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Insulator Protein CP190 Regulates Expression of Spermatocyte Differentiation Genes in *Drosophila melanogaster* Male Germline

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Abstract—CP190 protein is one of the key components of *Drosophila* insulator complexes, and its study is important for understanding the mechanisms of gene regulation during cell differentiation. However, *Cp190* mutants die before reaching adulthood, which significantly complicates the study of its functions in imago. To overcome this problem and to investigate the regulatory effects of CP190 in adult tissues development, we have designed a conditional rescue system for *Cp190* mutants. Using Cre/loxP-mediated recombination, the rescue construct containing *Cp190* coding sequence is effectively eliminated specifically in spermatocytes, allowing us to study the effect of the mutation in male germ cells. Using high-throughput transcriptome analysis we determined the function of CP190 on gene expression in germline cells. *Cp190* mutation was found to have opposite effects on tissue-specific genes, which expression is repressed by CP190, and housekeeping genes, that require CP190 for activation. Mutation of *Cp190* also promoted expression of a set of spermatocyte differentiation genes that are regulated by tMAC transcriptional complex. Our results indicate that the main function of CP190 in the process of spermatogenesis is the coordination of interactions between differentiation genes and their specific transcriptional activators.

INTRODUCTION

Specialization of cells during multicellular organism development is accompanied by an activation of developmental genes by enhancers [1] and insulator proteins play a key role in the regulation of promoter-enhancer interactions [2]. They form physical contacts between special DNA regions—insulators, thereby providing spatial interactions between promoters and *cis*-regulatory elements. In a similar way, insulator proteins block nonspecific promoter-enhancer interactions and establish barriers between adjacent domains on chromosomes.

In *Drosophila melanogaster*, nearly a dozen regulatory proteins that bind to insulators in various combinations have been found [3–6]. CP190, which interacts with most of the known insulators, is one of the key proteins among them. CP190 is involved in DNA contact formation, regulation of three-dimensional nuclear architecture, and blocking of promoter-enhancer interactions [7–9]. In some cases, CP190 acts as a transcription factor and attracts regulatory proteins and epigenetic modifiers to target genes [6, 10–12].

Loss of CP190 function is accompanied by transcriptional alterations that affect, among others, homeotic genes encoding key developmental regulators [8, 13, 14]. Null mutations of *Cp190* are recessive lethal, leading to the death of *D. melanogaster* at the pupal stage [15, 16], making it challenging to investigate the regulatory effects of CP190 in adult cells. Therefore, its effects on gene expression and nuclear morphology have been studied either in cultured cells or at early developmental stages [8, 13], giving rise to numerous limitations when interpreting results.

Here we used spermatogenesis of *D. melanogaster* as a model of cellular differentiation. During the differentiation of male germline cells, more than 1000 specific genes are activated under the effect of specialized transcription factors tMAC and tTAF [17, 18]. However, only a small portion of genomic tMAC and tTAF binding sites are in close proximity to target genes [18]. This may indicate the regulation of genes through a network of spatial interactions between the regulatory elements of genome involving insulator proteins. To study the effect of CP190 on gene regulation during cell differentiation, we developed a genetic system that allowed us to selectively examine the phenotype of lethal *Cp190* mutations in adult male germline cells. Using this approach, we evaluated the effect of CP190 protein on gene expression in testes and analyzed its

effect on the regulation of spermatocyte differentiation genes.

EXPERIMENTAL

Molecular cloning and *Drosophila* transformation. A rescue construct (GenBank ON783212) containing the coding sequence of the *Cp190* gene under the control of *Ubi63E* gene promoter was created based on the pUAST-attB vector, as described in RESULTS. PhiC31-mediated transgenesis of the $y^-w^-;P\{y[+t7.7]=CaryP\}attP40;M\{vas\text{-}int.B\}ZH\text{-}102D$ line harboring the *attP40* integration site in the second chromosome was performed using this construct, producing $y^1w^{67};CP190\text{-}mCD8\text{-}GFP$ line [19].

Fly stocks. All *D. melanogaster* lines were maintained at 25°C on standard feed. The following fly stocks were used for the experiments: $y^1w^{67};CP190\text{-}mCD8\text{-}GFP$ carried a rescue construct; $y^1w^{67};nanos\text{-}Cre$ harbored *nanos-Cre* transgene integrated into *attP40* site [18, 20], $y^1w^{67};+/+;Cp190^2,e^1/TM6B,Tb^1$ [15] and $y^1w^{67};+/+;Cp190^3,e^1/TM6,Tb^1$ [16], carried mutant alleles of *Cp190*. By standard genetic crosses, flies $y^1w^{67};CP190\text{-}mCD8\text{-}GFP;Cp190^2,e^1/TM6,Tb^1$ and $y^1w^{67};nanos\text{-}Cre;Cp190^3,e^1/TM6,Tb^1$ were obtained from these stocks. Males with Cre/loxP-mediated recombination were obtained by crossing *nanos-Cre* carried males with rescue construct carried females. When performing control experiments in RNA-seq assay, we used $y^1w^{67};\{Ubi>stop>mCD8\text{-}GFP\}$ line with $\{Ubi>stop>mCD8\text{-}GFP\}$ transgene integrated into *attP40* site [20].

RNA extraction and reverse transcription. For the expression analysis of whole testes, 25 pairs of testes were dissected and RNA was isolated using TRIZOL reagent (Invitrogen, USA, 15596018). 1 μg of total RNA was used for cDNA production; reverse transcription was performed using Superscript II Reverse Transcriptase (Invitrogen, 18080093) with dT_{20} oligomers as primers.

Quantitative PCR. Quantitative PCR was performed using BioMaster HS-qPCR SYBR Blue reaction mixture (Biolabmix, Russia, MHC030). The sequences of primers used for assay are listed in Table S1 (Supplementary Information on the web-site http://www.molecbio.ru/downloads/2023/1/supp_Romanov_engl). The efficacy of rescue cassette elimination was evaluated using ΔΔCt method, and *Cp190* expression was measured using absolute quantification. *Actin42A* gene primers were used as a reference in both cases. Serial dilutions of gDNA from larval imaginal discs of $y^1w^{67};CP190\text{-}mCD8\text{-}GFP$ females were used for standard curve producing.

Microscopy. For the analysis of live GFP fluorescence, testes were transferred to slides into a drop of PBS and covered with a cover glass, then analyzed immediately using Olympus BX-51 fluorescence microscope. For immunostaining, testes were fixed using standard method with 4% formaldehyde solution in PBST [21]. Primary rat anti-CP190 antibodies (kindly provided by A.K. Golovnin [22]) and secondary antibodies (anti-Rat Cross-Adsorbed A568, Thermo Fisher Scientific, A-11077) were used in the experiment. Immunostaining samples were analyzed using a Carl Zeiss LSM 710 (Germany) confocal microscope.

