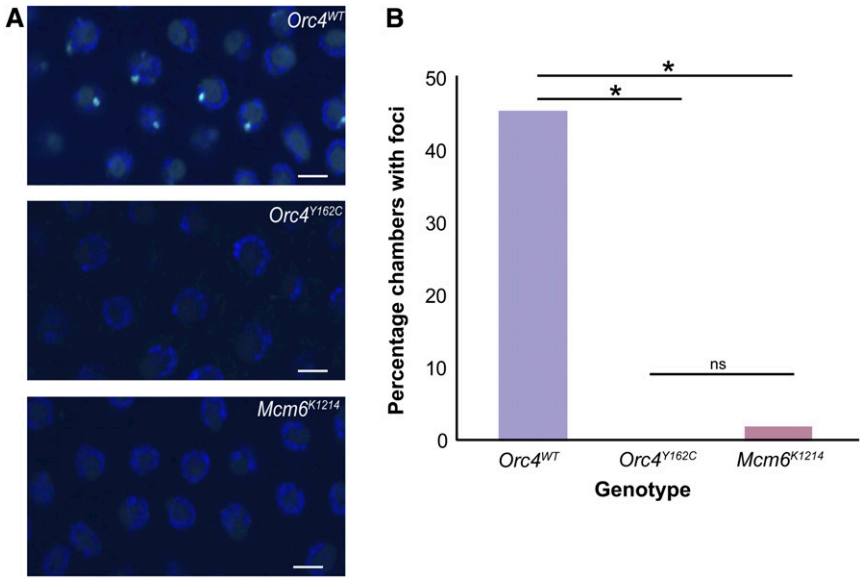

Figure 3 *Orc4*^{Y162C} females fail to replicate the chorion gene loci. (A) EdU staining of stage 10B egg chambers from *Orc4*^{WT}, *Orc4*^{Y162C}, and *Mcm6*^{K1214} females. EdU staining (cyan) marks the amplified chorion genes and was acquired with identical settings for all genotypes. DNA is stained with Hoechst (blue). Bar, 5 μm. (B) Quantification of stage 10 chambers with EdU foci from *Orc4*^{WT}, *Orc4*^{Y162C}, and *Mcm6*^{K1214} females. *Orc4*^{Y162C} and *Mcm6*^{K1214} animals fail to amplify the chorion genes as compared to *Orc4*^{WT} females. * $P < 1 \times 10^{-5}$ (Fisher's exact test). More than 50 stage 10 chambers were assayed for each genotype.

10 egg chambers of *Orc4*^{Y162C} females (Figure 3A). To quantify this replication defect, we imaged >50 stage 10 egg chambers for each genotype. There were large robust foci in 45% of the *Orc4*^{WT} stage 10 egg chambers, but none in *Orc4*^{Y162C} animals (Figure 3B). Demonstrating the robustness of our assays, ovaries from *Mcm6*^{K1214} females showed weak, fractured foci in 2% of the stage 10 egg chambers, similar to what has been previously published (Schwed *et al.* 2002). We noted that the Hoechst signal for the *Orc4*^{Y162C} animals was lower as compared to either the *Orc4*^{WT} or *Mcm6*^{K1214} animals (Figure S3). While this could be an issue with the staining, all the samples were stained at the same time with the same reagent mix. Thus, it is possible that this observation reflects a lower DNA content in the follicle cells of the *Orc4*^{Y162C} females as compared to control animals. Because the follicle cells themselves undergo a few rounds of total endoreplication cycles, it is possible this difference in Hoechst staining reflects a decrease in this endoreplication. Together these data provide evidence that the *Orc4*^{Y162C} mutation leads to a specific replication defect as females fail to amplify the chorion gene locus and this in turn results in the observed thin, fragile eggshells. Thus, while *Orc4*^{Y162C} mutant animals can replicate their DNA, DNA replication is defective during chorion gene amplification, when there may be a high demand for rapid origin licensing and firing (Lesly *et al.* 2017).

