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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37 Short title: MED28 homologue in *C. elegans*.

38 ABSTRACT

39 The evolutionarily conserved Mediator complex is a critical player in regulating transcription.

40 Comprised of approximately two dozen proteins, Mediator integrates diverse regulatory signals

41 through direct protein-protein interactions that, in turn, modulate the influence of Mediator on

42 RNA Polymerase II activity. One Mediator subunit, MED28, is known to interact with cytoplasmic

43 structural proteins, providing a potential direct link between cytoplasmic dynamics and the control

44 of gene transcription. Although identified in many animals and plants, MED28 is not present in

45 yeast; no bona fide MED28 has been described previously in *C. elegans*. Here, we identify

46   bioinformatically F28F8.5, an uncharacterized predicted protein, as the nematode homologue of

47   MED28. As in other metazoa, F28F8.5 has dual nuclear and cytoplasmic localization and plays

48   critical roles in the regulation of development. *F28F8.5* is a vital gene and its null mutants have

49   severely malformed gonads and do not reproduce. Our results indicate that F28F8.5 is a homologue

50   of MED28 and suggest that the potential to link cytoplasmic and nuclear events is conserved

51   between MED28 vertebrate and nematode homologues.

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55   Abbreviations: *F28F8.5* – gene coding for protein F28F8.5, *F28F8.5a* – splice form a, F28F8.5a

56   – protein form a, *F28F8.5b* – splice form b, F28F8.5b – protein form b, gDNA – genomic DNA,

57     $P_{F28F8.5}$*(V:15573749)::gfp::F28F8.5* – edited *F28F8.5* with *gfp* tagged to the N – terminus in the

58   position *V:15573749*, $P_{F28F8.5}$ *(V:15573749)::gfp::let858(stop)::SEC::F28F8.5* – edited F28F8.5

59   disrupted by GFP and SEC (Self Excision Cassette), $P_{F28F8.5(400\ bp)}$*::F28F8.5::gfp* - F28F8.5

60   tagged with *gfp* on its C-terminus regulated by its predicted internal promoter with size of 400 bp

61   upstream of ATG, GFP::F28F8.5 – protein F28F8.5 tagged on its N-terminus with GFP,

62   F28F8.5::GFP – protein F28F8.5 tagged on its C-terminus with GFP, *MED28* – vertebrate

63   Mediator complex subunit 28 gene, MED28 – vertebrate Mediator complex subunit 28 protein,

64   Med28 – Mediator complex subunit 28 in a general sense; this denomination is also used in

65   *Drosophila* and mouse gene nomenclature.

66

67   **INTRODUCTION**

68

69   The Mediator complex is a multiprotein assembly that is capable of integrating cellular

70   signals with the regulation of transcription through direct interaction with RNA Polymerase II.

71   The Mediator complex is found in all eukaryotic organisms. The Mediator complex is comprised

72   of 31 protein subunits in yeast and a similar number in mammals, each named MED followed by

73   a unique numerical designation (Poss et al. 2013; Allen & Taatjes 2015). The Mediator complex

74   likely co-evolved with basal transcription factors with a level of conservation between different

75   phyla that is relatively low (Poss et al. 2013; Allen & Taatjes 2015). While most Mediator subunits

76   are present in similar molar ratios and comprise the core complex, some subunits were found to be

77   present in variable amounts when complexes are isolated from tissue culture cells (Kulak et al.

78   2014). Quantification of proteomically analyzed Mediator subunits showed that distinct forms of

79   the complex have variable transcriptional activity (Paoletti et al. 2006) and analysis of Mediator

80   complex subunits in *Drosophila* indicated that some subunits are critical only for specific gene

81   transcription from endogenous genes but not for transcription from synthetic promoters (Kim et

82   al. 2004). This diversity in Mediator subunit composition allows variable interactions with

83   transcription factors and Pol II, greatly expanding its possible regulatory roles in controlling

84   transcription.

85   One of the Mediator complex subunits, MED28, is only found in higher eukaryotes and

86   has other distinguishing features among the MED subunits. *MED28* was originally identified as a

87   gene expressed in endothelial cells and was named EG-1 (Endothelial-derived Gene-1) (Liu et al.

88   2002); it was later shown to be part of the Mediator complex and named MED28 (Brower et al.

89   2002; Sato et al. 2004). MED28 has several cytoplasmic-associated interactions, in addition to

90   being part of Mediator and regulating transcription. MED28 was shown to associate with the actin

91   cytoskeleton and linked to the regulation of smooth muscle genes (Wiederhold et al. 2004).

92   MED28 has also been shown to associate with several Src-family kinases and is a target of their

93   phosphorylation (Lee et al. 2006). In addition, MED28 interacts at the plasma membrane with

94   Grb2 and Merlin (also called Neurofibromin 2 or Schwannomin), a membrane-cytoskeleton

95   scaffolding protein linking actin filaments to the cell membrane (McClatchey & Giovannini 2005;

96   McClatchey & Fehon 2009). These many and diverse cytoplasmic interactions suggest that

97   MED28 serves as a multi-faceted adaptor/scaffold to relay cellular signaling from the cytoskeleton

98   to transcription in the nucleus (Lee et al. 2006).

