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The nematode homologue of Mediator complex subunit 28, F28F8.5, is a critical regulator of *C. elegans* development

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The evolutionarily conserved Mediator complex is a critical player in regulating transcription. Comprised of approximately two dozen proteins, Mediator integrates diverse regulatory signals through direct protein-protein interactions that, in turn, modulate the influence of Mediator on RNA Polymerase II activity. One Mediator subunit, MED28, is known to interact with cytoplasmic structural proteins, providing a potential direct link between cytoplasmic dynamics and the control of gene transcription. Although identified in many animals and plants, MED28 is not present in yeast; no bona fide MED28 has been described previously in *C. elegans.* Here, we identify bioinformatically F28F8.5, an uncharacterized predicted protein, as the nematode homologue of MED28. As in other metazoa, F28F8.5 has dual nuclear and cytoplasmic localization and plays critical roles in the regulation of development. *F28F8.5* is a vital gene and its null mutants have severely malformed gonads and do not reproduce. Our results indicate that F28F8.5 is a homologue of MED28 and suggest that the potential to link cytoplasmic and nuclear events is conserved between MED28 vertebrate and nematode homologues.

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ABSTRACT

The evolutionarily conserved Mediator complex is a critical player in regulating transcription. Comprised of approximately two dozen proteins, Mediator integrates diverse regulatory signals through direct protein-protein interactions that, in turn, modulate the influence of Mediator on RNA Polymerase II activity. One Mediator subunit, MED28, is known to interact with cytoplasmic structural proteins, providing a potential direct link between cytoplasmic dynamics and the control of gene transcription. Although identified in many animals and plants, MED28 is not present in yeast; no bona fide MED28 has been described previously in *C. elegans.* Here, we identify

bioinformatically F28F8.5, an uncharacterized predicted protein, as the nematode homologue of MED28. As in other metazoa, F28F8.5 has dual nuclear and cytoplasmic localization and plays critical roles in the regulation of development. *F28F8.5* is a vital gene and its null mutants have severely malformed gonads and do not reproduce. Our results indicate that F28F8.5 is a homologue of MED28 and suggest that the potential to link cytoplasmic and nuclear events is conserved between MED28 vertebrate and nematode homologues.

Abbreviations: *F28F8.5* – gene coding for protein F28F8.5, *F28F8.5a* – splice form a, F28F8.5a – protein form a, *F28F8.5b* – splice form b, F28F8.5b – protein form b, gDNA – genomic DNA, $P_{F28F8.5}$*(V:15573749)::gfp::F28F8.5* – edited *F28F8.5* with *gfp* tagged to the N – terminus in the position *V:15573749*, $P_{F28F8.5}$ *(V:15573749)::gfp::let858(stop)::SEC::F28F8.5* – edited F28F8.5 disrupted by GFP and SEC (Self Excision Cassette), $P_{F28F8.5(400\ bp)}$*::F28F8.5::gfp* - F28F8.5 tagged with *gfp* on its C-terminus regulated by its predicted internal promoter with size of 400 bp upstream of ATG, GFP::F28F8.5 – protein F28F8.5 tagged on its N-terminus with GFP, F28F8.5::GFP – protein F28F8.5 tagged on its C-terminus with GFP, *MED28* – vertebrate Mediator complex subunit 28 gene, MED28 – vertebrate Mediator complex subunit 28 protein, Med28 – Mediator complex subunit 28 in a general sense; this denomination is also used in *Drosophila* and mouse gene nomenclature.

INTRODUCTION

The Mediator complex is a multiprotein assembly that is capable of integrating cellular signals with the regulation of transcription through direct interaction with RNA Polymerase II. The Mediator complex is found in all eukaryotic organisms. The Mediator complex is comprised of 31 protein subunits in yeast and a similar number in mammals, each named MED followed by a unique numerical designation (Poss et al. 2013; Allen & Taatjes 2015). The Mediator complex likely co-evolved with basal transcription factors with a level of conservation between different phyla that is relatively low (Poss et al. 2013; Allen & Taatjes 2015). While most Mediator subunits are present in similar molar ratios and comprise the core complex, some subunits were found to be present in variable amounts when complexes are isolated from tissue culture cells (Kulak et al. 2014). Quantification of proteomically analyzed Mediator subunits showed that distinct forms of the complex have variable transcriptional activity (Paoletti et al. 2006) and analysis of Mediator complex subunits in *Drosophila* indicated that some subunits are critical only for specific gene transcription from endogenous genes but not for transcription from synthetic promoters (Kim et al. 2004). This diversity in Mediator subunit composition allows variable interactions with transcription factors and Pol II, greatly expanding its possible regulatory roles in controlling transcription.

One of the Mediator complex subunits, MED28, is only found in higher eukaryotes and has other distinguishing features among the MED subunits. *MED28* was originally identified as a gene expressed in endothelial cells and was named EG-1 (Endothelial-derived Gene-1) (Liu et al. 2002); it was later shown to be part of the Mediator complex and named MED28 (Brower et al. 2002; Sato et al. 2004). MED28 has several cytoplasmic-associated interactions, in addition to being part of Mediator and regulating transcription. MED28 was shown to associate with the actin cytoskeleton and linked to the regulation of smooth muscle genes (Wiederhold et al. 2004).

MED28 has also been shown to associate with several Src-family kinases and is a target of their phosphorylation (Lee et al. 2006). In addition, MED28 interacts at the plasma membrane with Grb2 and Merlin (also called Neurofibromin 2 or Schwannomin), a membrane-cytoskeleton scaffolding protein linking actin filaments to the cell membrane (McClatchey & Giovannini 2005; McClatchey & Fehon 2009). These many and diverse cytoplasmic interactions suggest that MED28 serves as a multi-faceted adaptor/scaffold to relay cellular signaling from the cytoskeleton to transcription in the nucleus (Lee et al. 2006).

Although conserved between insects and mammals, a bona fide MED28 homologue had yet to be identified in nematodes. Our previous work showed that the protein previously identified as "MDT-28" (**Med**ia**t**or-28) in nematode and other databases is instead the nematode homologue of perilipin, a protein regulating lipid metabolism at the level of lipid droplets and is not related to MED28 (Chughtai et al. 2015). Thinking it was unlikely that a MED28 homologue would be absent in nematode genomes, we searched for it using conserved features of MED28 orthologues from various phyla. Herein we identify a previously uncharacterized protein, F28F8.5, as the closest MED28 homologue. We show that F28F8.5 is both a nuclear and a cytoplasmic protein present in most, if not all, tissues throughout development. Down-regulation by RNAi, or disruption of *F28F8.5* by deletion, results in multiple developmental defects during embryonic and larval development. Our work indicates that the homologue of Mediator complex subunit 28 exists in nematodes and suggests that the potential to link cytoplasmic and nuclear events is conserved between MED28 vertebrate and nematode homologues.