Animals inheriting maternal Orc4^{Y162C} *cannot complete embryogenesis*

Apart from endoreplication, another distinct developmental time point that requires rapid origin licensing and firing is during the synchronous nuclear divisions in the early embryo. Immediately following fertilization, development is controlled by maternally deposited products while the genome is reprogrammed. During this time, the nuclei are in a shared, syncytial cytoplasm and divide quickly with an abbreviated cycle comprised of only a synthesis (S) phase and mitosis. These divisions occur approximately every 10 min, with DNA being replicated in about half of this time. We hypothesized that if the tyrosine-to-cysteine mutation in Orc4 caused defects in tissues in which there was a high demand for DNA replication then embryos inheriting maternal *Orc4*^{Y162C} would fail to progress through the early stages of development, similar to what has previously been reported for *humpty dumpty* (Lesly *et al.* 2017). To address this issue, we used the FLP/FRT system to generate germline clones that are homozygous for the *Orc4*^{Y162C} mutation, while remaining largely heterozygous for the mutation in the somatic follicle cells (Chou and Perrimon 1996) (Figure S4). Because we combined this with the dominant female-sterile *ovo*^{D1} mutation (Chou *et al.* 1993), this strategy generated animals that only inherited *Orc4*^{Y162C} maternally, but that should be largely heterozygous for *Orc4*^{Y162C} in the somatic follicle cells. Females heterozygous for both the *ovo*^{D1} and the *Orc4*^{Y162C} mutations did not produce eggs, as expected. By contrast, heat-shocked females laid some eggs, and these eggs had dorsal appendages, a striking difference from the very few eggs laid by *Orc4*^{Y162C} homozygous females (Figure 4A). These data provide evidence that our strategy at least partially rescued eggshell production. We quantified the numbers of eggs laid by females in which the germline clones were generated. These females laid ~4 times more eggs than *Orc4*^{Y162C} females (Figure 4B). These embryos were largely rescued for eggshell production, but failed to complete embryogenesis and showed general morphological defects (Figure 4A). This embryonic lethality may be due to a failure to complete the rapid replication cycles required for the first few hours of embryogenesis. However, we were unable to specifically identify the stage at which these embryos died. Alternatively, given the observed defects in nurse cell number, inadequate nurse cell–mediated loading of the oocyte with maternal messenger RNAs could also contribute to the embryonic lethality.

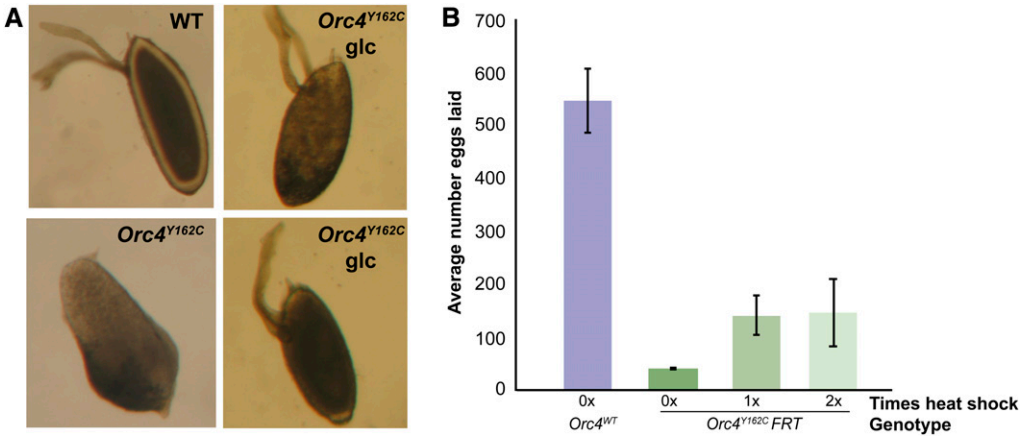

Figure 4 Embryos inheriting maternal *Orc4*^{Y162C} are inviable. (A) Images of embryos laid by mothers of the indicated genotype. Dorsal appendages are evident in embryos laid by wild-type females and females heterozygous for *Orc4*^{Y162C} in the follicle cells (*Orc4*^{Y162C} *glc*), but not from females homozygous for *Orc4*^{Y162C}. (B) Average numbers of eggs laid from 10 females of the indicated genotypes over 4 days are shown. Error bars indicate the SD from three biological replicates. *Orc4*^{Y162C}, *Orc4*^{Y162C} females; *Orc4*^{Y162C} *glc*, heat-shocked *hsFLP122*; FRTG13 *ovo*^{D1}/*FRTG13 Orc4*^{Y162C} females with germline clones; WT, wild-type females.