99   Although conserved between insects and mammals, a bona fide MED28 homologue had

100 yet to be identified in nematodes. Our previous work showed that the protein previously identified

101 as "MDT-28" (**Med**iat**o**r-28) in nematode and other databases is instead the nematode homologue

102 of perilipin, a protein regulating lipid metabolism at the level of lipid droplets and is not related to

103 MED28 (Chughtai et al. 2015). Thinking it was unlikely that a MED28 homologue would be

104 absent in nematode genomes, we searched for it using conserved features of MED28 orthologues

105 from various phyla. Herein we identify a previously uncharacterized protein, F28F8.5, as the

106 closest MED28 homologue. We show that F28F8.5 is both a nuclear and a cytoplasmic protein

107 present in most, if not all, tissues throughout development. Down-regulation by RNAi, or

108 disruption of *F28F8.5* by deletion, results in multiple developmental defects during embryonic

109 and larval development. Our work indicates that the homologue of Mediator complex subunit 28

110 exists in nematodes and suggests that the potential to link cytoplasmic and nuclear events is

111 conserved between MED28 vertebrate and nematode homologues.

112

113

114 **MATERIALS AND METHODS**

Sequence analysis

115

116   The UniProtKB (uniprot.org) and NCBI (ncbi.nlm.nih.gov) databases were searched with

117 BLAST, PSI-BLAST (Altschul et al. 1997), HHblits (Remmert et al. 2011) and HHpred (Soding

118 et al. 2005) programs. The protein sequences were identified with their UniProtKB identifiers and

119 the nucleotide sequences with their NCBI ones. The sequences were aligned with T-coffee

120 (Notredame et al. 2000; Di Tommaso et al. 2011) and PROMALS (Pei & Grishin 2007; Pei et al.

121 2007; Pei et al. 2008). The secondary structure predictions were performed with PSIPRED (Jones

122 1999; Cuff & Barton 2000; McGuffin et al. 2000). Multiple sequence alignments were displayed

123 and analyzed with Jalview (Clamp et al. 2004).

124

RNA isolation and cDNA synthesis

125

126   RNA and cDNA were prepared as described (Zima et al. 2015) with modifications.

127 Cultured nematodes were collected in water and pelleted by centrifugation for 5 min at 110 xg and

128 4 ºC. The excess of water was removed and the pellet was frozen at -80 °C. For the isolation of

129 RNA, the pellet was quickly melted and dissolved in 300 μl of resuspension buffer (10 mM Tris-

130 HCl; 10 mM EDTA, 5% 2- mercaptoethanol; 0.5% SDS; pH 7.5). After adding 8 μl of proteinase

131 K (20 mg/ml), the sample was mixed and incubated 1 h at 55 °C. RNA was isolated by phenol-

132 chloroform extraction and ethanol precipitation. The obtained RNA was incubated with RQ1

133 DNase (Promega, Fitchburg, WI, USA) and purified again by phenol-chloroform extraction and

134 ethanol precipitation. Complementary DNA (cDNA) was prepared with SuperScript III

135 (Invitrogen, Carlsbad, CA, USA) using random hexamers.

136

Strains, transgenic lines and genome editing

137

138   The *C. elegans* Bristol N2 strain was used whenever not specifically stated and maintained

139   as described (Brenner 1974).

140

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

142   For preparation of transgenic lines expressing F28F8.5::GFP from extrachromosomal

143   arrays under regulation of endogenous promoter, we used the PCR fusion-based technique

144   (Hobert 2002). Primers 7886 and 7888 were used for amplification of the genomic sequence of

145   *F28F8.5* (consisting of approximately 400 bp of the predicted promoter region preceding the

146   coding region of *F28F8.5*). The gene encoding GFP was amplified from the pPD95.75 vector with

147   primers 6232 and 6233. The complete construct was amplified with primers 7887 and 6234. The

148   resulting fusion construct contained the 3' UTR from pPD95.75 (originally from the *unc-54* gene).

149   The PCR mixture was injected into the gonads of young adult hermaphrodite animals together

150   with marker plasmid pRF4. The sequences of all primers used in the paper are in supplementary

151   information.

152

Genome editing

154   Lines with edited genomes were prepared from wild type N2 animals using the

155   CRISPR/Cas9 system as described (Dickinson et al. 2013; Dickinson et al. 2015; Ward 2015;

156   Dickinson & Goldstein 2016). Using this strategy, the *F28F8.5* gene was edited by insertion of a

157   construct including the coding sequence of GFP and a self-excision cassette containing the *sqt-*

158   *1(d)* gene (a visible selection marker leading to a Rol phenotype), *hs::Cre* (heat shock inducible

159   Cre recombinase) and hygR (hygromycin resistance) genes. The sgRNA sequence was targeted

160   near the start of the coding sequence for F28F8.5 gene using the pJW1219 plasmid (Addgene,

161   Cambridge, MA, USA) as the Cas9 vector (pMA007). It was prepared by PCR with primers 8403A

162   and 8333 and used in a concentration of 50ng/µl for microinjections. The plasmid pMA007 was

163   co-injected with the rescue repair template plasmid based upon pDD282 vector (pMA006) in a

164   concentration of 10 ng/µl and with 3 markers (see below). The repair template plasmid pMA006