Transcriptome analysis using RNA-seq. Testes of three-week-old imago $y^1w^{67};CP190\text{-}mCD8\text{-}GFP/nanos\text{-}Cre$ and $y^1w^{67};CP190\text{-}mCD8\text{-}GFP/nanos\text{-}Cre$ and $y^1w^{67};CP190\text{-}mCD8\text{-}GFP/nanos\text{-}Cre;Cp190^2/Cp190^3$ were used for experiment. Testes of $y^1w^{67};\{Ubi>stop>mCD8\text{-}GFP\}/nanos\text{-}Cre$ with $\{Ubi>stop>mCD8\text{-}GFP\}$ were used as controls. Total RNA was isolated from 25 paired testes per sample using TRIZOL. Sequencing libraries were prepared using TruSeq RNA Sample Preparation v2 Kit (Illumina, USA, RS-121-2002) and sequenced on the Illumina MiSeq platform using 2×75 bp paired-end mode. In total, from 3.7 to 6.5 millions of paired-end reads were obtained per sample.

Genomic alignment was performed using HISAT2 tool [23] (genome assembly *dm6*, Release 6 plus ISO1 MT, Aug. 2014) under standard parameters. Read counts for each gene were summarized using Feature-Counts [24] based on UCSC refGene genomic annotation (https://hgdownload.soe.ucsc.edu/goldenPath/dm6/bigZips/genes/dm6.refGene.gtf.gz). From two to three biological replicates for each genotype were prepared; gene expression profiles in samples of different genotypes showed a high correlation (Fig. S2, see Supplementary Information on the web-site http://www.molecbio.ru/downloads/2023/1/supp_Romanov_engl). Analysis of differential gene expression was performed using DESeq2 algorithm [25] with "ashr" [26] and "ihv" [27] options. Data on gene expression in whole testes of *can*, *comr*, *mip40*, and *bam* mutants have been obtained previously and are available in the Gene Expression Omnibus database (GSE97182) [18]. Data on gene expression in *Drosophila* tissues were obtained from the modENCODE database [28] and clustered using the Cluster 3.0 program [29]. The enrichment of tissue-specific genes was analyzed using the TissueEnrich package [30]. Sequencing data of testis transcriptome with single-cell resolution were obtained from Fly Cell Atlas database and analyzed using Seurat package [31, 32]. Sequencing results obtained in this work are available in the Gene Expression Omnibus database (PRJNA847720).

Binding of Can, Comr and Mip40 to CP190-dependent genes. Data on the binding of Can, Comr and Mip40 proteins with chromosomes of male germline were previously obtained using DamID-seq method (GSE97182) [18]. Protein binding sites were mapped using DamID-Seq software [33]. Every gene region in the genome was divided into a specified number of regions. According to DamID-seq data, protein enrichment in each region of certain gene set was cal-

culated by comparing the frequency of protein-bound GATC-fragment centers in each fragment of gene set with the same value for all *D. melanogaster* genes. Statistical significance of enrichment was assessed using Fisher's exact test.

RESULTS

Conditional Rescue System Ensures Viability of Cp190 Mutants

Mutants of insulator protein CP190 coding gene do not survive to the adult stage [15, 16], so the study of its function in tissues of adult *D. melanogaster* tissues is fraught with experimental difficulties. To overcome this problem and to establish the regulatory function of CP190 in adult male germ cells, we developed a genetic system for tissue-specific conditional rescue of the *Cp190* mutation (Fig. 1a).

The system is comprised of two genetic constructs. The first one is *CP190-mCD8-GFP* rescue construct that contains *Cp190* coding sequence (CDS) under the control of constitutive promoter *Ubi63E,* which has been successfully used previously to rescue *Cp190* mutants [31, 32]. CDS of *Cp190* is followed by hybrid terminator *HIS3-SV40* and coding sequence of mCD8-GFP chimeric reporter [33, 34]. Region spanning CDS of CP190 and terminator is floxed and comprise a rescue gene cassette. The second construct contains Cre recombinase gene under the control of *nanos* gene promoter, which ensures recombinase production specifically in germline cells (Fig. 1a) [18, 19]. If both constructs are combined in *Cp190*-mutant flies, rescue cassette would be eliminated in male germline, resulting in CP190-deficient cells marked with GFP, while mutant phenotype would be rescued in somatic cells. When the rescue cassette is removed in germline cells, coding sequence of mCD8-GFP chimeric reporter gene becomes close to *Ubi63E* promoter, resulting in GFP-labeled *Cp190*-deficient cells (Fig. 1b).

Using *phiC31*-mediated transgenesis, *CP190-mCD8-GFP* genetic construct was integrated into *attP40* site within second chromosome [19]. Transgenic flies were viable and fertile with both one and two copies of the construct (data not shown). At the same time, one copy was sufficient to rescue lethal phenotype of *Cp190²/Cp190³* compound, which do not produce normal CP190 protein [15, 16]. Expression of one or two copies of rescue construct didn't cause developmental defects under *Cp190²/Cp190³* or wild-type background. This indicates a moderate level of ectopic CP190 expression, since an overexpression of CP190-encoding transgenes was shown to cause severe developmental defects [34].

A series of genetic crosses produced males carrying both *CP190-mCD8-GFP* and *nanos-Cre* constructs against mutant *Cp190* (*y¹w⁶⁷; CP190-mCD8-GFP/nanos-Cre*; *Cp190²/Cp190³*). Activation of the mCD8-GFP reporter protein starting from the early spermatocyte stage was detected in the germline cells, indicating the removal of rescue cassette (Fig. 1c). Obtained flies possessed normal viability and exhibited no visible spermatogenesis abnormalities. GFP-negative spermatocytes and GFP-positive somatic envelope cells were not visually detected, indicating a high efficiency and specificity of rescue construct elimination.

In *y¹w⁶⁷* control testes, as well as testes carrying rescue construct in the absence of Cre recombinase, only background fluorescence of tracheal tubules and spermatid fragments was present (Fig. 1c). This confirms robust repression of reporter protein production in all cell types of testes.

An additional validation of the efficiency of rescue cassette elimination from the germline cell genome with quantitative PCR showed that rescue construct is remaining in 49.6% of whole testis cells (Fig. 2a). This value corresponds to the proportion of somatic cells in testis (50.4%), which were estimated using single cell transcriptome sequencing data [31]. This result indicates that the rescue cassette is eliminated in the vast majority of germ cells.

Conditional Rescue System Significantly Reduces Ectopic Cp190 Expression in Adult Male Germline

We have analyzed expression levels of endogenous and transgenic Cp190 mRNA by RT-qPCR. For this purpose, primers on *Cp190* coding and 3'-untranslated regions of endogenous *Cp190* were selected (Fig. 2b). The former pair of primers enabled us to measure the expression level of both endogenous and transgenic *Cp190*, while the latter pair captured only endogenous *Cp190* mRNA. It was found that rescue cassette elimination under *nanos-Cre* resulted in a 3.8-fold decrease in *Cp190* CDS expression in whole testes (Fig. 2c). However the level of *Cp190* CDS was still 2.4-fold higher compared to wild-type, which is likely due to increased expression of transgenic *Cp190* over endogenous in somatic cells as well as remaining rescue cassette in single spermatocytes (Fig. 2d).

