Transgenic alternative-splicing reporters reveal tissue-specific expression profiles and regulation mechanisms *in vivo*

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Alternative splicing of pre-mRNAs allows multicellular organisms to create a huge diversity of proteomes from a finite number of genes. But extensive studies *in vitro* or in cultured cells have not fully explained the regulation mechanisms of tissue-specific or developmentally regulated alternative splicing in living organisms. Here we report a transgenic reporter system that allows visualization of expression profiles of mutually exclusive exons in *Caenorhabditis elegans*. Reporters for *egl-15* exons 5A and 5B showed tissue-specific profiles, and we isolated mutants defective in the tissue specificity. We identified alternative-splicing defective-1 (*asd-1*), encoding a new RNA-binding protein of the evolutionarily conserved Fox-1 family, as a regulator of the *egl-15* reporter. Furthermore, an *asd-1; fox-1* double mutant was defective in the expression of endogenous *egl-15 (5A)* and phenocopied *egl-15 (5A)* mutant. This transgenic reporter system can be a powerful experimental tool for the comprehensive study of expression profiles and regulation mechanisms of alternative splicing in metazoans.

The importance of the alternative splicing of pre-mRNAs on the structure and function of proteins, as well as on cellular processes, has been discussed[1–3]. Recent global studies on cDNA sequences or microarray data have predicted that as many as two-thirds of human genes have multiple isoforms of mature mRNAs[4,5], and experiments with alternative-splicing microarrays revealed that many alternative splicing events are controlled in tissue- and cell type– and/or developmental stage–dependent manners[6,7]. These findings indicate that unidentified 'cellular codes' underlie the regulation of alternative splicing of many genes in living organisms[8–11]. Experimental elucidation of the expression profiles and regulation mechanisms of alternative splicing would lead to a better understanding of genome functions and cellular identities of multicellular organisms.

Regulation mechanisms of alternative splicing have been experimentally studied mostly *in vitro* and in cultured cells[12,13]. General *cis*-acting enhancer and silencer elements as well as *trans*-acting factors involved in the regulation of both constitutive and alternative exons have been well characterized by analyzing model genes. Expression cloning strategies have permitted the global collection of putative sequence elements that function in cultured cells, and bioinformatic analyses have identified putative *cis* elements within exons and introns. Recently, however, conditional knockouts of *trans*-acting SR protein families revealed that alternative splicing of only a few target genes are crucially dependent on a specific protein in cardiac muscles, even though many more genes expressed in this tissue have typical *cis* elements[14]. This indicates that we cannot precisely predict the alternative splicing patterns in each tissue or cell without the assessment of the regulation mechanisms in living organisms.

Some cell type–specific *trans*-acting alternative splicing factors and their target genes have been identified in conventional genetic studies on *C. elegans* and *Drosophila melanogaster*[15,16], suggesting feasibility of using the genetic approach for comprehensive studies on regulation of alternative splicing. As in vertebrates, most genes in *C. elegans* have introns[17], and ~5% of protein-coding genes have several different forms of their corresponding mature mRNAs[18]. Because of its advantage in morphological and genetic studies, we intended to generate a transgenic reporter system in *C. elegans*. Here we demonstrate that the reporter system allowed us to monitor the expression profiles of alternatively spliced exons at a single-cell resolution *in vivo* and can be used for identification of *cis*-acting elements and *trans*-acting factors regulating the expression of the reporter. We also demonstrate that regulation mechanisms identified using this reporter system can be applied to the endogenous gene *in vivo*, and that the regulation mechanisms of tissue-specific alternative splicing recently characterized in vertebrates are also conserved in nematodes.