MATERIALS AND METHODS

Sequence analysis

The UniProtKB (uniprot.org) and NCBI (ncbi.nlm.nih.gov) databases were searched with BLAST, PSI-BLAST (Altschul et al. 1997), HHblits (Remmert et al. 2011) and HHpred (Soding et al. 2005) programs. The protein sequences were identified with their UniProtKB identifiers and the nucleotide sequences with their NCBI ones. The sequences were aligned with T-coffee (Notredame et al. 2000; Di Tommaso et al. 2011) and PROMALS (Pei & Grishin 2007; Pei et al. 2007; Pei et al. 2008). The secondary structure predictions were performed with PSIPRED (Jones 1999; Cuff & Barton 2000; McGuffin et al. 2000). Multiple sequence alignments were displayed and analyzed with Jalview (Clamp et al. 2004).

RNA isolation and cDNA synthesis

RNA and cDNA were prepared as described (Zima et al. 2015) with modifications. Cultured nematodes were collected in water and pelleted by centrifugation for 5 min at 110 xg and 4 ºC. The excess of water was removed and the pellet was frozen at -80 °C. For the isolation of RNA, the pellet was quickly melted and dissolved in 300 µl of resuspension buffer (10 mM Tris-HCl; 10 mM EDTA, 5% 2- mercaptoethanol; 0.5% SDS; pH 7.5). After adding 8 µl of proteinase K (20 mg/ml), the sample was mixed and incubated 1 h at 55 °C. RNA was isolated by phenol-chloroform extraction and ethanol precipitation. The obtained RNA was incubated with RQ1 DNase (Promega, Fitchburg, WI, USA) and purified again by phenol-chloroform extraction and ethanol precipitation. Complementary DNA (cDNA) was prepared with SuperScript III (Invitrogen, Carlsbad, CA, USA) using random hexamers.

Strains, transgenic lines and genome editing

The *C. elegans* Bristol N2 strain was used whenever not specifically stated and maintained as described (Brenner 1974).

Preparation of $P_{F28F8.5(400bp)}$::*F28F8.5::gfp*

For preparation of transgenic lines expressing F28F8.5::GFP from extrachromosomal arrays under regulation of endogenous promoter, we used the PCR fusion-based technique (Hobert 2002). Primers 7886 and 7888 were used for amplification of the genomic sequence of *F28F8.5* (consisting of approximately 400 bp of the predicted promoter region preceding the coding region of *F28F8.5*). The gene encoding GFP was amplified from the pPD95.75 vector with primers 6232 and 6233. The complete construct was amplified with primers 7887 and 6234. The resulting fusion construct contained the 3' UTR from pPD95.75 (originally from the *unc-54* gene). The PCR mixture was injected into the gonads of young adult hermaphrodite animals together with marker plasmid pRF4. The sequences of all primers used in the paper are in supplementary information.

Genome editing

Lines with edited genomes were prepared from wild type N2 animals using the CRISPR/Cas9 system as described (Dickinson et al. 2013; Dickinson et al. 2015; Ward 2015; Dickinson & Goldstein 2016). Using this strategy, the *F28F8.5* gene was edited by insertion of a construct including the coding sequence of GFP and a self-excision cassette containing the *sqt-1(d)* gene (a visible selection marker leading to a Rol phenotype), *hs::Cre* (heat shock inducible Cre recombinase) and hygR (hygromycin resistance) genes. The sgRNA sequence was targeted near the start of the coding sequence for F28F8.5 gene using the pJW1219 plasmid (Addgene,

Cambridge, MA, USA) as the Cas9 vector (pMA007). It was prepared by PCR with primers 8403A and 8333 and used in a concentration of 50ng/µl for microinjections. The plasmid pMA007 was co-injected with the rescue repair template plasmid based upon pDD282 vector (pMA006) in a concentration of 10 ng/µl and with 3 markers (see below). The repair template plasmid pMA006 was prepared in two steps. First the plasmid pMA005 was prepared from gDNA of *F28F8.5* (containing both repair arms) and amplified by PCR with primers 8404 and 8405 and cloned into pCU19 backbone. The plasmid pMA005 was subsequently modified – the FP-SEC segment was added and the CRISPR/Cas9 site was altered to protect against Cas9 attack. The linear PCR product of pMA005 was prepared using primers 8406 and 8407 with overlapping regions for Gibson assembly (New England *BioLabs*, Ipswich, MA, USA). The primer 8406 was prepared with alternate codons for protection against CRISPR/Cas9 site. Linear insert of FP-SEC was prepared by PCR from pDD282 plasmid (Addgene) with primers 8408 and 8409. Primers were prepared with overlapping parts for cloning into linear pMA005 plasmid by Gibson assembly and the final rescue plasmid pMA006 was prepared. Plasmids pGH8 (10 ng/µl), pCFJ104 (5 ng/µl) and pCJ90 (2.5 ng/µl) (Addgene) were used as fluorescent coinjection markers. After microinjections the population of nematodes were grown for 3 days at 25º C and Hygromycin (Invitrogen) was added in a final concentration of 250 µg/ml. After 3 days integrated nematodes were selected according to the rolling phenotype and loss of extrachromosomal arrays.

Using this strategy, we obtained heterozygous lines with a disrupted *F28F8.5* gene with an inserted *gfp* regulated by the endogenous promoter of *F28F8.5* in one allele. Homozygous animals for $P_{F28F8.5}$*::gfp* (edited F28F8.5 – $P_{F28F8.5}$ *(V:15573749)::gfp::let858(stop)::SEC::F28F8.5*) with disrupted *F28F8.5* on both alleles and expressing GFP under the regulation of the endogenous promoter) were clearly distinguishable by their phenotypes and the presence of *rol* marker. These

184 animals were sterile and had severe developmental defects (see results). The particular genotypes
185 were controlled by single worm PCR of representative animals after their microscopic analysis.
186 The excision of the Self Excision Cassette segment was achieved by a 4 hours heat shock
187 at 34º C. Three different lines were obtained: animals with one WT allele and one allele of
188 integrated *gfp::F28F8.5* in its normal genomic position ($P_{F28F8.5}$*(V:15573749)::gfp::F28F8.5* –
189 edited *F28F8.5* with *gfp* tagged to the N – terminus in the position *V:15573749*, homozygous
190 animals with both alleles carrying integrated *gfp::F28F8.5*, and animals with both WT alleles of
191 *F28F8.5.*
192 The presence of knock-in of *gfp* was confirmed by single nematode PCR with primers 7887
193 and 8454. The PCR product was purified and sequenced with primers 8455 and 8456. PCR was
194 done by REDTaq ReadyMix PCR reaction (Sigma-Aldrich, St. Louis, Missouri, USA) and or by
195 Phusion High/Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA).
196