Production of CP190-depleted germline cells was confirmed using immunostaining (Fig. 2d). In *y¹w⁶⁷;CP190-mCD8-GFP/nanos-cre;Cp190²/Cp190³* testes, CP190 was detected in nuclei of cells from apical end, testis sheath and in single spermatocytes. At the same time, CP190 expression was observed only in single nuclei of spermatocytes. In wild-type testes, a stable level of CP190 expression was observed in both somatic and germ cells. Thus, our system enables rescue of *Cp190* mutant phenotype in somatic cell lineage with simultaneous elimination of rescue cassette in male germline, which allows us to reproduce mutant phenotype specifically in adult sex cells.
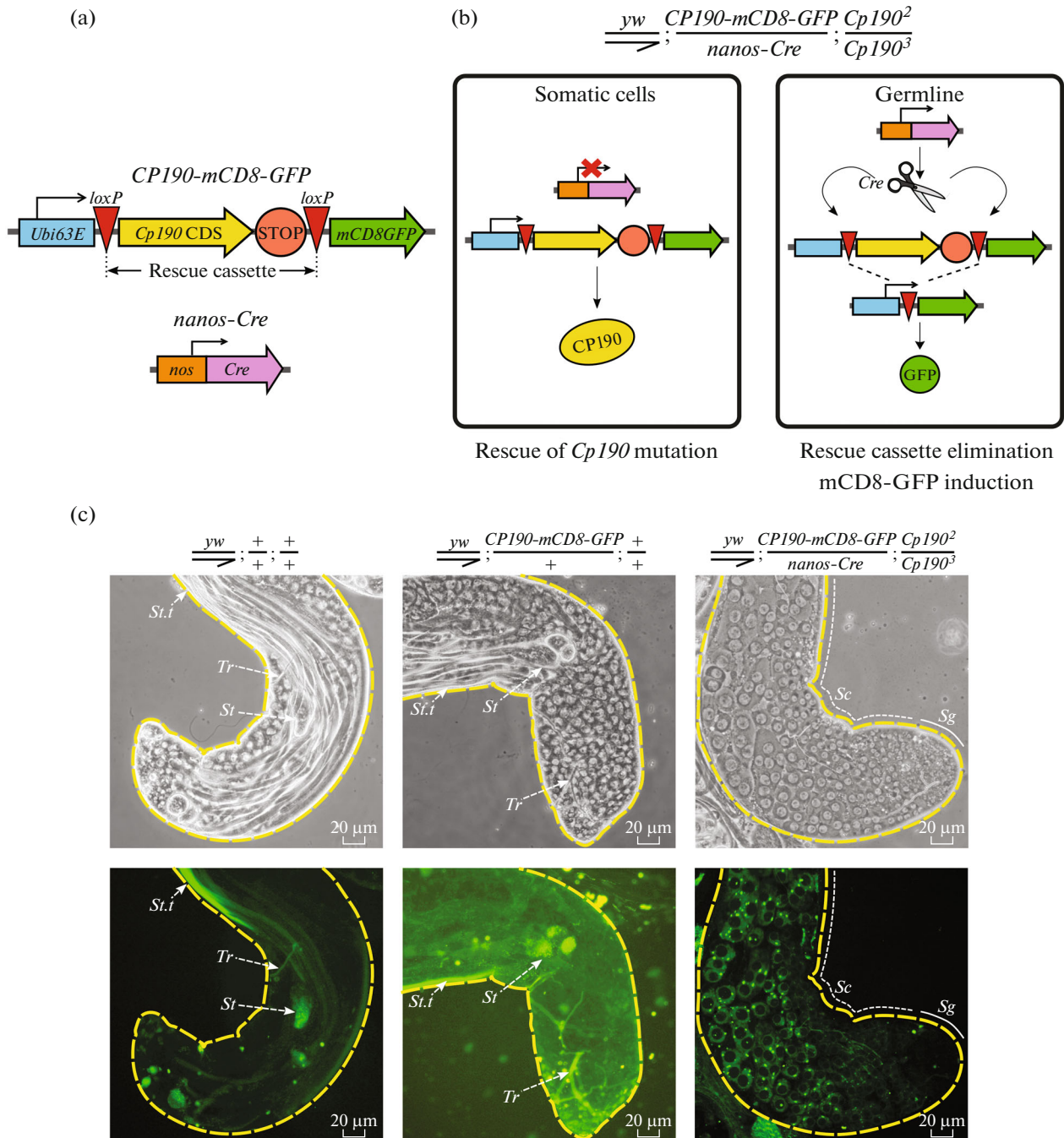
Fig. 1. Genetic system for conditional rescue of *Cp190* loss of function mutants. (a) Schematic representation of *Cp190-mCD8-GFP* rescue construct and *nanos-Cre* construct for Cre recombinase expression in *Drosophila* germline. (b) Conceptual scheme of *Cp190²/Cp190³* mutants conditional rescue (see explanations in the text). (c) Imaging of testes not carrying the rescue construct, with a single dose of one and with conditional rescue of *Cp190* mutation. Phase contrast (top row) and GFP fluorescence detection (bottom row). Yellow dotted lines indicate the boundaries of testes. Legend: *St.t*—spermatid tails; *St*—spermatid heads; Tr—tracheal tubes; *Sg*—area of testis containing spermatogonia (continuous line); *Sc*—area of testis containing spermatocytes (dashed line).

Mutation of Cp190 Gene Affects the Expression of Differentiation Genes in Drosophila Male Germline