RESULTS

Generation of transgenic reporter worms

As a model gene, we used *egl-15*, which encodes the sole homolog of fibroblast growth factor receptor (FGFR) in *C. elegans*. Its mutually exclusive exons 5A and 5B correspond to an insert within the extracellular domain[19]. EGL-15(5B) and its ligand,

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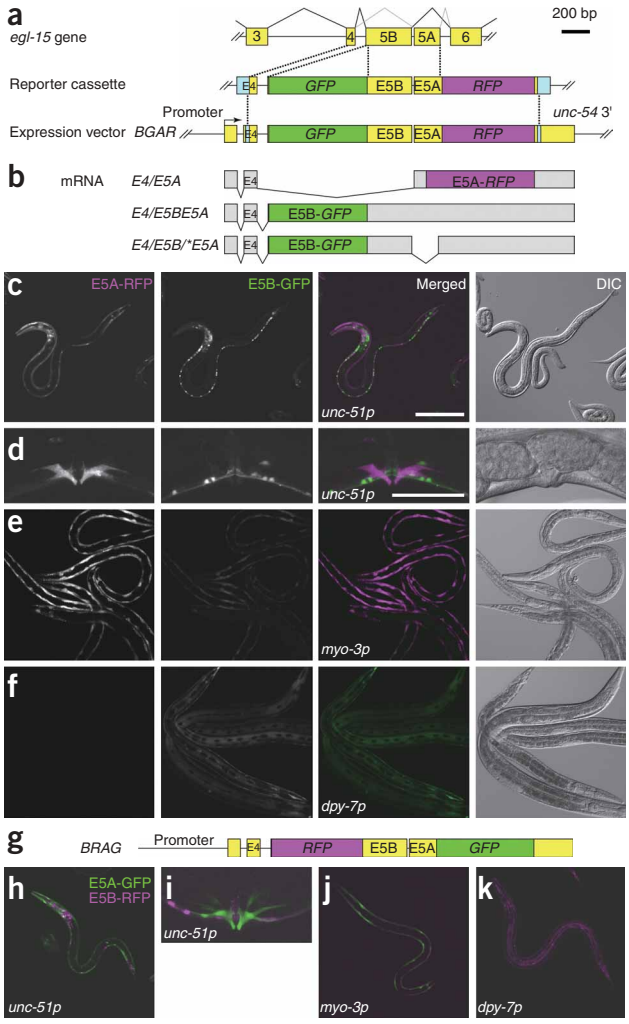

Figure 1 | Alternative splicing reporter worms. (**a**) Construction of *egl-15* reporter E5B-*GFP*–E5A-*RFP* (*BGAR*). Genomic fragment of *egl-15* exon 4 through 5A was amplified, and cDNAs for *GFP* and *RFP* were inserted to construct the reporter cassette. The reporter cassette was introduced between the promoter and 3′ cassette by homologous recombination. Boxes indicate exons. (**b**) Schematic representation of mRNAs derived from the *BGAR* reporter in **a**. Constitution of mRNAs was analyzed by cloning and sequencing RT-PCR products. Predicted ORFs are colored in green for E5B-*GFP* and in magenta for E5A-*RFP*. *E5A, cryptic 3′-splice site within E5A. (**c**–**f**) Transgenic worms expressing *BGAR* reporter driven by *unc-51* (**c**,**d**), *myo-3* (**e**) and *dpy-7* (**f**) promoters. Projection images of E5A-RFP, E5B-GFP, merged and DIC images of the same fields are shown. (**g**) Schematic representation of *BRAG* mini-genes. (**h**–**k**) Merged views of confocal images of reporter worms expressing *BRAG* reporter under *unc-51* (**h**,**i**), *myo-3* (**j**) and *dpy-7* (**k**) promoters. L2–L3 larvae (**c**,**e**,**f**,**h**,**j**,**k**). Lateral view of an adult vulva (**d**,**i**). Scale bars in **c**,**e**,**f**,**h**,**j**,**k**, 100 μm; in **d**,**i**, 50 μm.

LET-756(FGF), exert essential functions, whereas EGL-15(5A) is specifically expressed in sex myoblasts, and is required for the directed migration of the cells toward EGL-17(FGF)–releasing cells[19].