197 **Down-regulation of gene expression by RNA interference**

198 For RNAi using microinjecitons, *F28F8.5* cDNA was prepared from total cDNA using
199 primers 7889 and 7890. The plasmid pPCRII(Topo) (Invitrogen, Carlsbad, Ca, USA) containing
200 *F28F8.5b* cDNA was linearized using restriction enzymes NotI / SacI. The dsRNA was prepared
201 by in vitro transcription using SP6/T7 Riboprobe® in vitro Transcription Systems (Promega,
202 Madison, WI, USA) from opposing promoters synthesizing complementary single stranded RNA
203 (ssRNA) for both strands of F28F8.5 cDNA and its complementary strand. After in vitro
204 transcription (~2 hours) equal volumes of sense and antisense RNA were mixed, incubated at 75
205 °C for 10 min and slowly cooled to room temperature during 30 min. Control RNAi was prepared
206 from the promoter region of *nhr-60* as previously described (Simeckova et al. 2007). The dsRNA

207 concentration was measured using a UV spectrophotometer and diluted to the concentration of ~2
208 µg/µl that was used for injections (Tabara et al. 1999; Timmons et al. 2001; Vohanka et al. 2010).
209 For feeding RNAi, Standard Nematode Growth Medium (NGM) agar plates were prepared
210 and supplemented with Ampicillin (100 µg/ml final concentration) and isopropyl β-D-
211 1-thiogalactopyranoside (IPTG,1.5 mM final concentration). *E. coli* strain HT115 was transformed
212 with plasmids containing cDNA of *F28F8.5* in L4440 vector or only empty L4440 vector (for
213 control). After transformation, a single colony from each culture was grown in liquid culture
214 containing LB medium with Ampicillin (100µg/ml final concentration) and IPTG (1.5 mM final
215 concentration). The culture was grown to $OD_{600} \approx 1.0$ and 1ml of culture was spread over NGM
216 agar plates to completely cover the surface and excess (300 µl) was removed. The bacteria were
217 allowed to grow and were induced overnight at room temperate (~22 °C).
218

219 ## Microinjections

220 Microinjections of plasmids, DNA amplicons or dsRNA into gonads of young adult
221 hermaphrodites were done using an Olympus IX70 microscope equipped with a Narishige
222 microinjection system (Olympus, Tokyo, Japan). The plasmids were injected into the gonads of
223 young hermaphrodites as described (Tabara et al. 1999; Timmons et al. 2001; Vohanka et al. 2010).
224

225 ## Microscopy

226 Fluorescence microscopy and Nomarski optics microscopy were done using an Olympus
227 BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). Animals were
228 analyzed on microscopic glass slides with thin layer of 2% agarose and immobilized by 1mM
229 levamizole (Sigma-Aldrich, St. Louis, MO, USA). Confocal microscopy of live homozygous

animals with edited F28F8.5 expressing GFP::F28F8.5 was performed using an inverted Leica SP8 TCS SMD FLIM system equipped with a 63x 1.2 NA water immersion objective, a pulsed white light laser (470-670 nm), AOBS and two internal hybrid single photon counting detectors, and operated by Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany). The GFP fluorescence was excited at a wavelength of 488 nm and the emitted light was simultaneously recorded in two spectral ranges (Channel 1 – 495 nm to 525 nm, Channel 2 – 525 nm to 580 nm; the two channel setup was used to help resolve between spectrally different autofluorescence and GFP fluorescence signals).

Fluorescence-lifetime imaging microscopy (FLIM)

For FLIM acquisitions the single photon counting signal from the internal hybrid detectors, acquired during confocal acquisitions, was simultaneously processed by HydraHarp400 TCSPC electronics (PicoQuant, Berlin, Germany) and information about the arrival times of all photons was stored to a hard-drive in TTTR data format. The fast FLIM images were reconstructed using home written software "TTTR data analysis". The signal from both time synchronized channels was added up. The false color scale (1 to 3 ns) is based on the average photon arrival time, with blue color representing to the short lifetime and red color to the long lifetime fluorescence.

Single nematode PCR

Single animal PCR was used for verification of all transgenic lines. Following the microscopy examination, selected animals were removed from microscopic slides and transferred into caps of PCR tubes. The sample was diluted in a solution of Proteinase K (20 mg/ml) diluted in 1:333 in

Barstead Buffer (resulting in Barstead Lysis Buffer) and was kept on ice. The tube was sealed in bottom-up position and the sample transferred to the bottom of the tube by centrifugation. The tube was frozen for 10 min. at -70º C. Next, the tube was heated for 1 hour at 60º C and additional 15 min. at 95º C. The resulting sample was used immediately for amplification of DNA by PCR or stored at -80º C before further analysis. Barstead Buffer consists of 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% (v/v) NP40 (Nonidet P-40), 0.45% (v/v) Tween-20, 0.01% (w/v). Proteinase K was diluted immediately before use as a 20 mg/ml stock solution which was kept on ice and diluted to final working solution at concentration of 200 µg/ml. Similarly, from selected nematode culture plates genomic DNA was prepared and used for further screening by PCR and sequencing.

The resulting precipitated DNA was dissolved in 10 µl of deionized water and used for amplification by PCR using primers outside the edited genomic regions. Specificity of amplification was controlled by DNA sequencing.

Similarly, homozygous animals with edited F28F8.5 (with gfp inserted behind the START codon) were analyzed by single worm PCR with primers #7887 and #8454.

RESULTS

Identification of the closest homologue of vertebrate Mediator complex subunit 28 in *C. elegans*

To identify a *C. elegans* homologue of MED28, we queried protein databases with curated SwissProt sequences from UniProtKB. They comprised several mammalian and insect proteins

(e.g. human MED28_HUMAN and *D. melanogaster* MED28_DROME). The more sensitive profile-to-profile HHblitz and HHpred algorithms provided hits to a *C. elegans* annotated protein F28F8.5a and b with highly significant E-values. According to Wormbase (WS 248), two protein isoforms are produced from the *F28F8.5* gene, isoform a with the length of 200 amino acids and isoform b that has a two amino acid insertion at position 20 of the N-terminal evolutionarily non-conserved region. The best results were obtained when pre-aligned vertebrate and insect MED28 paralogues were used as query in 3 iterations ($E<10^{-48}$ and the probability of true positive >99.99%). When the pre-aligned nematode sequences homologous to F28F8.5 were used to query profiles of human or *Drosophila* sequences in reciprocal searches, MED28 proteins were obtained with equally significant scores. BLAST and PSI-BLAST searches in their standard settings were not able to reveal a significant hit ($E<10^{-3}$); the only nematode hit was a *Trichinella spiralis* protein (E5RZQ1). However, when the searches in protein databases were limited to sequences from *Ecdysozoa* with *Insecta* excluded (conservative inclusion threshold $E<10^{-6}$) in the first two iterations, F28F8.5 provided human and *Drosophila* MED28. PSI-BLAST with *T. spiralis* query sequence in database limited to *Ecdysozoa* in the first 2 iterations provided both human and *Drosophila* MED28 and F28F8.5 in one run ($E<10^{-8}$). We concluded from these searches that F28F8.5 is a homologue of MED28 and very likely its previously unrecognized orthologue.