To determine the effect of CP190 on gene expression in adult testes, we performed high-throughput transcriptome sequencing (RNA-seq) followed by differential gene expression analysis (table S2, see Supplementary Information on the web-site http://www.molecbio.ru/downloads/2023/1/supp_Romanov_engl). In the experiment we analyzed testes of three-week-
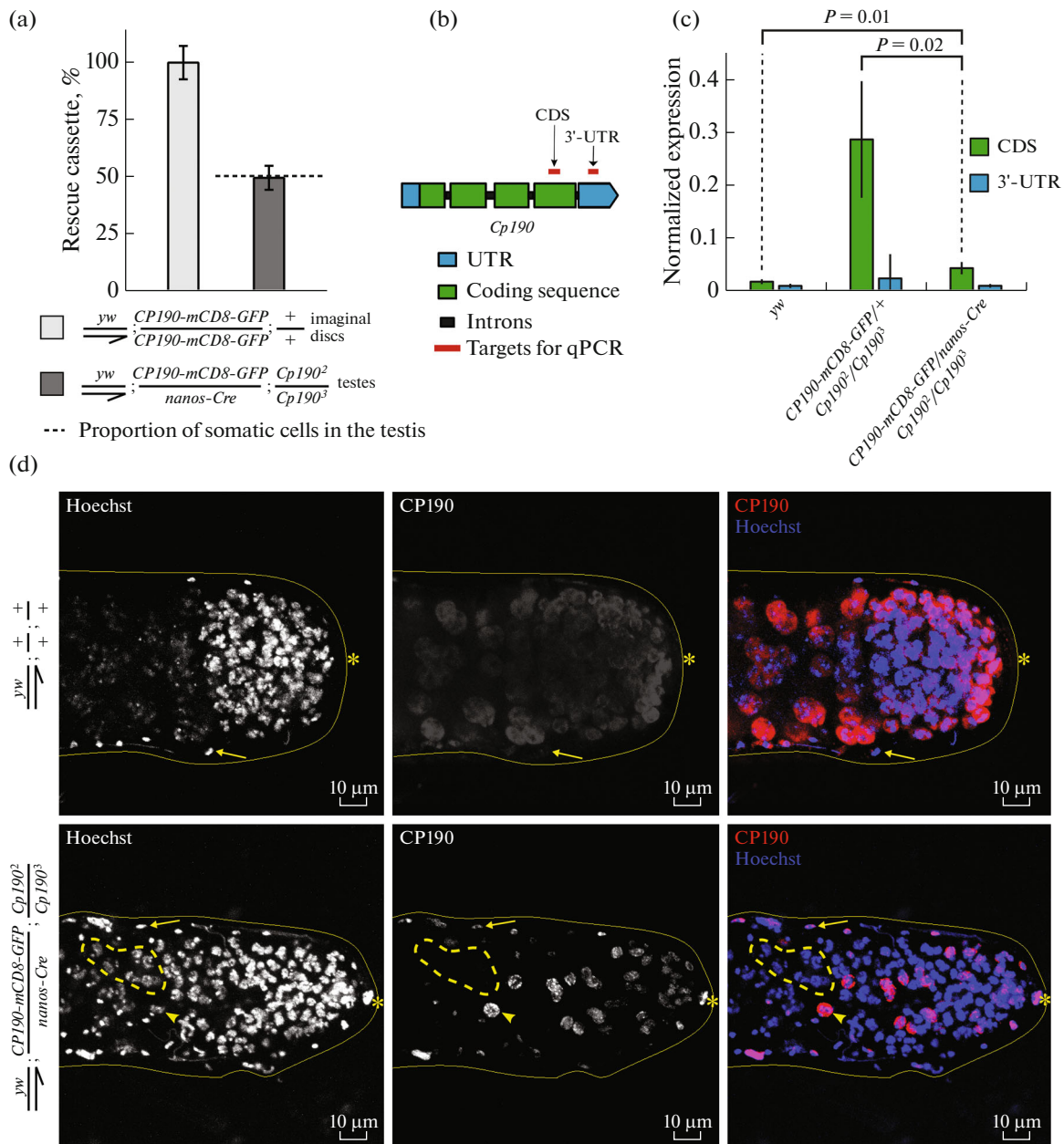
Fig. 2. Cre-mediated recombination results in elimination of rescue cassette and loss of CP190 in cells of the male germline. (a) The number of copies of the rescue cassette in testes with conditional rescue was compared with the imaginal discs of larvae carrying a double dose of the CP190-mCD8-GFP construct. The number of copies of the rescue cassette was normalized to the dose of the construct. The dotted line indicates the proportion of somatic cells in *Drosophila* testes according to single cell transcriptome sequencing [31]. (b) Location of targets for RT-qPCR inside *Cp190* gene. Corresponding primers were used to measure the expression of endogenous and transgenic *Cp190* mRNA. (c) Normalized expression of *Cp190* in of three-day-old adult's testes with *CP190-mCD8-GFP* and *nanos-Cre* constructs. Expression was measured using primers to the loci indicated in the diagram (b). Expression was normalized to the activity of the *Actin42A* housekeeping gene. *P*-value was obtained using Student's T-test with a two-sided distribution. (d) Confocal images of testes immunostaining using anti-CP190. In the upper row is the testis of the wild type (*yw*). The bottom row is the testis of males under conditional rescue. DNA was stained with Hoechst dye. The boundaries of testes are underlined with a continuous yellow line. An asterisk marks the apical end of the testes. A long arrow shows somatic cells of the sheath. A dotted line shows a cluster of CP190-negative spermatocytes. The short arrow shows a CP190-positive spermatocyte. The scale is 10 μm.

old imago as *y¹w⁶⁷;CP190-mCD8-GFP/nanos-Cre* and *y¹w⁶⁷;CP190-mCD8-GFP/nanos-Cre;Cp190²/Cp190³* testes showed equivalent level of *Cp190* expression at that age (Fig. S2, see Supplementary Information on the web-site http://www.molecbio.ru/downloads/2023/1/supp_Romanov_engl). It is important to note that the activity of rescue cassette is controlled by the *Ubi63E* gene promoter, which makes level of *Cp190*

transcription higher than the endogenous one (Fig. 2c), thus can affect overall gene expression. To accurately account for this effect in our experiment, we evaluated the effect of increased *Cp190* expression in somatic cells on the transcriptome of whole testes.

For this purpose, we measured gene expression in testes of *y^{1}w^{67};CP190-mCD8-GFP/nanos-Cre*;+/+ males, in which endogenous *Cp190* gene were functional while the rescue cassette were active only in somatic cells. We used gene expression in testes of control line (see Experimental) for comparison. The presence of rescue construct resulted in moderate changes in transcription: expression of only 12 genes changed more than 8-fold (Fig. 3a). In contrast, analysis of testes with conditional rescue of *Cp190* mutation in somatic cells (*y^{1}w^{67};CP190-mCD8-GFP/nanos-cre*;*Cp190^2/Cp190^3*) revealed 89 genes whose expression changed more than 8-fold compared to the control (Fig. 3a).

Moreover, among 226 genes with differential expression under conditional rescue (154 increased expression, 72 decreased), only 43 were also altered under *Cp190* overexpression in somatic cells (Fig. 3b). These 43 genes were excluded from further analysis, which was focused on isolating the specific effect of a *Cp190* mutation on gene expression in germline.

After this correction, we found 120 genes which had increased expression under mutant *Cp190* background (Set 1) as well as 63 genes which were downregulated (Set 2) (Fig. 3b). To characterize these sets, we analyzed tissue-specific expression patterns using modENCODE data on fly tissue transcriptomes [26]. It turned out that among the genes in Set 1, three major clusters can be distinguished, represented by those active in male reproductive, nervous, and digestive systems (Fig. 3c). The genes in Set 2 are expressed ubiquitously, and only 14 of them can be classified as testis-specific. Analysis of gene expression patterns in various *Drosophila* tissues using the TissueEnrich tool [30] confirmed the prevalence of testis-specific genes in Set 1 but not in Set 2 (Fig. 3d). Thus, CP190 protein can be involved both in maintaining the expression of ubiquitously active genes in testes and in repression or modulation of the activity of tissue-specific genes, including ones involved in spermatogenesis.

At the same time, it should be taken into account that the cellular composition of the testes is quite diverse; it is represented by several types of somatic cells, as well as germline cells at different stages of differentiation. Various cell types have distinguished features in the expression profile, which is confirmed by the data of transcriptome sequencing of single testes cells. The characterized expression profiles of individual cell populations make it possible to identify specific marker genes and to determine in which cells a particular gene is normally active.