165   was prepared in two steps. First the plasmid pMA005 was prepared from gDNA of *F28F8.5*

166   (containing both repair arms) and amplified by PCR with primers 8404 and 8405 and cloned into

167   pCU19 backbone. The plasmid pMA005 was subsequently modified – the FP-SEC segment was

168   added and the CRISPR/Cas9 site was altered to protect against Cas9 attack. The linear PCR

169   product of pMA005 was prepared using primers 8406 and 8407 with overlapping regions for

170   Gibson assembly (New England *BioLabs*, Ipswich, MA, USA). The primer 8406 was prepared

171   with alternate codons for protection against CRISPR/Cas9 site. Linear insert of FP-SEC was

172   prepared by PCR from pDD282 plasmid (Addgene) with primers 8408 and 8409. Primers were

173   prepared with overlapping parts for cloning into linear pMA005 plasmid by Gibson assembly and

174   the final rescue plasmid pMA006 was prepared. Plasmids pGH8 (10 ng/µl), pCFJ104 (5 ng/µl)

175   and pCJ90 (2.5 ng/µl) (Addgene) were used as fluorescent coinjection markers. After

176   microinjections the population of nematodes were grown for 3 days at 25º C and Hygromycin

177   (Invitrogen) was added in a final concentration of 250 µg/ml. After 3 days integrated nematodes

178   were selected according to the rolling phenotype and loss of extrachromosomal arrays.

179   Using this strategy, we obtained heterozygous lines with a disrupted *F28F8.5* gene with an

180   inserted *gfp* regulated by the endogenous promoter of *F28F8.5* in one allele. Homozygous animals

181   for $P_{F28F8.5}$*::gfp* (edited F28F8.5 – $P_{F28F8.5}$ *(V:15573749)::gfp::let858(stop)::SEC::F28F8.5*) with

182   disrupted *F28F8.5* on both alleles and expressing GFP under the regulation of the endogenous

183   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

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

230 animals with edited F28F8.5 expressing GFP::F28F8.5 was performed using an inverted Leica

231 SP8 TCS SMD FLIM system equipped with a 63x 1.2 NA water immersion objective, a pulsed

232 white light laser (470-670 nm), AOBS and two internal hybrid single photon counting detectors,

233 and operated by Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany).

234 The GFP fluorescence was excited at a wavelength of 488 nm and the emitted light was

235 simultaneously recorded in two spectral ranges (Channel 1 – 495 nm to 525 nm, Channel 2 – 525

236 nm to 580 nm; the two channel setup was used to help resolve between spectrally different

237 autofluorescence and GFP fluorescence signals).

238

Fluorescence-lifetime imaging microscopy (FLIM)

240   For FLIM acquisitions the single photon counting signal from the internal hybrid detectors,

241 acquired during confocal acquisitions, was simultaneously processed by HydraHarp400 TCSPC

242 electronics (PicoQuant, Berlin, Germany) and information about the arrival times of all photons

243 was stored to a hard-drive in TTTR data format. The fast FLIM images were reconstructed using

244 home written software "TTTR data analysis". The signal from both time synchronized channels

245 was added up. The false color scale (1 to 3 ns) is based on the average photon arrival time, with

246 blue color representing to the short lifetime and red color to the long lifetime fluorescence.

247

Single nematode PCR

249 Single animal PCR was used for verification of all transgenic lines. Following the microscopy

250 examination, selected animals were removed from microscopic slides and transferred into caps of

251 PCR tubes. The sample was diluted in a solution of Proteinase K (20 mg/ml) diluted in 1:333 in

252 Barstead Buffer (resulting in Barstead Lysis Buffer) and was kept on ice. The tube was sealed in

253 bottom-up position and the sample transferred to the bottom of the tube by centrifugation. The

254 tube was frozen for 10 min. at -70º C. Next, the tube was heated for 1 hour at 60º C and additional

255 15 min. at 95º C. The resulting sample was used immediately for amplification of DNA by PCR

256 or stored at -80º C before further analysis. Barstead Buffer consists of 50 mM KCl, 10 mM Tris

257 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).

258 Proteinase K was diluted immediately before use as a 20 mg/ml stock solution which was kept on

259 ice and diluted to final working solution at concentration of 200 µg/ml. Similarly, from selected

260 nematode culture plates genomic DNA was prepared and used for further screening by PCR and

261 sequencing.

262   The resulting precipitated DNA was dissolved in 10 µl of deionized water and used for

263 amplification by PCR using primers outside the edited genomic regions. Specificity of

264 amplification was controlled by DNA sequencing.

265   Similarly, homozygous animals with edited F28F8.5 (with gfp inserted behind the START

266 codon) were analyzed by single worm PCR with primers #7887 and #8454.