All PSI-BLAST MED28 homologues possess variable N- and C-termini of 3 to 80 amino acids showing no conservation across *metazoa*. This conservation is loose even just within *Drosophilae* or *Caenorhabditae* sequences. Only the central core of about 110 amino acids is preserved in metazoan evolution. **Fig. 1** shows a sequence alignment of this conserved core of selected MED28 homologues. All sequences are predicted to fold into 3 helices forming a putative coiled coil fold (UniProt annotation). Submitting the alignment shown in **Fig. 1** to HHpred for 3D

structure recognition reveals a structural fold of yeast MED21 (PDB identifier 1ykh_B). It is indeed a 3-helix coiled coil forming a heterodimer with MED7. It can be expected that MED28 forms a very similar fold interacting with a yet to be determined subunit of the MED complex.

F28F8.5 is a nuclear as well as a cytoplasmic protein

Information available in WormBase indicates that the *F28F8.5* gene can be expressed as both an individual and multigene transcript, located as the last gene in a four gene operon that is both SL-1 and SL-2 trans-spliced. To determine the intracellular localization of F28F8.5, we edited the *F28F8.5* gene using CRISPR/Cas9 technology. We inserted the gene coding for GFP directly after the first codon. The arrangement used in our experiment (based on (Dickinson et al. 2013; Dickinson et al. 2015; Ward 2015; Dickinson & Goldstein 2016)) employed a self-excision cassette added after *gfp*. This strategy initially created a disrupted *F28F8.5* gene and putative null allele that can be detected by expression of GFP alone regulated by the endogenous promoter elements of *F28F8.5*, including those of the entire operon. We found that only heterozygous animals could be propagated due to the sterility of homozygotes tagged in this manner. Assuming this tag is not deleterious to the expression of other genes in the operon, this result suggests that *F28F8.5* is an essential gene.

After removal of the self-excision cassette from this edited *F28F8.5* gene induced by heat shock (visualized by continuous expression of GFP::F28F8.5 fusion protein and loss of the rolling phenotypic marker), the endogenous locus had an N-terminus GFP-tagged F28F8.5 gene that we maintained as homozygous animals, demonstrating this edited allele is fully functional. Note that both known protein isoforms of F28F8.5 (a and b) would be tagged on their N-terminus with GFP by this method.

The GFP::F28F8.5 pattern was both nuclear and cytoplasmic from embryos to adults (**Fig. 2**). Prominent nuclear localization was found in oocytes, zygotes, larvae, and adults. Cells with clear nuclear accumulation of GFP::F28F8.5 included epidermal, intestinal, pharyngeal, uterine and vulval muscle cells (**Fig. 2**). The gonad expressed *gfp::F28F8.5* and mitotic as well as meiotic nuclei accumulated GFP::F28F8.5 (**Fig. 2**).

Selected animals were analyzed by confocal microscopy for determination of subcellular distribution of GFP::F28F8.5. Scanning through several focal planes revealed signal in the GFP excitation/emission range in nuclei as well as in the cytoplasm of embryos, all larval stages and adults (**Fig. 3**). Structures resembling gut granules were also strongly positive in the GFP recording mode. In order to distinguish between GFP-specific fluorescence and autofluorescence from the intestinal cells, we applied FLIM with an expectation that autofluorescence (such as that from gut granules) is likely to produce a signal with a short fluorescence life time opposed to GFP-specific fluorescence. Structures such as gut granules could be clearly detected (**Fig. 3**, panels O, Q, S, T and U, blue color) while fluorescence with a longer life time expected for GFP::F28F8.5 was detected in the germline, in oocytes and embryos and in most somatic nuclei of larvae as well as adult animals (**Fig. 3**, panels O, Q, S, T and U, red and yellow colors).

We also generated transgenic lines expressing F28F8.5::GFP from extrachromosomal arrays consisting of endogenous internal *F28F8.5* promoter regulating a fusion gene with *gfp* attached to *F28F8.5* on its 3' terminus. As with the N-terminally tagged F28F8.5, F28F8.5::GFP showed both nuclear and cytoplasmic localization. As expected for an extrachromosomal transgene, the expression of *F28F8.5::gfp* was not detected in the germline. This reporter was expressed in embryos starting at the 2-fold stage and continued throughout development (**Supplementary Fig. S1).** We did notice that *F28F8.5::gfp* was expressed in the excretory cell

and its channels (**Supplementary Fig. S1** S and T), a pattern not observed with the endogenously edited GFP-tagged gene.

F28F8.5 regulates development

To achieve loss-of-function, RNAi was used to down-regulate *F28F8.5* expression. Analysis of 2567 progeny of 17 young adult hermaphrodites inhibited for *F28F8.5* function by microinjection of dsRNA into the syncytial gonad revealed that F28F8.5 was essential for proper development (**Fig. 4**). From the total progeny, 1127 animals were affected (44 %) exhibiting embryonic and larval arrest and a range of less severe phenotypes, including defective molting, protruding vulvae that often burst, male tail ray defects, and uncoordinated (Unc) movement (**Fig. 4**).

Complete loss of F28F8.5 that occurred in homozygous animals with both disrupted alleles of F28F8.5 gene (that are found among the progeny of heterozygous animals carrying one disrupted allele and one edited allele *gfp::F28F8.5*) resulted in sterility. However, these animals were able to develop to adults. This demonstrates that a single maternal allele provides sufficient gene product to get homozygous null animals through much of development, but was insufficient for proper somatic and germline gonad development (**Fig. 5**). The gonad was irregular, contained masses of undifferentiated tissue and showed problems of directional growth. Defective vulva formation was also observed.

DISCUSSION

Mediator complex as a multiprotein structure is able to interact with a large number of additional proteins and integrate regulatory signals from several cell signaling cascades (Allen & Taatjes 2015). The steric organization of the Mediator complex is dynamic and allows variable arrangement of its subunits (Poss et al. 2013). Individual subunits of the Mediator complex can be divided into essential subunits, present in many or perhaps all Mediator complexes and proteins that are present only in some complexes and participate in specific more restricted transcription regulation (Paoletti et al. 2006; Kulak et al. 2014). Med28 is a subunit that belongs to the latter group and has a special position in-between Mediator subunit proteins for its dual regulatory role, one as a Mediator subunit (Sato et al. 2004; Beyer et al. 2007) and the second which is cytoplasmic at the level of the cytoskeleton (Gonzalez-Agosti et al. 1996; Wiederhold et al. 2004; Lee et al. 2006; Lu et al. 2006; Huang et al. 2012). This suggests that Med28 may be able to bring cytoplasmic regulatory interactions towards the regulation of gene expression.