The MGS mutation in Orc4 does not disrupt ORC chromatin association

Having demonstrated multiple tissue-specific defects in animals homozygous for the MGS-associated tyrosine-to-cysteine substitution in Orc4, we wanted to determine if these defects arose from a failure of ORC to form or to associate with chromatin. Unfortunately, we were unable to obtain reagents that allowed us to address these issues using our *Drosophila* model. Thus, we used Cas9 genome-editing to make the analogous mutation in *S. cerevisiae* (Y232C) as we had previously generated monoclonal antibodies that allowed us to detect various ORC components (Bose *et al.* 2004). Using our validated monoclonal antibodies, we demonstrated the Y232C substitution did not destabilize Orc4 or the largest ORC subunit, Orc1 (Figure 5, A and B). Consistent with these data, we observed similar expression levels of epitope-tagged wild type or Y162C-containing Orc4 in *Drosophila* tissue culture cells (Figure S1).

Biochemical data with a reconstituted human complex containing the orthologous Y164C mutation suggested that this substitution did not inhibit complex formation (Tocilj *et al.* 2017). To test if the Orc4 Y232C substitution disrupted the ability of ORC to associate with chromatin, we assayed whether either Orc4 itself or an additional subunit of ORC that directly interacts with Orc4, Orc1, associated with chromatin under low (100 mM) and high (750 mM) salt washes (Figure 5C). As a control, we performed the chromatin-association assay with yeast cells carrying a mutation in *orc1* that deletes the bromo-adjacent homology (BAH) domain. The *orc1*$^{BAH\Delta}$ allele has previously been shown to disrupt ORC chromatin association (Müller *et al.* 2010). In the strain carrying the Orc4 MGS-associated mutation, both Orc4 and Orc1 associated with chromatin and remained in the pellet upon low-salt wash but not high-salt wash, similar to the association in wild-type cells (Figure 5C). By contrast, in the *orc1*$^{BAH\Delta}$ strain chromatin association of both ORC subunits was disrupted even under low-salt conditions (Figure 5C). Thus, the tyrosine-to-cysteine substitution in Orc4 does not disrupt the ability of ORC to bind to chromatin. Combined with prior *in vitro* assays demonstrating a disruption in ATPase activity of ORC carrying this substitution (Tocilj *et al.* 2017), our data support a model in which in MGS patients ORC forms and can associate with chromatin but has decreased activity that is specifically detrimental in tissues with high-replication demand.

Discussion

Despite multiple studies of MGS-causing mutations in DNA replication proteins, it remains unclear whether and how different cell types within a multicellular organism have distinct sensitivities to defects in these essential proteins. While addressing these interesting challenges will require a variety of approaches and insights, an important tool will be controlled metazoan models that can facilitate future comprehensive experimental analyses. In this report, we used Cas9-mediated mutagenesis to engineer the endogenous *Orc4* locus and establish a metazoan model for MGS. Based on the data generated from this new model, molecular experiments in a *S. cerevisiae* model, and published biochemical experiments with both yeast and human ORC, we conclude that the MGS-causing mutation in *Orc4* results in tissue-specific DNA-replication defects without perturbing ORC stability or its association with chromatin.

Our *Drosophila* model demonstrated that the *Orc4*^{Y162C} mutation was a hypomorphic allele that resulted in defects in tissues with high-replication demand, including both the follicle cells and early embryo. Similar to flies homozygous for other replication-defective alleles, *Orc4*^{Y162C} females are sterile and fail to replicate the chorion gene loci. Yeast with the corresponding tyrosine-to-cysteine mutation (Y232C) similarly show defects in DNA replication, particularly at the rDNA locus origin (Sanchez *et al.* 2017). Our analysis of