To determine in which particular testis cells Cp190-dependent genes are normally active, we used marker genes of different *Drosophila* testis cell populations from the DRscDB database [31, 38]. In Set 1, only single somatic cell marker genes were identified, whereas spermatocyte markers accounted for 10% in total (Fig. 4a). In Set 2, fat and pigment cell markers comprised 22% of genes, while germline cell markers were sporadic (Fig. 4a). Clustering of Set 1 genes by the expression level in different testis cell subpopulations using Fly Cell Atlas single cell transcriptome dataset [31] revealed that 66% of genes were spermatocyte-specific (Fig. 4b). At least 60% of expression of such genes is provided by germline cells, which allows us to refer the majority of Set 1 genes to spermatocyte differentiation genes (Fig. 4b). In contrast, only one-fifth of Set 2 genes can be identified as spermatocyte differentiation genes, and their total number is 5.7 times lower than in Set 1. Taken together, this suggests that CP190 has a predominantly repressive effect with respect to the spermatocyte differentiation genes.

The main part of Set 2 genes is normally active predominantly in somatic cells of testes (Fig. 4b). At the same time, it was still unclear whether the expression of these genes changed in germline cells under *Cp190* depletion. Using single cell transcriptome data [31] to estimate the contribution of individual testis cell populations to gene expression, we modeled two expression profiles: in the first profile, Set 2 genes were inactivated exclusively in germline, while in the second profile—exclusively in somatic cells. In the next step, we compared the observed changes in gene expression of Set 2 with two profiles. Inactivation of genes with somatic specialization in somatic cells alone or in germline alone does not allow us to simulate the same severe drop in transcription that we observed in testes with conditional rescue ($P < 5 \times 10^{-8}$, Wilcoxon's test) (Fig. 4c). In contrast, the estimate made for spermatocyte differentiation genes under hypothesis of germline-specific inactivation did not differ from the observed values ($P = 0.23$, Wilcoxon's test) (Fig. 4c). This may indicate that the drop in the transcription level of genes from somatic specialization group of Set 2 occurs in both germline and somatic cells.

It is interesting to note that similar regulatory effects of CP190 were found in *Drosophila* somatic cell culture. Using previously obtained data from transcriptome analysis of Kc167 cells under *Cp190* knockdown by RNA interference, we found an upregulation of 820 genes and a downregulation of only 260 (Fig. 5a) [13]. Similarly, upregulated genes were represented predominantly by genes specific for testes, brains, and digestive system (Figs. 5b, 5c). On the other hand, genes which were downregulated under *Cp190* depletion had nonspecific expression profiles (Figs. 5b, 5c).

Cp190 Depletion Disrupts Expression of tMAC Targets

In testes activation of differentiation genes occurs in a coordinated manner during the growth phase of spermatocytes I. Several mutations that disrupt this
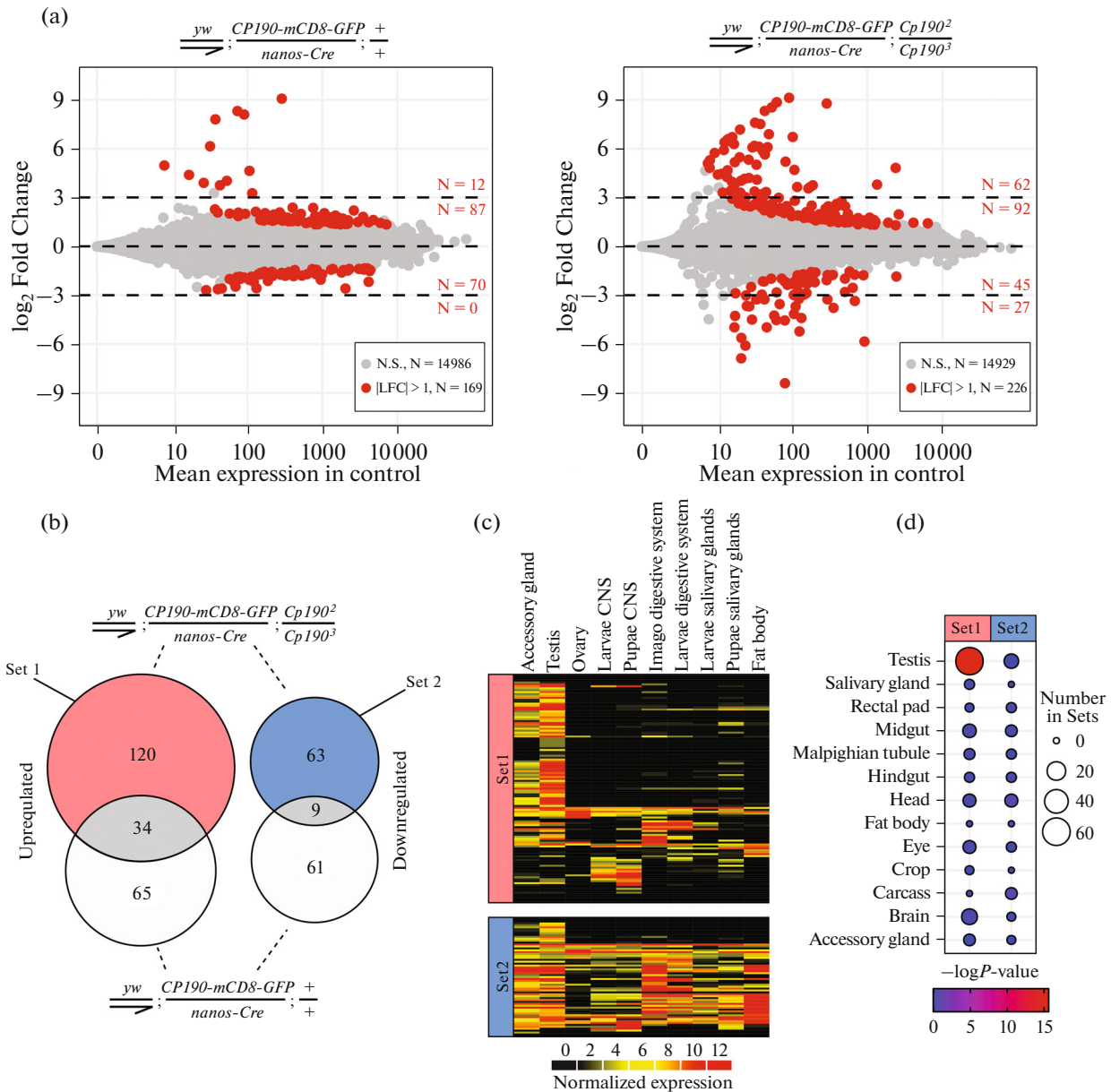
(a)

(b)

(c)

(d)

Fig. 3. Depletion of CP190 in male germline is accompanied by activation of testis-specific genes and inactivation of housekeeping genes. (a) Differential gene expression in testes with increased CP190 expression in somatic cells (left diagram) or in testes with conditional rescue (right diagram) in RNA-seq. Log$_2$ FoldChanges (LFC) of transgenic testes compared to the control is plotted on the vertical axis. On the horizontal axis, the normalized mean expression in control testes is plotted. Genes with a statistically significant differential expression ($|LFC| > 1$, $P < 0.05$) are marked in red. LFC values of genes with insignificant expression change (gray dots, N.S.) were truncated using an empirical Bayesian approach for clarity. Dotted lines indicate LFC values +3 and 0. Red indicates the number of red dots with LFC values between the closest dotted lines. (b) Intersection between sets of genes whose expression significantly increased and decreased in testes with conditional rescue or in testes with increased expression of CP190 in somatic cells (red dots in diagram a). Genes whose expression changed only in the background of conditional rescue are designated as Set 1 and Set 2. (c) Normalized expression of genes from Sets 1 and 2 in different Drosophila tissues according to modENCODE [28]. (d) Analysis of tissue-specific expression of CP190-dependent genes with expression in the male germline cells.

process have been described [17]. For example, mutation in *bam* gene aborts spermatogenesis progression at spermatogonia stage, resulting in the enrichment of the testes with undifferentiated germline cells [39]. Meiosis arrest genes *mip40*, *comr* and *can* encode tran-

scription factors, and their mutations lead to spermatogenesis arrest at spermatocyte I stage [17].