In this work, we identified an uncharacterized predicted protein F28F8.5 as the nematode homologue of MED28. Our experiments support the fundamental role of F28F8.5 in developmental events. Keeping with its expected function as a component of the Mediator complex, downregulation of F28F8.5 by RNAi led to a wide range of defects during embryonic as well as larval development. In contrast to downregulation of F28F8.5 by RNAi, null mutants with disrupted *F28F8.5* gene found in the progeny of heterozygous animals with one disrupted allele and one WT allele or one disrupted allele and one edited allele coding for GFP::F28F8.5 were able to reach adulthood. RNAi is a powerful tool for gene downregulation that can be applied to studied animals at specific developmental times and affects the gene expression in a wide range of cells including the germline. While heterozygous animals with one functional allele of *F28F8.5* supply their embryos with maternal transcripts, the embryos in the progeny of parents with F28F8.5

downregulated by RNAi are devoid of this maternal load. This shows that the normal or edited F28F8.5 supplied as maternal load from heterozygous parents is sufficient for null mutant animals to proceed throughout most of developmental events vitally dependent on F28F8.5. The amount of F28F8.5 inherited as maternal load is, however, not sufficient for normal development of gonad and sex organs and the homozygous null mutant animals do not produce viable embryos. Most phenotypes that we observed in our RNAi experiments are listed in Wormbase (WS 254) based on high throughput screens (Kamath & Ahringer 2003; Simmer et al. 2003; Frand et al. 2005; Sonnichsen et al. 2005). Nuclear localization and the wide expression of F28F8.5 was previously reported and is also listed in Wormbase (WS254) (Mounsey et al. 2002; Matus et al. 2010). The indispensability of F28F8.5 during embryonic development is similar to findings reported for Med28 (Li et al. 2015). The wide expression pattern of F28F8.5 is also keeping with the data reported in the Mouse Genome Database (http://www.mousephenotype.org/data/genes/MGI:1914249, accessed on October 5, 2016) (Eppig et al. 2015) and in Human Protein Atlas (http://www.proteinatlas.org, accessed on October 7, 2016) (Uhlen et al. 2015).

Based on the closest sequence similarity of F28F8.5 to MED28 that can be detected informatically in nematode genomes, conserved dual nuclear and cytoplasmic expression and involvement in a wide range of developmental processes, F28F8.5 is named (with WormBase approval) as MDT-28. W01A8.1, which was originally denominated also MDT-28 is re-named as PLIN-1 (Chughtai et al. 2015).

MED28 is a candidate Mediator complex subunit linking cytoplasmic structural signals towards the core of transcription regulation. The connection between cytoplasmic events and

412 regulation of gene expression can be seen at frequent situations. Numerous transcription factors
413 are regulated by their spatial restriction, binding or incorporation into cytoplasmic structures and
414 organelles. Many proteins that have primarily cytoplasmic structural functions were shown to
415 possess transcription regulating activity (e.g. proteins interacting with steroid receptors (Biddie &
416 Hager 2009), FOX transcription factors (Gan et al. 2005; Wang et al. 2015), and BIR-1/Survivin
417 (Kostrouch et al. 2014)).

418 The direct link between effector proteins and the regulation of transcription can be traced
419 to Eubacteria and Archaea. Lrp/AsnC proteins, metabolic effectors in Archaea and related Lrs14
420 proteins are serving as multipotent (Lrp) and specific (Asn) regulators of gene expression. Lrs14
421 has a clear negative autoregulatory potential illustrating the ancient origin of the transcriptional
422 function of effector proteins (Bell & Jackson 2000; Thaw et al. 2006; Orell et al. 2013). Similarities
423 between the core transcriptional machinery of Eukaryotes and Archaea can be clearly found
424 (Hirata & Murakami 2009). While the archaeal transcriptional complex seems to be sufficiently
425 dependent on two basal transcriptional regulators, TBP and TFB, Pol II dependent transcription,
426 in higher eukaryotes requires 5 (or 6) general transcription factors (reviewed in (Burton et al.
427 2016)) and the modular assembly of the Mediator complex at the promoters of regulated genes.
428 This modular complex is capable of linking the informatic network necessary for cells
429 differentiated to multiple cell types (or in other words multiple structural cell states) with gene
430 expression. MED28 homologues are thus likely to be able to bring cytoplasmic proteins to the core
431 of gene transcription. This may explain why MED28 evolved in multicellular eukaryotes
432 containing structurally differentiated cells.

433 In conclusion, MED28 homologues in vertebrates, insects and nematodes share similarities
434 indicating their conserved roles in cytoplasmic and nuclear events. It can be hypothesized that

435 many proteins that are primarily building blocks of cellular structures and structure-associated
436 proteins are likely to be part of regulatory loops that regulate gene expression. Similarly, as is the
437 case of evolution of operons in Rhabditida that are formed during evolution if they are biologically
438 tolerated for the sake of other regulatory or energetic gains (Qian & Zhang 2008; Blumenthal
439 2012), regulation by structural proteins may also be evolving for a limited number of structural
440 proteins leaving other structure-forming proteins available for evolution of other functions. The
441 homologues of MED28 in mammals, insects and nematodes therefore may be a link between
442 cellular structural states and regulation of gene expression.
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Figure 1

Multiple sequence alignment of selected metazoan homologues of MED28 compared with F28F8.5.

Aligned with PROMALS (http://prodata.swmed.edu/promals/promals.php), variable C- and N-termini not shown, amino acid residue types colored according to Clustal scheme in Jalview, red bars indicate consensus positions of predicted α-helices. Sequences from top to bottom (organism, identifier): *Caenorhabditis elegans*, O18692; *Trichinella spiralis*, E5RZQ1; *Wuchereria bancrofti*, EJW84794.1; *Pristionchus pacificus*, translated contig of CN657719.1 FG102945.1 CN657262.1 CN656622.1; *Ancylostoma ceylanicum*, A0A016SKV7; *Globodera pallida*, translated CV578368.1; *Panagrolaimus davidi*, translated JZ658977.1; *Ixodes scapularis*, B7PAW5; *Drosophila melanogaster*, MED28_DROME; *Homo sapiens*, MED28_HUMAN. Readers with specific color preferences may download the compared sequences from (**Supplementary File S1**) and create the Clustal scheme with different color specifications using the Jalview program (http://www.jalview.org/)