267

268

269 **RESULTS**

270 **Identification of the closest homologue of vertebrate Mediator complex subunit**

271 **28 in *C. elegans***

272

273   To identify a *C. elegans* homologue of MED28, we queried protein databases with curated

274 SwissProt sequences from UniProtKB. They comprised several mammalian and insect proteins

275   (e.g. human MED28_HUMAN and *D. melanogaster* MED28_DROME). The more sensitive

276   profile-to-profile HHblitz and HHpred algorithms provided hits to a *C. elegans* annotated protein

277   F28F8.5a and b with highly significant E-values. According to Wormbase (WS 248), two protein

278   isoforms are produced from the *F28F8.5* gene, isoform a with the length of 200 amino acids and

279   isoform b that has a two amino acid insertion at position 20 of the N-terminal evolutionarily non-

280   conserved region. The best results were obtained when pre-aligned vertebrate and insect MED28

281   paralogues were used as query in 3 iterations (E<10^{-48} and the probability of true positive

282   >99.99%). When the pre-aligned nematode sequences homologous to F28F8.5 were used to query

283   profiles of human or *Drosophila* sequences in reciprocal searches, MED28 proteins were obtained

284   with equally significant scores. BLAST and PSI-BLAST searches in their standard settings were

285   not able to reveal a significant hit (E<10^{-3}); the only nematode hit was a *Trichinella spiralis* protein

286   (E5RZQ1). However, when the searches in protein databases were limited to sequences from

287   *Ecdysozoa* with *Insecta* excluded (conservative inclusion threshold E<10^{-6}) in the first two

288   iterations, F28F8.5 provided human and *Drosophila* MED28. PSI-BLAST with *T. spiralis* query

289   sequence in database limited to *Ecdysozoa* in the first 2 iterations provided both human and

290   *Drosophila* MED28 and F28F8.5 in one run (E<10^{-8}). We concluded from these searches that

291   F28F8.5 is a homologue of MED28 and very likely its previously unrecognized orthologue.

292   All PSI-BLAST MED28 homologues possess variable N- and C-termini of 3 to 80 amino

293   acids showing no conservation across *metazoa*. This conservation is loose even just within

294   *Drosophilae* or *Caenorhabditae* sequences. Only the central core of about 110 amino acids is

295   preserved in metazoan evolution. **Fig. 1** shows a sequence alignment of this conserved core of

296   selected MED28 homologues. All sequences are predicted to fold into 3 helices forming a putative

297   coiled coil fold (UniProt annotation). Submitting the alignment shown in **Fig. 1** to HHpred for 3D

298 structure recognition reveals a structural fold of yeast MED21 (PDB identifier 1ykh_B). It is

299 indeed a 3-helix coiled coil forming a heterodimer with MED7. It can be expected that MED28

300 forms a very similar fold interacting with a yet to be determined subunit of the MED complex.

301

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

303 Information available in WormBase indicates that the *F28F8.5* gene can be expressed as

304 both an individual and multigene transcript, located as the last gene in a four gene operon that is

305 both SL-1 and SL-2 trans-spliced. To determine the intracellular localization of F28F8.5, we edited

306 the *F28F8.5* gene using CRISPR/Cas9 technology. We inserted the gene coding for GFP directly

307 after the first codon. The arrangement used in our experiment (based on (Dickinson et al. 2013;

308 Dickinson et al. 2015; Ward 2015; Dickinson & Goldstein 2016)) employed a self-excision

309 cassette added after *gfp*. This strategy initially created a disrupted *F28F8.5* gene and putative null

310 allele that can be detected by expression of GFP alone regulated by the endogenous promoter

311 elements of *F28F8.5*, including those of the entire operon. We found that only heterozygous

312 animals could be propagated due to the sterility of homozygotes tagged in this manner. Assuming

313 this tag is not deleterious to the expression of other genes in the operon, this result suggests that

314 *F28F8.5* is an essential gene.

315 After removal of the self-excision cassette from this edited *F28F8.5* gene induced by heat

316 shock (visualized by continuous expression of GFP::F28F8.5 fusion protein and loss of the rolling

317 phenotypic marker), the endogenous locus had an N-terminus GFP-tagged F28F8.5 gene that we

318 maintained as homozygous animals, demonstrating this edited allele is fully functional. Note that

319 both known protein isoforms of F28F8.5 (a and b) would be tagged on their N-terminus with GFP

320 by this method.

321   The GFP::F28F8.5 pattern was both nuclear and cytoplasmic from embryos to adults (**Fig.**

322   **2**). Prominent nuclear localization was found in oocytes, zygotes, larvae, and adults. Cells with

323   clear nuclear accumulation of GFP::F28F8.5 included epidermal, intestinal, pharyngeal, uterine

324   and vulval muscle cells (**Fig. 2**). The gonad expressed *gfp::F28F8.5* and mitotic as well as meiotic

325   nuclei accumulated GFP::F28F8.5 (**Fig. 2**).

326   Selected animals were analyzed by confocal microscopy for determination of subcellular

327   distribution of GFP::F28F8.5. Scanning through several focal planes revealed signal in the GFP

328   excitation/emission range in nuclei as well as in the cytoplasm of embryos, all larval stages and

329   adults (**Fig. 3**). Structures resembling gut granules were also strongly positive in the GFP recording

330   mode. In order to distinguish between GFP-specific fluorescence and autofluorescence from the

331   intestinal cells, we applied FLIM with an expectation that autofluorescence (such as that from gut

332   granules) is likely to produce a signal with a short fluorescence life time opposed to GFP-specific

333   fluorescence. Structures such as gut granules could be clearly detected (**Fig. 3**, panels O, Q, S, T

334   and U, blue color) while fluorescence with a longer life time expected for GFP::F28F8.5 was

335   detected in the germline, in oocytes and embryos and in most somatic nuclei of larvae as well as

336   adult animals (**Fig. 3**, panels O, Q, S, T and U, red and yellow colors).