Defects in Mip40 and Comr, the components of testis meiosis arrest complex tMAC, cause dramatic
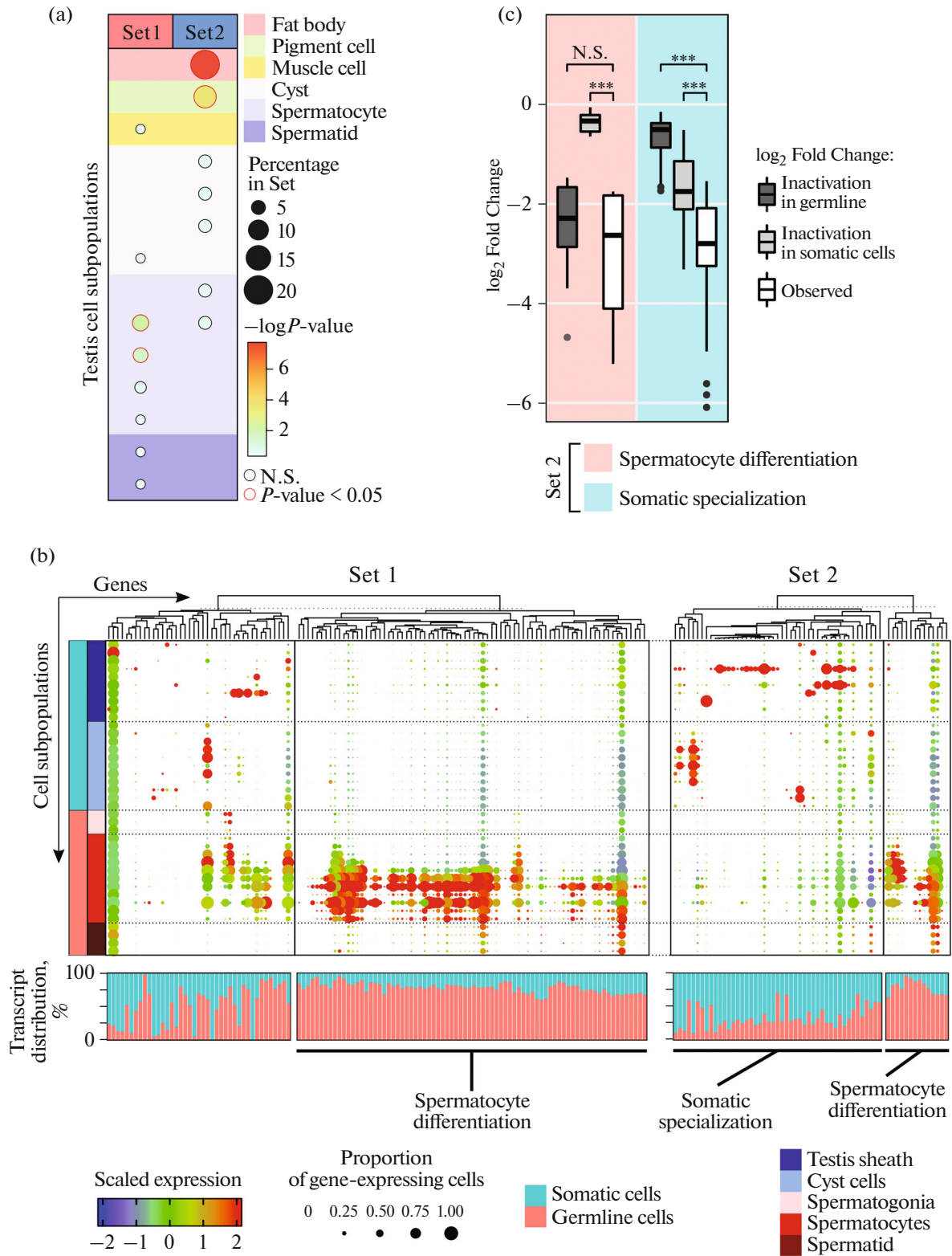
(a)

(b)

(c)

Fig. 4. Loss of CP190 in male germline cells leads to impaired activity of spermatocyte differentiation genes. (a) Enrichment of cell type marker genes in Sets 1 and 2 according to DRscDB data [38]. Cell types were annotated in the single cell transcriptome sequencing experiment [31], in some cases including several subpopulations with their own subset of marker genes. The subpopulations cell types are indicated by colored rectangles. The size of the circles characterizes the representation of markers in the whole set. Statistical significance of enrichment was calculated by Fisher's exact test. Markers with significant enrichment are encircled with red. (b) Gene expression from Sets 1 and 2 in testis cell subpopulations according to Fly Cell Atlas [31]. The identity of populations according cell type and anatomical structures is encoded by colored rectangles. The color of the dots characterizes the scaled level of gene expression compared to the average expression in the testis cells. The size of the dots describes the proportion of cells in the population in which transcripts of a given gene are detected. The bar chart shows the contribution of germline and somatic cells to the total transcription of each gene in the whole testis. Using hierarchical clustering by expression levels in cell subpopulations, genes in each set were divided into two clusters. In Set 1, genes with high activity in spermatocytes (spermatocyte differentiation genes) are clearly distinguished. In Set 2, the clusters can be characterized as somatic specialization genes and spermatocyte differentiation genes. (c) Comparison of expression change estimates in two clusters from Set 2. $\log_2$ FoldChange estimates were obtained separately for spermatocyte differentiation genes (red) and somatic specialization genes (blue) from the assumption that, in testes with conditional rescue, inactivation occurred only in germline cells (dark gray rectangles) or only in somatic cells (light gray rectangles). The observed $\log_2$ FoldChange values are shown in white rectangles. ***$P < 5 \times 10^{-8}$, Wilcoxon test, N.S.—$P > 0.05$.