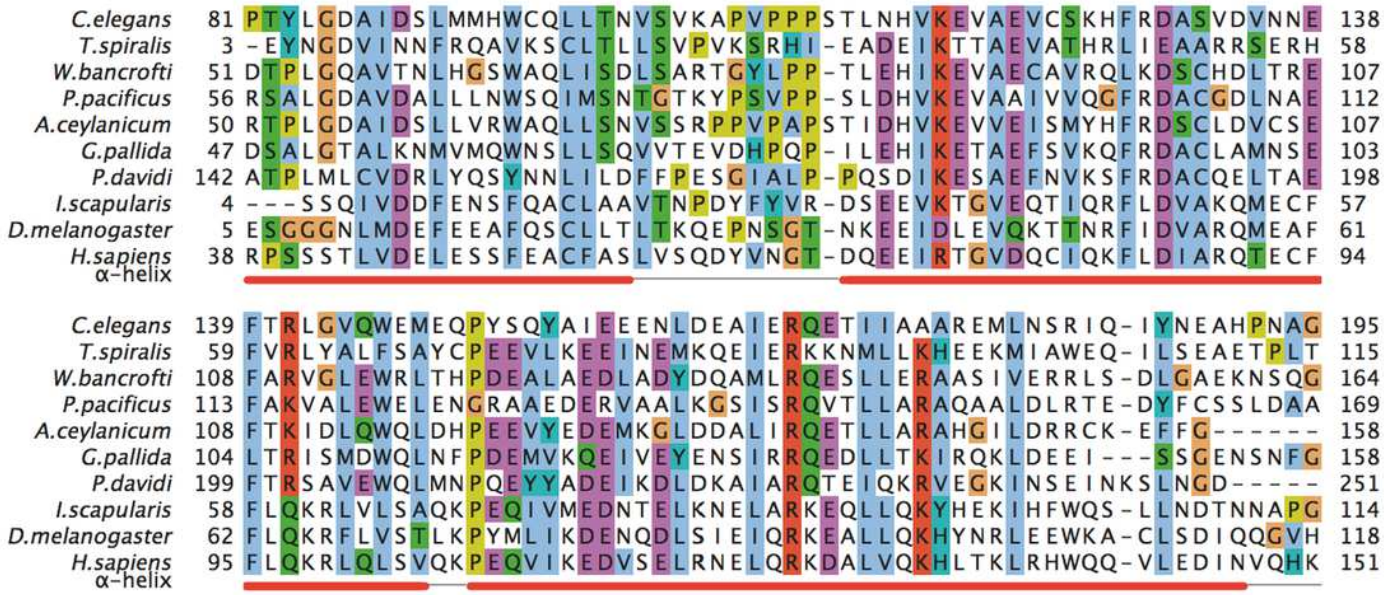

Figure 2

Expression pattern of GFP::F28F8.5 in homozygous animals with edited *F28F8.5* gene

GFP tagged to F28F8.5 at its N-terminus using CRISPR/Cas9 technology visualized the expression of F28F8.5 in the gonads (Panels B and D, arrows) in mitotic nuclei and continues throughout the embryonic development (Panels F, H, J and L). The expression of the edited gene is relatively faint and panels B, D, F, H, J and L are shown after contrast enhancement by the Auto Contrast tool of Adobe Photoshop program 7.0. The wide and likely ubiquitous expression of GFP::F28F8.5 continues during larval stages (larvae L3 and L4 are shown in panels M, N and O, P, respectively) as well as in adults (panels Q and R). Expression of the edited gene in the nuclei of the developing vulva is indicated by the arrow in panel N. Panels A, C, E, G, I, K, M, O and Q show larvae in Nomarski optics and panels B, D, F, H, J, L, N, P and R in GFP fluorescence. Bar represent 50 µm in panels A, B, E, F, M, N, O, P, Q, R and 10 µm in panels C, D, G, H, I, J, K, and L.

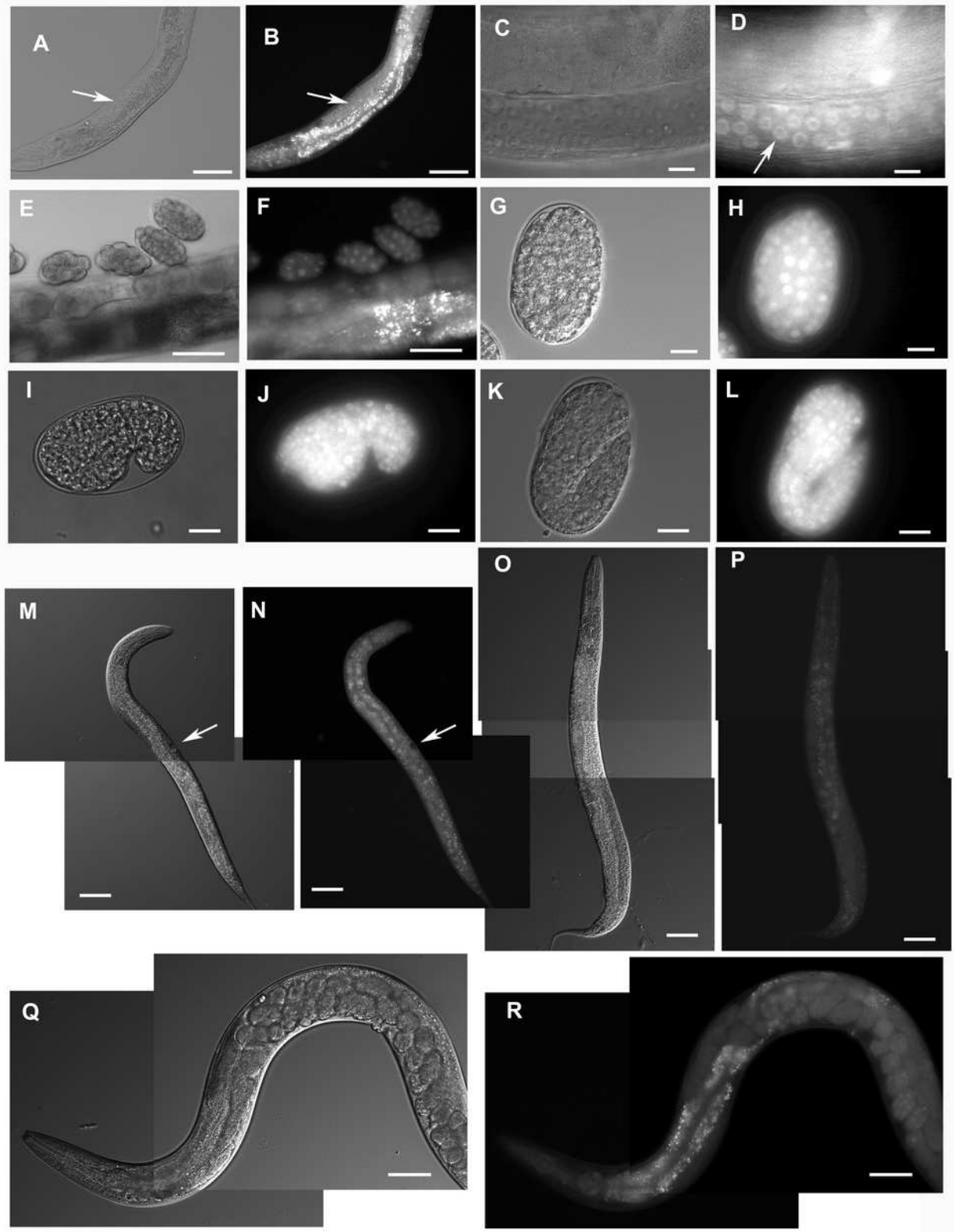

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Figure 3

Analysis of GFP::F28F8.5 expression in homozygous animals with edited *F28F8.5* gene by confocal microscopy and fluorescence lifetime imaging microscopy (FLIM).