337   We also generated transgenic lines expressing F28F8.5::GFP from extrachromosomal

338   arrays consisting of endogenous internal *F28F8.5* promoter regulating a fusion gene with *gfp*

339   attached to *F28F8.5* on its 3' terminus. As with the N-terminally tagged F28F8.5, F28F8.5::GFP

340   showed both nuclear and cytoplasmic localization. As expected for an extrachromosomal

341   transgene, the expression of *F28F8.5::gfp* was not detected in the germline. This reporter was

342   expressed in embryos starting at the 2-fold stage and continued throughout development

343   (**Supplementary Fig. S1).** We did notice that *F28F8.5::gfp* was expressed in the excretory cell

344   and its channels (**Supplementary Fig. S1** S and T), a pattern not observed with the endogenously

345   edited GFP-tagged gene.

346

347   **F28F8.5 regulates development**

348   To achieve loss-of-function, RNAi was used to down-regulate *F28F8.5* expression.

349   Analysis of 2567 progeny of 17 young adult hermaphrodites inhibited for *F28F8.5* function by

350   microinjection of dsRNA into the syncytial gonad revealed that F28F8.5 was essential for proper

351   development (**Fig. 4**). From the total progeny, 1127 animals were affected (44 %) exhibiting

352   embryonic and larval arrest and a range of less severe phenotypes, including defective molting,

353   protruding vulvae that often burst, male tail ray defects, and uncoordinated (Unc) movement (**Fig.**

354   **4**).

355   Complete loss of F28F8.5 that occurred in homozygous animals with both disrupted alleles

356   of F28F8.5 gene (that are found among the progeny of heterozygous animals carrying one

357   disrupted allele and one edited allele *gfp::F28F8.5*) resulted in sterility. However, these animals

358   were able to develop to adults. This demonstrates that a single maternal allele provides sufficient

359   gene product to get homozygous null animals through much of development, but was insufficient

360   for proper somatic and germline gonad development (**Fig. 5**). The gonad was irregular, contained

361   masses of undifferentiated tissue and showed problems of directional growth. Defective vulva

362   formation was also observed.

363

364

365   **DISCUSSION**

366   Mediator complex as a multiprotein structure is able to interact with a large number of

367   additional proteins and integrate regulatory signals from several cell signaling cascades (Allen &

368   Taatjes 2015). The steric organization of the Mediator complex is dynamic and allows variable

369   arrangement of its subunits (Poss et al. 2013). Individual subunits of the Mediator complex can be

370   divided into essential subunits, present in many or perhaps all Mediator complexes and proteins

371   that are present only in some complexes and participate in specific more restricted transcription

372   regulation (Paoletti et al. 2006; Kulak et al. 2014). Med28 is a subunit that belongs to the latter

373   group and has a special position in-between Mediator subunit proteins for its dual regulatory role,

374   one as a Mediator subunit (Sato et al. 2004; Beyer et al. 2007) and the second which is cytoplasmic

375   at the level of the cytoskeleton (Gonzalez-Agosti et al. 1996; Wiederhold et al. 2004; Lee et al.

376   2006; Lu et al. 2006; Huang et al. 2012). This suggests that Med28 may be able to bring

377   cytoplasmic regulatory interactions towards the regulation of gene expression.

378   In this work, we identified an uncharacterized predicted protein F28F8.5 as the nematode

379   homologue of MED28. Our experiments support the fundamental role of F28F8.5 in

380   developmental events. Keeping with its expected function as a component of the Mediator

381   complex, downregulation of F28F8.5 by RNAi led to a wide range of defects during embryonic as

382   well as larval development. In contrast to downregulation of F28F8.5 by RNAi, null mutants with

383   disrupted *F28F8.5* gene found in the progeny of heterozygous animals with one disrupted allele

384   and one WT allele or one disrupted allele and one edited allele coding for GFP::F28F8.5 were able

385   to reach adulthood. RNAi is a powerful tool for gene downregulation that can be applied to studied

386   animals at specific developmental times and affects the gene expression in a wide range of cells

387   including the germline. While heterozygous animals with one functional allele of *F28F8.5* supply

388   their embryos with maternal transcripts, the embryos in the progeny of parents with F28F8.5

389   downregulated by RNAi are devoid of this maternal load. This shows that the normal or edited

390   F28F8.5 supplied as maternal load from heterozygous parents is sufficient for null mutant animals

391   to proceed throughout most of developmental events vitally dependent on F28F8.5. The amount

392   of F28F8.5 inherited as maternal load is, however, not sufficient for normal development of gonad

393   and sex organs and the homozygous null mutant animals do not produce viable embryos. Most

394   phenotypes that we observed in our RNAi experiments are listed in Wormbase (WS 254) based on

395   high throughput screens (Kamath & Ahringer 2003; Simmer et al. 2003; Frand et al. 2005;

396   Sonnichsen et al. 2005). Nuclear localization and the wide expression of F28F8.5 was previously

397   reported and is also listed in Wormbase (WS254) (Mounsey et al. 2002; Matus et al. 2010). The

398   indispensability of F28F8.5 during embryonic development is similar to findings reported for

399   Med28 (Li et al. 2015). The wide expression pattern of F28F8.5 is also keeping with the data

400   reported   in   the   Mouse   Genome   Database

401   (http://www.mousephenotype.org/data/genes/MGI:1914249, accessed on October 5, 2016) (Eppig

402   et al. 2015) and in Human Protein Atlas (http://www.proteinatlas.org, accessed on October 7,

403   2016) (Uhlen et al. 2015).