alterations in testis gene expression [17, 18]. On the other hand, impaired function of testis TBP-associated factors tTAFs, including Can, causes less severe disruption of transcriptome. Together, tTAF and tMAC promote the activation of spermatocyte differentiation program. However, only a fraction of spermatocyte differentiation genes are directly regulated by tTAFs and tMAC [18].
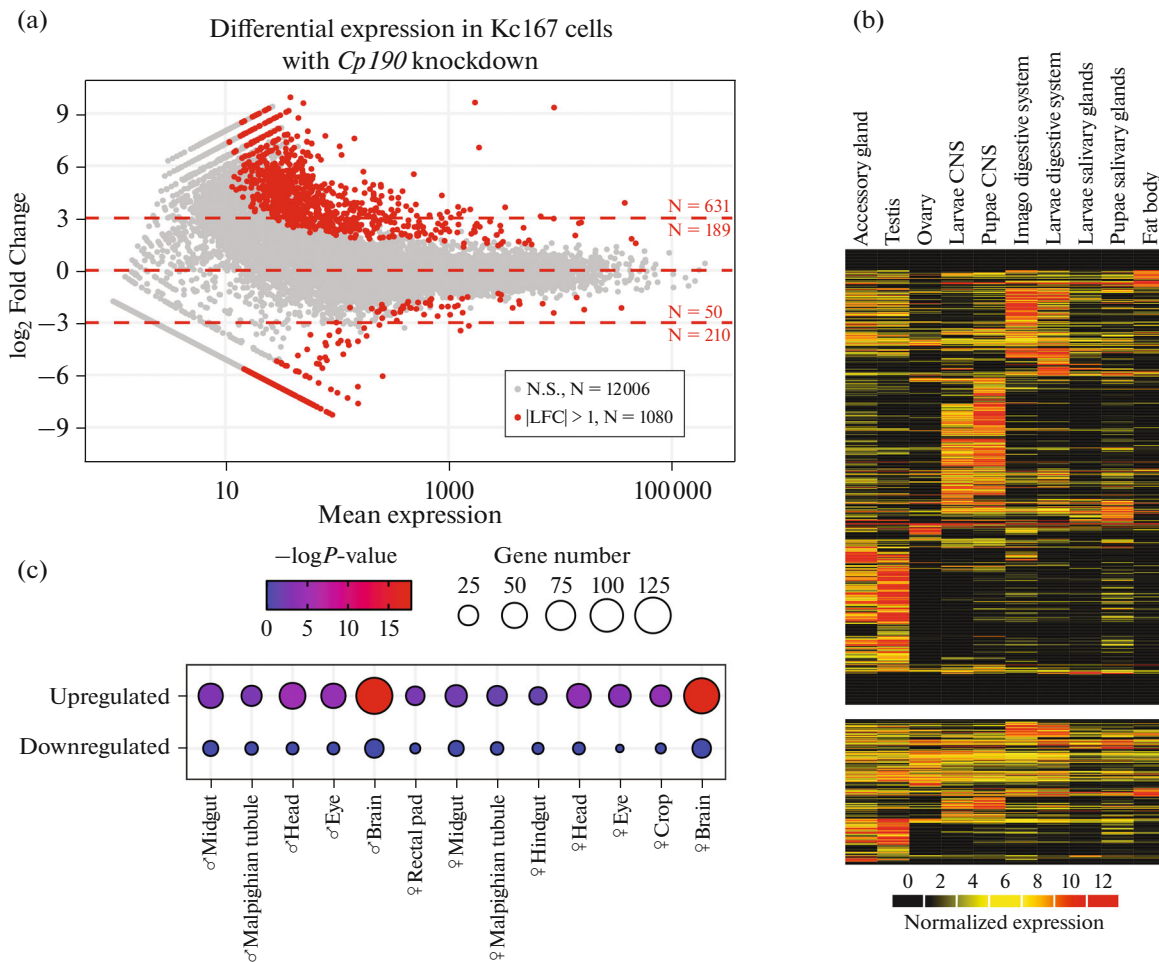

Fig. 5. CP190 downregulates tissue-specific genes and promotes the activation of housekeeping genes in Kc167 cells. (a) The effect of Cp190 inactivation in Kc167 cells according to published data [13]. Differential expression was analyzed in the same manner as in Fig. 3a. (b) Expression of CP190-dependent genes (red dots in diagram (a) in *Drosophila* tissues according to modENCODE [28]. (c) Analysis of tissue-specific expression for CP190-dependent genes from Kc167 cells.
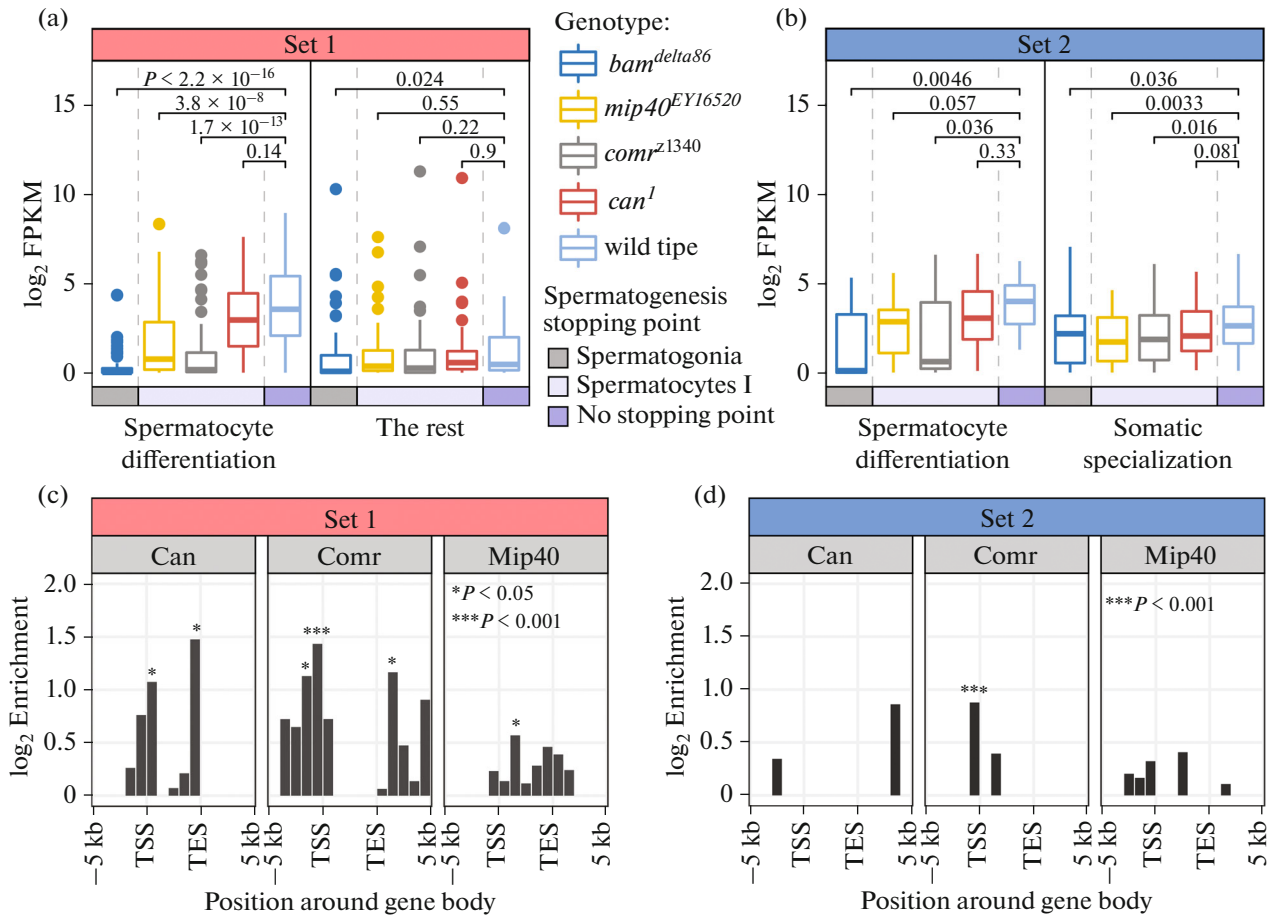
Fig. 6. Depletion of CP190 in male germline cells leads to impaired activity of tMAC-dependent spermatocyte differentiation genes. (a) Expression of genes from two clusters of Set 1 in testes of wild type and mutants with impaired differentiation germline cells. Colored rectangles show the stage of spermatogenesis arrest in testes of each genotype. Genes *mip40* and *comr* encode components of testis-specific transcriptional activator tMAC. Gene *can* encodes one of the tTAF proteins. Statistical significance of differences in gene expression in testes between mutants and wild type was established by Wilcoxon's test. (b) Enrichment of Set 1 wuth genes bound by Can, Comr and Mip40 in specific region in chromosomes of male germline [18]. Statistical significance of enrichment was measured using Fisher's exact test. TSS—transcription start site; TES—transcription termination site. (c) Same as in (a), for genes from the two clusters of Set 2. (d) Same as (b), for genes from Set 2.