All confocal images of GFP fluorescence (panels B, D, G, I, L,N and O) are recorded in Channel 1 (495 to 525 nm). The contrast of figures was enhanced by the Auto contrast tool of Adobe Photoshop program. Unprocessed images are accessible in Supplementary files. FLIM images (panels C, E, J, R, S, T, U and V) are calculated from merged recordings in Channel 1 and Channel 2 (525 nm to 585 nm). Panels A, F, H, K, M, P and Q are images in Nomarski optics at same optical focus as in corresponding confocal images of GFP fluorescence and FLIM. Panels A to E show control images of a WT embryo and the distal part of a WT L4 larva. Panels B and D show two confocal planes of GFP fluorescence and panels C and E corresponding FLIM images that show mostly short life time fluorescence in the cytoplasm of embryonic cells as well as cells and subcellular structures in the adult animal. Arrowheads pointing at the embryo in panels B and C indicate weak autofluorescence in the cytoplasm of all cells. Arrows in panels A, B and C indicate the turn of gonad and arrowheads in the distal part of the shown hermaphrodite animal in panels B and C indicate nuclei of an enterocyte which is devoid of almost all fluorescence. Panels G to V show animals with edited *F28F8.5* (*gfp::F28F8.5*) in Nomarski microscopy, confocal fluorescence microscopy and FLIM analysis. Panels F and G present Nomarski microscopy and a confocal image of GFP fluorescence of an embryo expressing GFP::F28F8.5 ubiquitously in nuclei and in the cytoplasm. Panels H to J show a distal part of an adult hermaphrodite animal at recording settings identical with that used in the control sample shown in panels B to E. FLIM analysis shows a long lifetime fluorescence in nuclei and in the cytoplasm of most cells that contrasts with the low level of fluorescence seen in the control sample. Arrowheads indicate nuclei of enterocytes. Panels K and L show an L1 larva with edited *F28F8.5* (panel K, Nomarski optics, panel L in confocal microscopy) with GFP signal seen ubiquitously in nuclei and in cytoplasm. Panel M (Nomarski

optics) and N (GFP confocal image) shows an L4 larva with GFP signal in uterine and vulval cells. The position of the vulva is indicated by arrowheads in panels M and N. Panel O shows a fluorescence confocal image of the central part of an adult hermaphrodite animal with two L1 larvae on both of its sides. Panels P and Q show two different focal planes in Nomarski optics of the same area as in panel O. Panels R, S, T, U and V show several confocal planes in FLIM analysis of the same areas. Panels P and Q pair with panels S and T. Panel W shows the calibration table for FLIM in the range of 1 to 3 ns used in all panels presenting FLIM analysis. Blue areas shown in FLIM pictures represent short life time fluorescence presumably corresponding to autofluorescence (blue arrows in panels O, R, S, T and V). Arrowheads in panels O, R, S and U indicate nuclei of enterocytes and in panel T nuclei of early embryos with long life time fluorescence characteristic for GFP. Bars represent 50 µm.

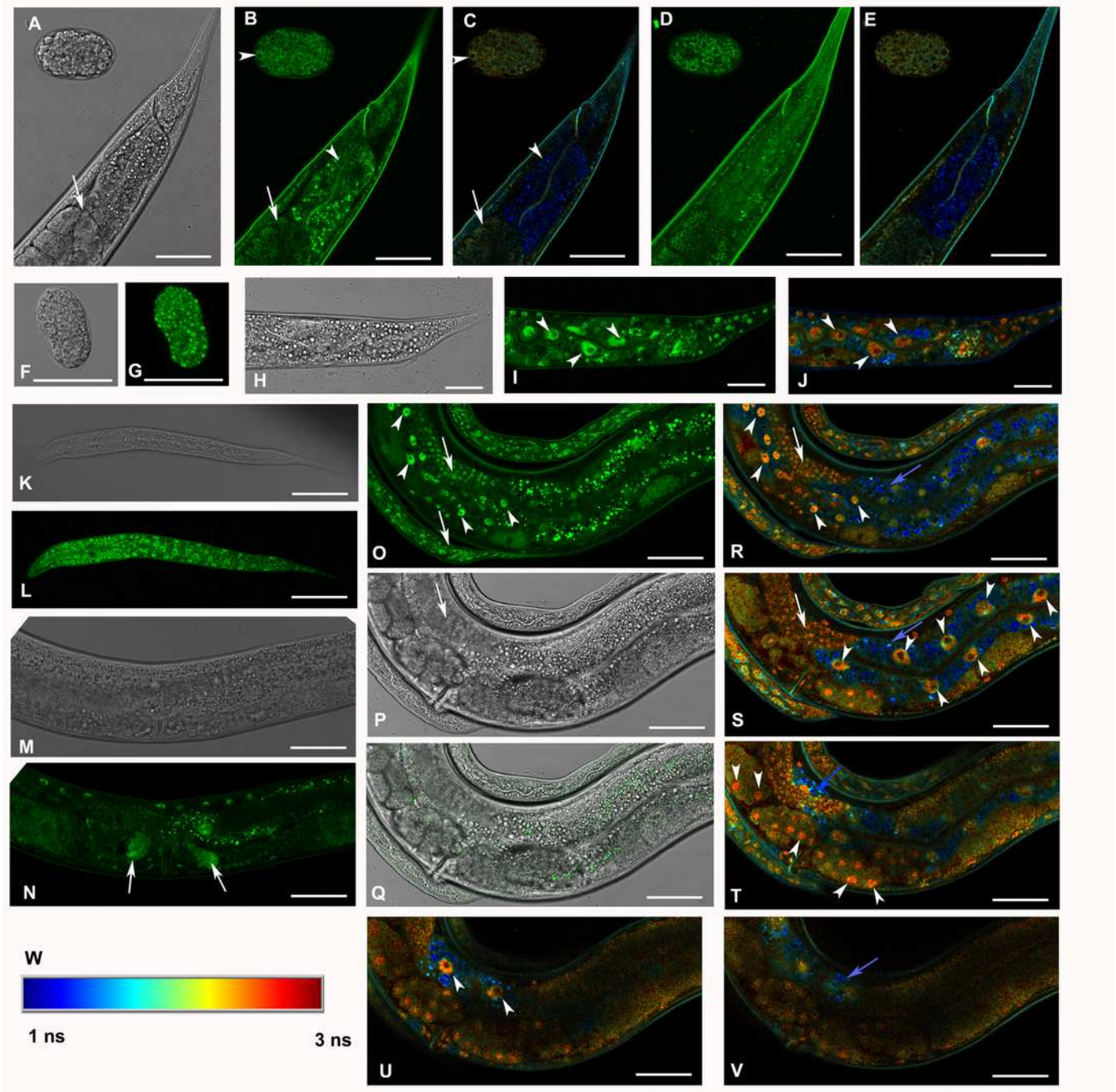

Figure 4

Down-regulation of *F28F8.5* by RNAi induces developmental defects.