To monitor the usage of mutually exclusive exons, we first introduced cDNAs for *RFP* and *GFP* downstream of exon 5A and 5B, respectively, in separate mini-genes (**Supplementary Note 1** online). When expressed under the *unc-51* promoter, which drives expression in a variety of tissues, the transgenic worms showed differential expression patterns of RFP and GFP corresponding to alternative splicing patterns (see **Supplementary Note 1** and below). Next we constructed a unified reporter (**Fig. 1**), in which cDNAs for *RFP* and *GFP* were introduced into exon 5A–derived exon (E5A) and exon 5B–derived exon (E5B), respectively. When expressed under the *unc-51* promoter, the transgenic worms also showed a differential expression pattern of GFP and RFP (**Fig. 1c**), which was essentially the same pattern as that in a combination of *E5AR* and *E5BG* (**Supplementary Note 1**). The composition of mRNAs derived from the unified reporter transgene was consistent with the alternative selection of E5A and E5B (**Fig. 1b**), and predicted open reading frames (ORFs) for each mRNA isoform corresponded to an RFP-fusion protein (E5A-RFP) and a GFP-fusion protein (E5B-GFP), respectively (**Fig. 1b**). Consistent with the specific expression of endogenous exon 5A in sex myoblasts[19], the vulval muscles, which derived from the sex myoblasts, exclusively expressed E5A-RFP (**Fig. 1d**). When we interchanged the positions of cDNAs encoding GFP and RFP in the reporter mini-gene (**Fig. 1g**), the expression patterns of GFP and RFP completely reversed (**Fig. 1h,i**), indicating that the tissue-dependent differential expression of GFP and RFP was not due to the differential stability of GFP- or RFP-encoding mRNAs or of these proteins in different tissues.

To analyze expression profiles of the *egl-15* reporter, we generated several transgenic worms with various tissue-specific promoters driving expression of the reporter mini-gene. The *myo-3* promoter predominantly drove the expression of E5A-RFP in body-wall muscles (**Fig. 1e**) and vulval muscles (data not shown). E5B-GFP was exclusively expressed in the hypodermis under the *dpy-7* promoter (**Fig. 1f**). When we interchanged the positions of cDNAs encoding GFP and RFP (**Fig. 1g**), the expression patterns of GFP and RFP completely reversed (**Fig. 1j,k**). Further results summarized in **Table 1** demonstrated that alternative splicing of the *egl-15* reporter was regulated in a tissue-dependent manner *in vivo*.

Screening for mutants with altered expression profiles

To examine the applicability of our transgenic reporter system to study regulation mechanisms of alternative splicing *in vivo*, we screened for mutants defective in the tissue-specific expression of the *egl-15* reporter (**Fig. 2**). We mutagenized the transgenic worms that predominantly expressed E5A-RFP in body-wall muscles (**Fig. 2c**) and isolated worms expressing higher amounts of GFP than the parental strain using a fluorescence-assisted worm sorter (**Fig. 2b**). Of the $\sim 5 \times 10^4$ genomes screened, we isolated 18 independent alleles with various expression profiles (**Fig. 2d–i**). We classified these mutants according to their reporter expression profiles and SNP-based chromosome mapping (**Fig. 2k**). Two groups with the 'orange' phenotype expressed both E5B-GFP and E5A-RFP throughout the body-wall muscles (**Fig. 2e,g**) and were mapped to chromosome I. In another group with the 'chimera' phenotype, some body-wall muscle cells predominantly expressed E5B-GFP, whereas other body-wall muscle cells preferentially expressed E5A-RFP with a complementary pattern (**Fig. 2d**), and the gene was mapped to chromosome III. The other alleles showed chimeric expression profiles and mapped to chromosome X (**Fig. 2j**). With one allele, E5B-GFP was predominantly expressed

Table 1 | Expression profiles of *egl-15* reporter

	Muscles				Epidermis		Nervous system		
Promoter	Pharyngeal muscle	Body-wall muscle	Vulval muscle	Anal muscle	Hypodermis	Intestine	Amphid neurons	Mechanosensory neurons	Motor neurons
unc-51	E5A	E5A	E5A	E5A	E5B	–	E5B > E5A	E5B > E5A	E5B > E5A
myo-2	E5A > E5B	–	–	–	–	–	–	–	–
myo-3	–	E5A > E5B	E5A > E5B	–	–	–	–	–	–
dpy-7	–	–	–	–	E5B	–	–	–	–
elt-2	–	–	–	–	–	E5B	–	–	–
F25B3.3	–	–	–	–	–	–	E5B > E5A	E5B > E5A	E5B > E5A
mec-7	–	–	–	–	–	–	–	E5B > E5A	–
unc-4	–	–	–	–	–	–	–	–	E5B