Given the association of CP190 with differentiation genes, we proposed a functional interaction between CP190, tTAF, and tMAC. We previously obtained gene expression profiles in testes of *bam*, *mip40*, *comr*, and *can* mutants, and mapped binding sites of Mip40, Comr, and Can in chromosomes of male germline cells [18]. Thus, we compared expression of Set 1 genes, which were upregulated under conditional rescue, in testes of wild-type and *bam*, *mip40*, *comr*, and *can* mutants (Fig. 6a).

The activity of spermatocyte differentiation genes from Set 1 (Fig. 4b) is significantly reduced in *bam*, *mip40*, and *comr* mutants, but not in *can* mutants (Fig. 6a). The rest genes of Set 1 is almost unaffected by *mip40*, *comr* and *can* mutations, except *bam* mutation, which leads to a slight decrease in their expression, which can be explained by the presence of genes with high activity in spermatocytes in that subset (Fig. 4b).

Thus, CP190 limits the activity of tMAC-dependent spermatocyte differentiation genes.

The distribution of Comr, Mip40, and Can on chromosomes show that these proteins bind to genes from Set 1 more frequently than expected, while Comr binding was most significant (Fig. 6b). Thus, *Cp190* mutation affects the activity of genes directly regulated by the tMAC complex.

The expression of Set 2 genes is characterized by less pronounced differences between *bam*, *mip40*, *comr*, and *can* mutants and *wild-type* (Fig. 6c), although both mutants with tMAC disruption and *bam* mutants have reduced expression of spermatocyte differentiation genes and genes specific to somatic cells. Set 2 genes had some association with transcription factor Comr (Fig. 6d), indicating that their expression can be directly activated by tMAC complex, and CP190 protein is somehow involved in this process.

DISCUSSION

In this work, we studied in detail the effect of the CP190 protein on gene activity in *D. melanogaster*. The main effect of the CP190 mutation was observed on the subpopulation of spermatocyte differentiation genes, which are regulated by tMAC transcription complex. Interestingly, in the absence of CP190 protein, the activity of those genes increases 2-fold or more, which allows us to conclude that their activity is modulated by this protein in normal conditions, limiting the level of their expression.

In general, *Drosophila* cell cultures have shown moderate changes in the transcriptome under depletion of insulator protein CP190 [8, 13, 40]. We also identified only 183 genes which expression depends on CP190 in spermatocytes. The expression of 120 CP190-dependant genes was upregulated upon *Cp190* mutation and 63 were downregulated, suggesting a prevailing repressor function of CP190. It was also shown on cell cultures that CP190, like other insulator proteins, tends to suppress transcription, despite its simultaneous activating functions [13, 41].

We found that CP190 affects the activity of tissue-specific genes and housekeeping genes in opposite ways, indicating the possibility that there are several mechanisms by which CP190 controls the activity of these genes. It can be assumed that CP190 is capable of acting as a transcription factor for genes whose activation depends on CP190. This is indirectly confirmed by the fact that the cooperative action of CP190 and the factor M1BP, a housekeeping gene regulator, is required to activate CP190-dependent genes [13]. Moreover, promoters of genes whose expression is downregulated upon knockdown in cells are enriched with CP190, while the repressor effect of CP190 is most likely not to be directly implemented [13].

It is important to note that the activation of spermatocyte differentiation program is accompanied by a significant rearrangement of chromatin architecture [42]. Thus, the number of distal contacts decreases at the spermatocyte stage, and promoters of active differentiation genes appear in an isolated environment [42]. For CP190, along with the Chro protein, involvement in the formation and maintenance of topologically associated domains (TADs) has been shown [41]. Moreover, it was found that disruption of gene expression resulting from CP190 knockdown correlates with alteration of boundaries of surrounding TADs [41]. Moreover, it was found that TADs containing housekeeping genes tended to be more stable [41]. This suggests that observed differential effect of CP190 on the expression of tissue-specific and housekeeping genes may be caused by divergent regulation at the level of chromatin structure, making housekeeping genes to be activated by CP190, while tissue-specific genes to be repressed.

Given the absence of an effect on male germline cell differentiation, the normal fertility of mutant males, and the moderate influence on gene expression pattern changes, the CP190 insulator protein can hardly be considered as a key regulatory factor of spermatogenesis. In comparison, the *comr* (tMAC) mutation affects the expression of over 2500 genes and causes a delay in meiosis [43]. However, given the key role of CP190 in the regulation of nuclear architecture, it can be assumed that CP190 deficiency leads to failures in the communication of specific transcription factors and their regulatory elements and general alterations in chromatin structure, which causes disruptions in the fine-tuning mechanisms of expression regulation.

In our study, we found that CP190 depletion leads to inactivation of tMAC-dependent differentiation genes. Notably, in *Drosophila* somatic cells, repressor complex dREAM, homologous to tMAC, whose main function is to regulate differentiation and cell cycle genes, functions as partner of CP190 [44–47]. The interaction of CP190 and dREAM in cell cultures plays an important role in the control of divergent gene pairs [46]. CP190-dependent recruitment of dREAM to divergent genes in *Drosophila* enables the reduction of downregulation of single gene in pair. The presence of common subunits (e.g. Mip40 and Caf1) in dREAM and tMAC, as well as the relationship between CP190 and tMAC-dependent genes, allows us to speculate that CP190 plays a role in tMAC recruitment to chromosomes. Future studies in this direction could explain why the main effect of CP190 depletion in male germline is to inhibit the activity of spermatocyte differentiation genes.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. The conditions in which *Drosophila melanogaster* was kept complied with the standards specified in Order of the Ministry of Health of Russia no. 267 of June 19, 2003 "On Approval of the Rules of Laboratory Practice in the Russian Federation."

SUPPLEMENTARY INFORMATION

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ADDITIONAL INFORMATION

The text was submitted by the author(s) in English.

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