Animals developed from parents injected with dsRNA specific for *F28F8.5* show retention of embryos (panel A), vacuoles (panels A and C, arrows) and herniation through the vulva (panels B and C, arrowheads). Defects of male specific structures - missing rays were seen (panel D and E, arrow). Some larvae were found atrophic, with signs of narrow enterocytes (panel F shows an L3 larva with dilated gut lumen (arrows). The dumpy phenotype with masses of tissue and vacuoles (panel G, arrows) were also common in the progeny of microinjected parents. Panel H shows an L3 or L4 larva with severe dilatation of gut lumen (arrows) and thin enterocytes (arrowheads). Panels I and J show a very frequent burst through vulva phenotype (arrowheads) and defective development of gonad (panel J). Panel K shows a hermaphrodite animal with retention of malformed embryos (arrow). Other phenotypes seen included molting defects indicated by arrows in panel L and cellular defects (indicated by arrowheads). Panel M shows an animal with defects of gonad (arrows) and small cellular defects (arrowheads). Bars represent 50 µm.

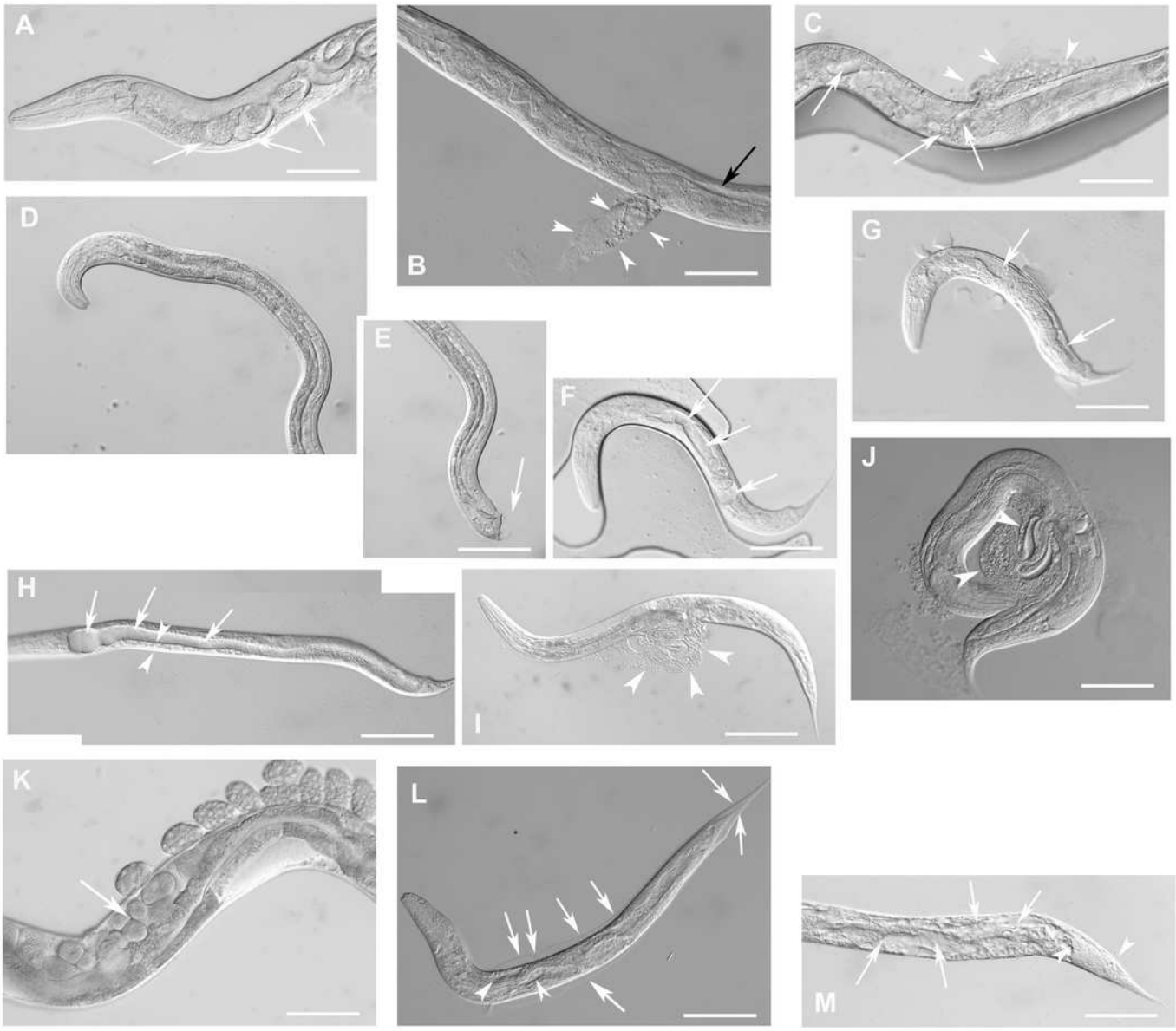

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Figure 5

Disruption of *F28F8.5* by CRISP/Cas9 technique.

Animals with disrupted *F28F8.5* on both alleles express GFP under the regulation of *F28F8.5* promoter. Panels A to J show paired images of animals in Nomarski optics and in GFP fluorescence. Panels A and B show an adult hermaphrodite animal with diffuse fluorescence in cells in the head area including anterior arms of the excretory cell (arrowheads). Panels C and D show a malformed larva probably in L3 stage with a Dpy phenotype and diffuse fluorescence in a malformed gonad (arrows) and the intestine (arrowheads). Panels E and F show an adult hermaphrodite animal with diffuse fluorescence in gut, pharyngeal cells and severely malformed gonad containing irregular structures (arrows). Panels G, H, I and J show an adult animal with a malformed gonad, Pvul phenotype, dense gut and diffuse GFP fluorescence throughout the body. Panels K and L show the central part of the body of a hermaphrodite with the Pvul phenotype (arrowhead) and malformation of gonad (arrow). Panel M is composed of three consecutive images showing an adult hermaphrodite animal with severely malformed gonad (arrows), missing uterus and malformed vulva. The fluorescence images show that unlike GFP::F28F8.5, GFP alone localizes diffusely in the cytoplasm and is not found in nuclei. Bars represent 50 µm.