Expression of E5A and E5B was monitored by expression of corresponding fluorescent proteins from the transgenic reporters *BGAR* and/or *BRAG* driven under the indicated promoters. E5A and E5B, exclusively expressing E5A or E5B. E5A > E5B and E5B > E5A, predominantly expressing E5A and E5B, respectively. –, not expressed.

in most body-wall muscle cells and a few of these cells expressed E5A-RFP (**Fig. 2h**); with another allele, E5A-RFP–expressing cells predominated (**Fig. 2i**). The expression profiles were reproduced with 100% of penetrance in all these alleles. We confirmed altered profiles of alternative splicing by reverse transcriptase (RT)-PCR analysis (**Fig. 2k**). Thus these transgenic worms allowed us to efficiently screen splicing mutants.

Identification of *asd-1* gene

We identified a gene corresponding to the 'chimera' phenotype (**Fig. 2d**). By sequencing genomic DNAs from the mutants, we identified mutations in a predicted gene, R74.5, from all seven alleles classified into the group, and we named the gene *asd-1*. ASD-1 protein has a single RNA recognition motif (RRM) and belongs to the evolutionarily conserved Fox-1 family (**Fig. 3a**). Vertebrates have three genes that belong to this family, and fruit flies have one. In *C. elegans*, FOX-1 (ref. 20) and SPN-4 (ref. 21) belong to this family. The sequence alignment of the RRMs is shown in **Figure 3b**. Members of the Fox-1 family have recently been identified as alternative splicing regulators with sequence-specific RNA binding properties[22], and structural analyses have confirmed the binding specificity of the RRM of mammalian Fox-1 and Fox-2 to UGCAUGU sequence[23]. The critical residues for recognition of the UGCAUG sequence are well conserved in the Fox-1 family, including ASD-1 and FOX-1 (**Fig. 3b**). Four alleles of the *asd-1* mutants have nonsense mutations, and three alleles have mis-sense mutations within the conserved amino-acid residues in the RRM (**Fig. 3a**,**b**). All of these alleles of *asd-1* mutants had essentially the same phenotype.

Next we examined the genetic interaction between *asd-1* and the other Fox-1 family genes. *asd-1* and *fox-1* had distinct but dosage-dependent effects on *egl-15* reporter expression (**Fig. 3c–f**): *asd-1; fox-1* double homozygotes almost exclusively expressed E5B-GFP (**Fig. 3f**), whereas homozygotes in *fox-1* alone were indistinguishable from wild-type background worms (**Fig. 3c**,**d**), and heterozygotes of one gene in the homozygous background of the other had an intermediate phenotype (**Fig. 3d**,**e**). RT-PCR analysis confirmed the effects of *asd-1* and *fox-1* mutations on the splicing patterns of the *egl-15* reporter (**Fig. 3g**). In contrast, mutation in *spn-4* did not affect *egl-15* reporter expression, and *spn-4* had no apparent genetic interaction with *asd-1* and *fox-1* (data not shown).