404   Based on the closest sequence similarity of F28F8.5 to MED28 that can be detected

405   informatically in nematode genomes, conserved dual nuclear and cytoplasmic expression and

406   involvement in a wide range of developmental processes, we suggest to name F28F8.5 as MDTH-

407   28 (MED28 homologue) because MDT-28 already exists in databases (but shown to be a

408   homologue of perilipin (Chughtai et al. 2015), its duplicate use would cause confusion and MED

409   denomination is used in nematode nomenclature for a different class of proteins.

410   MED28 is a candidate Mediator complex subunit linking cytoplasmic structural signals

411   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.

443

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622

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/)
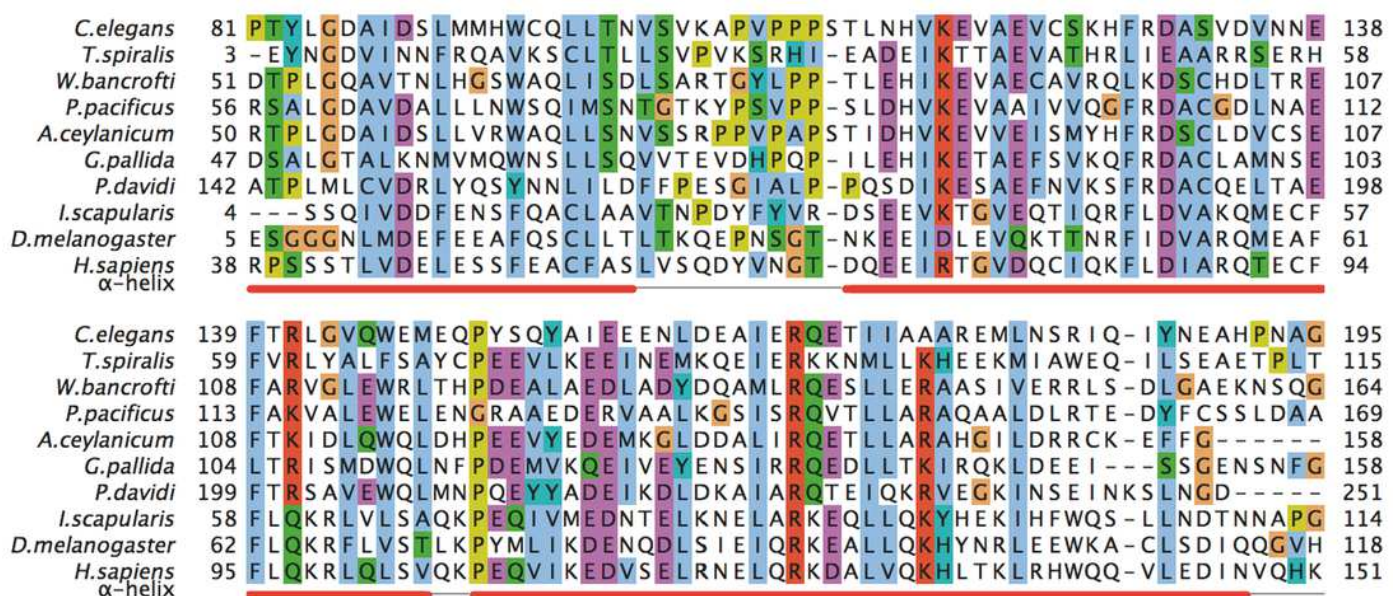
Figure 2