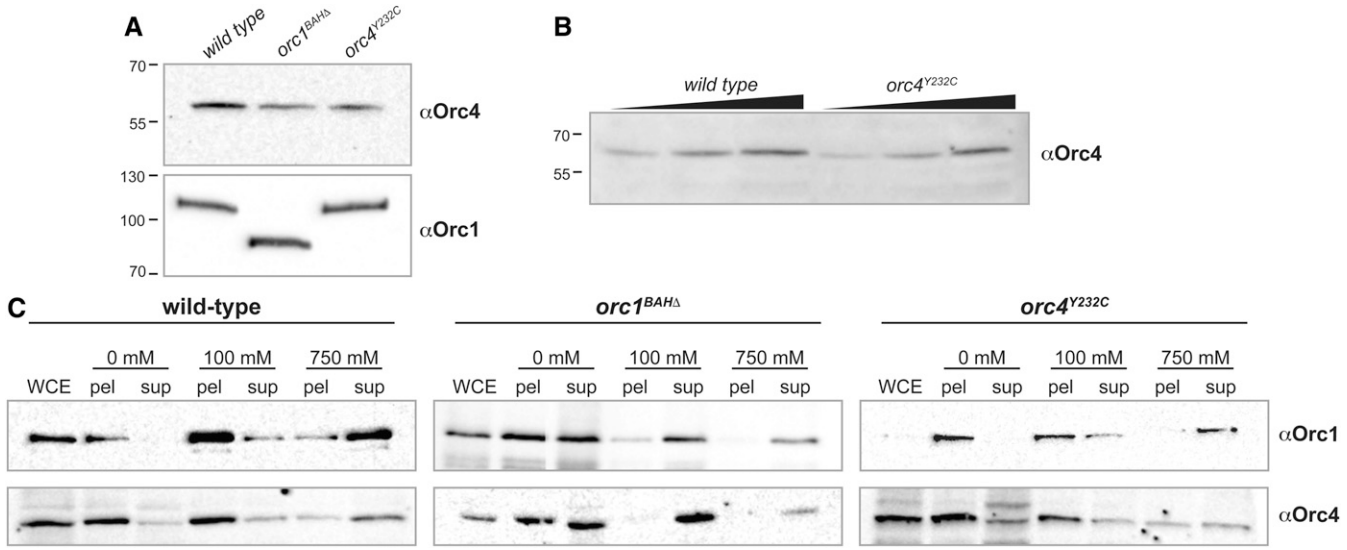

Figure 5 The MGS-associated tyrosine-to-cysteine mutation in Orc4 does not reduce Orc1 or Orc4 levels or ORC chromatin association. (A) Immunoblots of Orc4 and Orc1 protein levels in wild-type, *orc1*$^{BAH\Delta}$, and *orc4*^{Y232C} strains. (B) Immunoblots of increasing amounts of wild-type and *orc4*^{Y232C} extract. (C) Immunoblots of Orc4 and Orc1 identifying chromatin association in wild-type, *orc1*$^{BAH\Delta}$, and *orc4*^{Y232C} strains as indicated. Chromatin was pelleted and relative protein levels were assayed by immunoblot on the chromatin-associated pellet (pel) and supernatant (sup) in yeast extract (0 mM) and upon low (100 mM) and high (750 mM) NaCl washes. WCE, whole-cell pellet.

the yeast model showed that the resulting mutant ORC retained an association with chromatin that is similar to wild type. Thus, the MGS-causing tyrosine-to-cysteine substitution supports DNA replication in a number of tissues but results in tissue- or locus-specific replication defects. These observations are consistent with recent structural and biochemical studies of the human MGS-causing Y174C substitution in Orc4. The region of human Orc4 surrounding and including Y174 interacts with the ATPase domain of Orc1 (Tocilj *et al.* 2017). This region of Orc4 is highly conserved across species, showing significant evolutionary constraint that is likely reflective of the requirement for the Orc4-Orc1 interaction in the essential ATPase activity of ORC (Bowers *et al.* 2004). Indeed, Y174C in human Orc4 results in altered ORC ATPase activity *in vitro* (Tocilj *et al.* 2017). Taken together, these data provide support for a model wherein the MGS-associated tyrosine-to-cysteine substitution in Orc4 leads to a functional, but compromised ORC that either reduces recruitment of the MCM hexamer to chromatin and/or reduces the efficiency of a post-MCM loading step that is required to generate a sufficient number of functional chromosomal origins. Because most cells license more origins of replication than are necessary to replicate the genome, a reduction in MCM loading can likely be tolerated in many tissues, and thus a MGS mutant supports development. However, based on emerging data, including data in this study, we propose that in tissues that require rapid or efficient rounds of replication, the reduction in origin function caused by the tyrosine-to-cysteine substitution in Orc4 reduces replication enough to prevent these tissues from developing normally.

We showed that embryos inheriting only maternal *Orc4*^{Y162C} failed to develop normally, providing evidence that *Orc4*^{Y162C} is insufficient to support the rapid division cycles required for early embryogenesis. However, because there are nurse cell defects in females homozygous for *Orc4*^{Y162C}, we cannot rule out the possibility that the defects we observed in embryos generated by germline clones may result from these embryos receiving a deficient number of other maternally supplied molecules deposited by these nurse cells into the developing oocyte. The nurse cells are generated from the same progenitor cell that gives rise to the oocyte. Thus, it is not possible to create embryos deficient for maternal Orc4 while retaining wild-type Orc4 in the nurse cells. Nonetheless, the shared replication-associated phenotypes in both the follicle cells and the early embryo of loss-of-function alleles in two distinct MGS-associated genes, *humpty dumpty* (*DONSON*) and *Orc4*, supports a causative role of replication defects in driving at least some of the tissue-specific MGS phenotypes (Bandura *et al.* 2005; Lesly *et al.* 2017).