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.
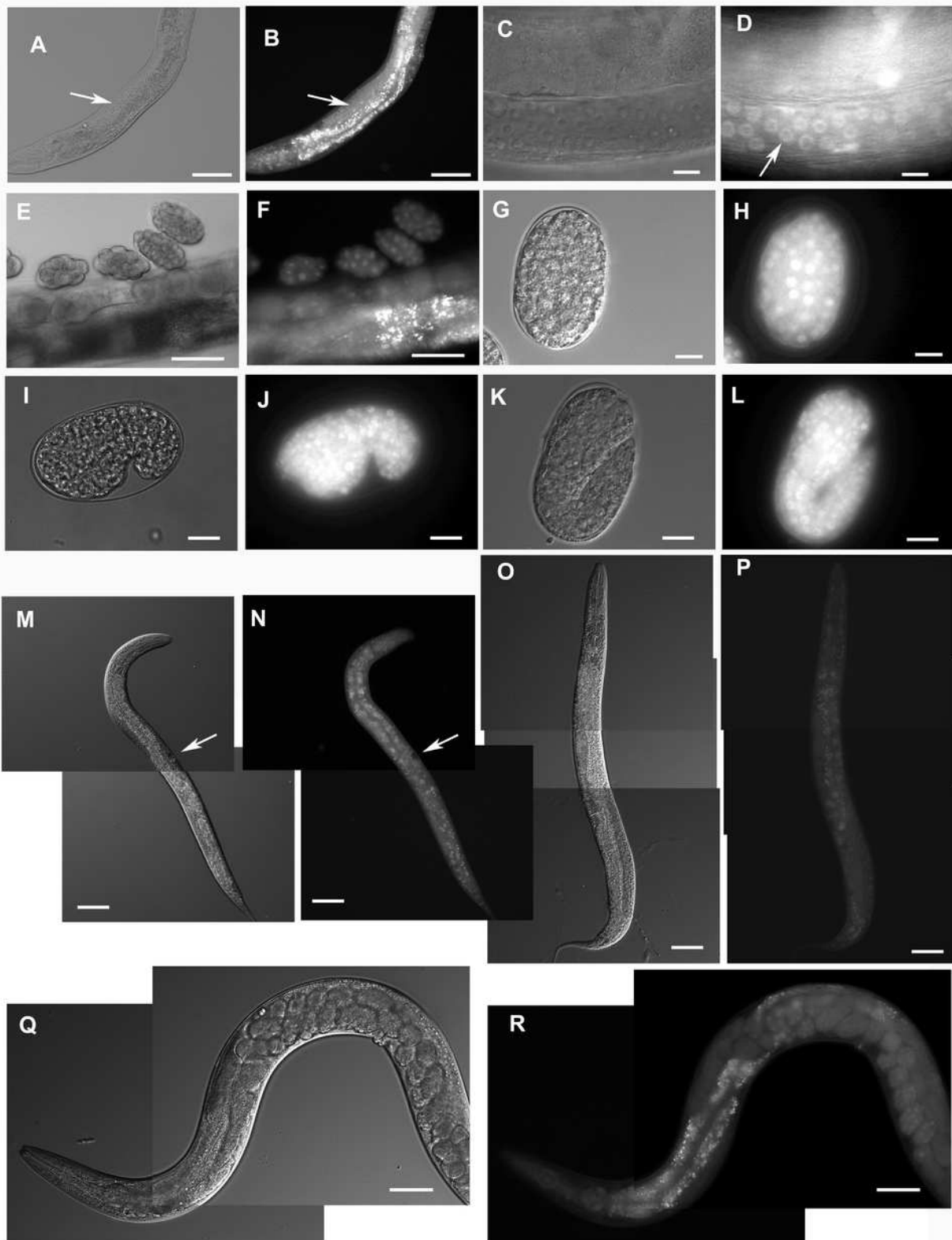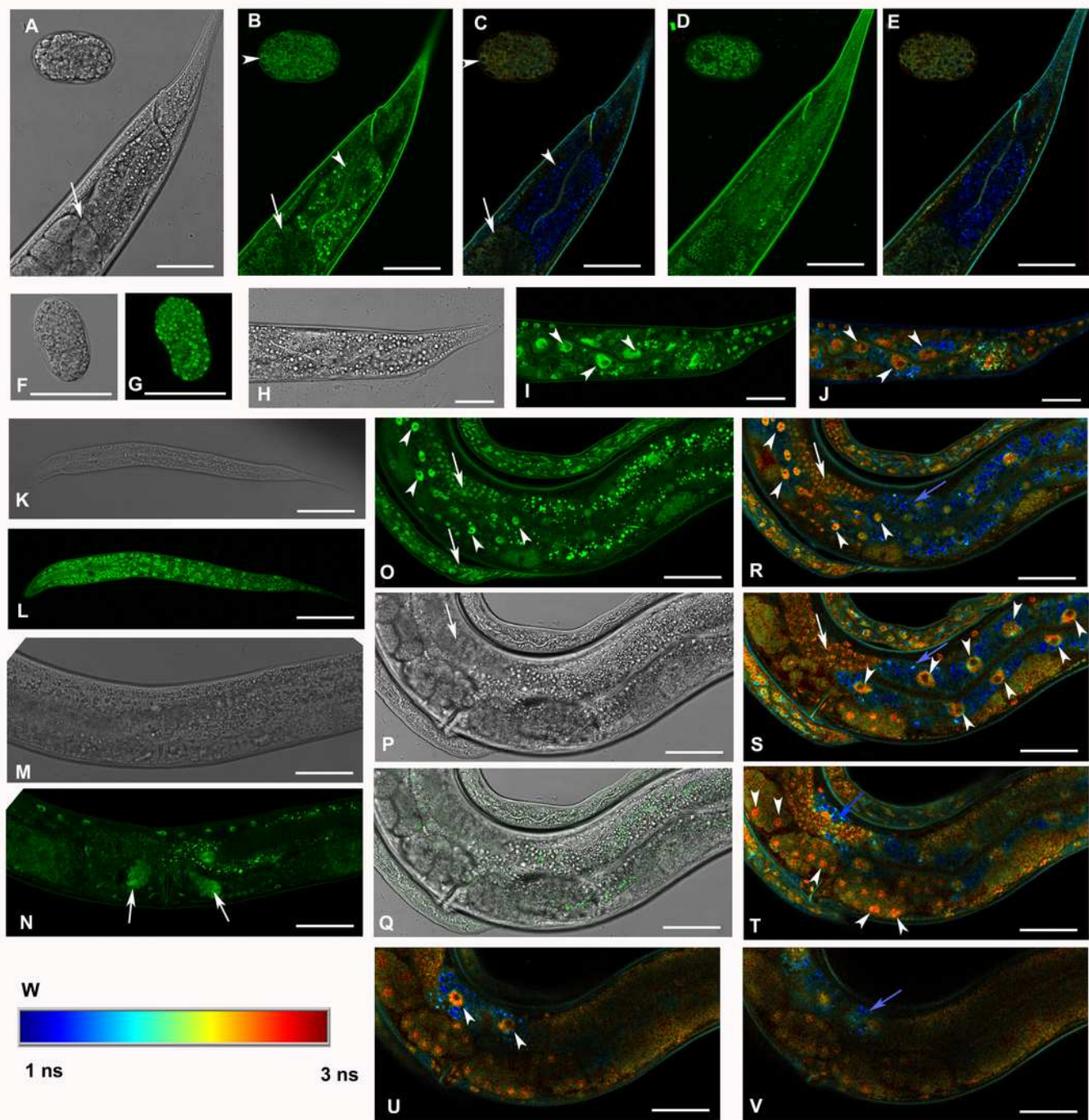
Figure 3

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.
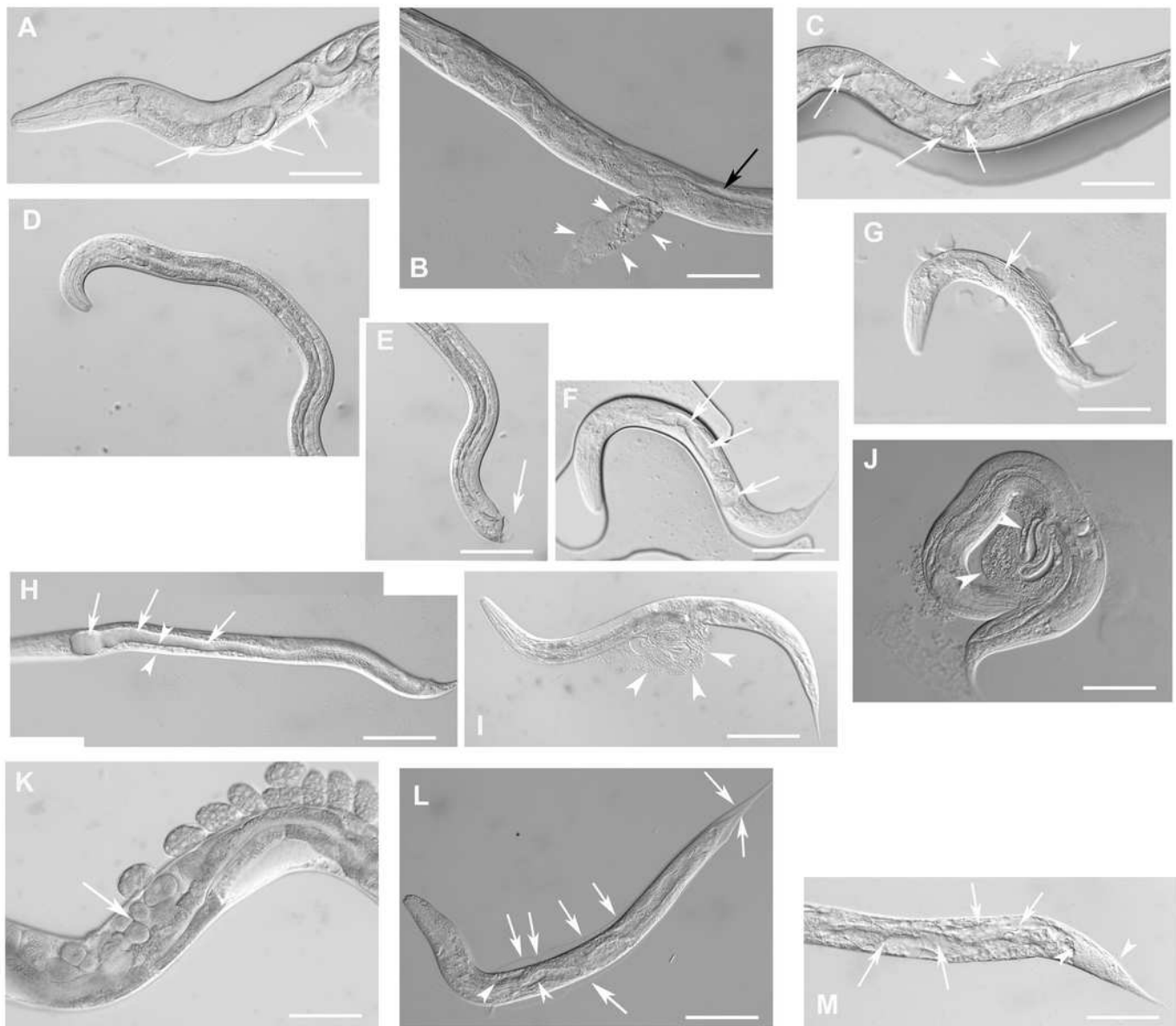
Figure 5

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.