While we propose that replication efficiency is a critical factor that leads to the phenotypic defects in *Orc4*^{Y162C} animals, we cannot exclude the possibility that the defects are due to the noncanonical cell cycles in the tissues assayed. In the adult fly a limited number of tissues, notably the nurse and follicle cells of the ovary and the external cells of the mechanosensory bristles, undergo multiple rounds of replication (Zielke *et al.* 2013). In the follicle cells, in addition to undergoing limited rounds of nearly complete genome endoreplication, the chorion gene loci undergo multiple rapid rounds of reinitiation of DNA synthesis. Similarly, in the early embryo the nuclear division cycle is a series of rapid synthesis and mitosis phases without gap phases. Thus, while all of these tissues likely have a high-replication demand relative to other tissues, they also rely on noncanonical cell

cycles. Further experiments will be required to determine if phenotypes caused by MGS mutations are due specifically to defects in tissues with cycles of rapid DNA replication, nonstandard cycles of DNA replication, or both.

While data from multiple model systems provide evidence that individuals with the *Orc4*^{Y174C} MGS mutation have an insufficient number of origins to support rapidly replicating tissues, it remains unclear if MGS mutations in other ORC subunits similarly result in decreased replication capacity in specific tissues. Biochemical and phenotypic data from *Drosophila* suggest that the MGS mutation in *Orc6* results in a replication defect caused by a destabilization of ORC (Bleichert *et al.* 2013; Balasov *et al.* 2015). Similar to the model we propose for *Orc4*^{Y174C}, this destabilized ORC reduces MCM recruitment to chromatin (Bleichert *et al.* 2013). Modeling of the *Orc1* MGS mutations in zebrafish generates small fish with morphological defects, similar to the phenotypes in MGS individuals. In contrast to the models proposed for Orc4 and Orc6, the phenotypes in the zebrafish model of *Orc1* MGS mutations have been suggested to arise from defects in cilia formation (Bicknell *et al.* 2011b; Yao *et al.* 2017; Maerz *et al.* 2019). We observed bristle defects in *Orc4*^{Y162C} homozygous adults, but not in adults defective in either MCM6 or Chiffon (DBF4), indicating that not every replication-defective hypomorphic allele causes this phenotype. However, it is worth noting the scutellar bristle phenotype we observed in *Orc4*^{Y162C} adults is shared with a *Drosophila* model of an MGS mutation in *Orc6* (Balasov *et al.* 2015). These external cuticular structures arise from shaft and socket cells that have undergone endocycles, and the size of this structure is correlated with the number of endocycles, suggesting a possible defect in endoreplication in these MGS mutants (Audibert *et al.* 2005; Szuplewski *et al.* 2009). If replication defects underlie the observed bristle phenotype, perhaps the *Mcm6* and *chiffon* alleles failed to cause this defect simply because they provide for a replication capacity in the relevant cells above a required threshold whereas the MGS-associated *Orc* alleles do not. It is possible that individual tissues have a distinct threshold of sensitivity to reductions in replication capacity and even to reductions in different replication proteins, depending on the expression level of those proteins or other cell-specific attributes that affect replication efficiency. Nevertheless, the different phenotypes resulting from distinct mutations in proteins required for replication raises the possibility that roles for ORC proteins outside of origin licensing may affect MGS phenotypes. Future work in multiple organisms will be needed to identify how each MGS mutation leads to the disease phenotypes. Our results indicate that a comprehensive survey of multiple replication-defective and MGS-associated alleles in a single-model system may be particularly informative.

In summary, our data clearly demonstrate tissue-specific replication defects caused by the MGS-associated mutation in Orc4, and along with previously published work support the model that the tissue-specific defects in MGS patients may arise because cells within these tissues have specialized replication demands (Lesly *et al.* 2017). Furthermore, our data demonstrate the ability to gain mechanistic insights into disease phenotypes by combining rapid and precise editing of the *Drosophila* genome with the wealth of tools and knowledge derived from over a century of studying this powerful model metazoan.

Acknowledgments

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