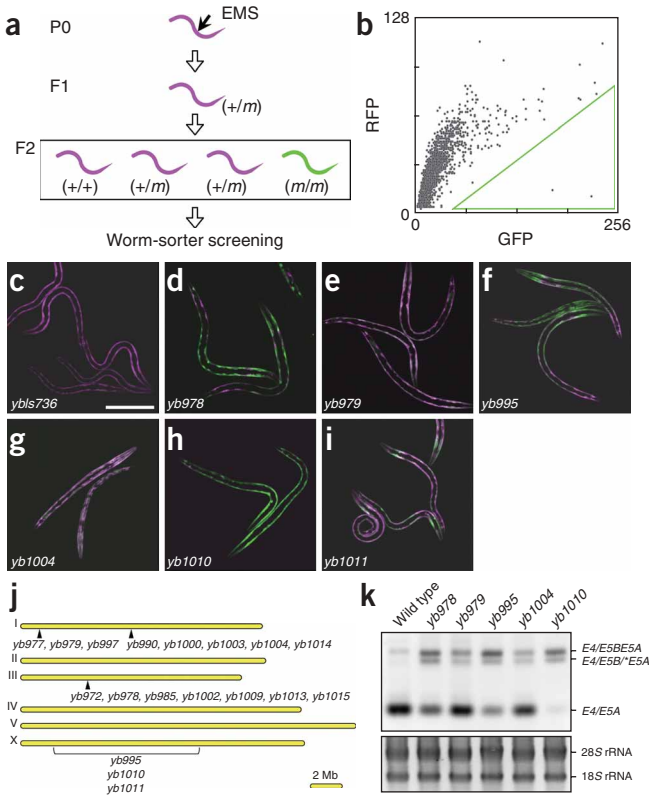

Figure 2 | Screening for mutants defective in tissue-specific alternative splicing of *egl-15* reporter. (**a**) Schematic view of mutant screening. P0 hermaphrodite worms were treated with EMS and F2 worms were pooled and subjected to worm-sorter screening. +, wild-type allele. *m*, mutant allele. (**b**) An example of fluorescence profiles in mutant screening with a worm sorter. Each dot represents a single F2 worm. Worms (in a green triangle) that express more GFP than the parental strain were isolated. (**c–i**) Confocal images of a parental and mutant strains (GFP, green; RFP, magenta). A parental strain *ybIs736 [myo-3::EGL-15BGAR] X* expressing the *BGAR* reporter in the body-wall muscles under the *myo-3* promoter (**c**). Mutant strains; alleles are indicated (**d–i**). Scale bar, 100 μm. (**j**) Summary of chromosome mapping. Genomic loci of all alleles examined are indicated. (**k**) RT-PCR analysis of mRNAs derived from the *egl-15* reporter transgene (top) and total RNA (bottom). Genetic backgrounds of *ybIs736* reporter are indicated. Each band corresponds to mRNAs schematically illustrated in **Figure 1b**.

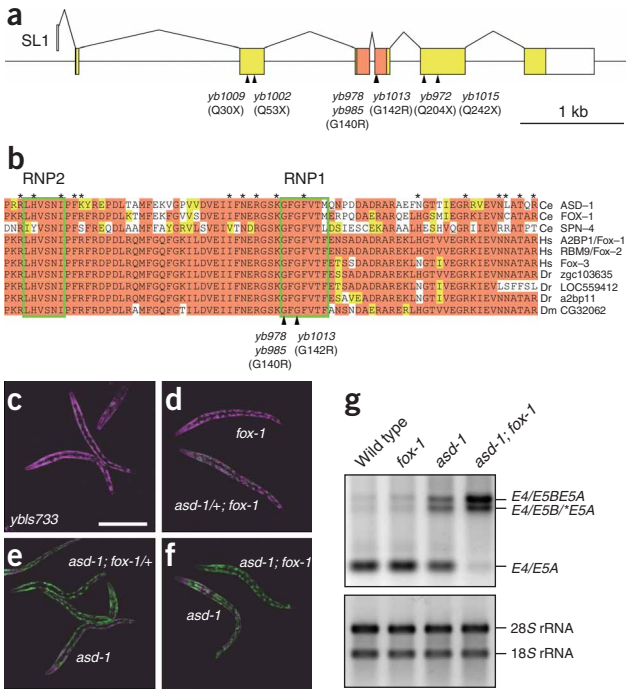

Figure 3 | Identification of *asd-1* gene. (**a**) Schematic representation of *asd-1* gene and mutations identified in mutant alleles. Boxes indicate exons. ORFs are in yellow, and RRMs are in orange. SL1, spliced leader. (**b**) Amino-acid sequence alignment of RRMs of Fox-1 family RNA-binding proteins in worms (Ce; FOX-1, ASD-1 and SPN-4), mammals (Hs; A2BP1/Fox-1, RBM9/Fox-2 and LOC339162/D11Bwg0517e/Fox-3), zebrafish (Dr; zgc103635, LOC559412 and a2bp1l) and fruit fly (Dm; CG32062). Identical amino-acid residues are shaded in orange and residues with similar properties are shaded in yellow. Conserved RNP1 and RNP2 motifs are boxed in green. Positions of mis-sense mutations identified in *asd-1* mutant alleles are indicated. (**c**–**f**) Merged view of E5A-RFP and E5B-GFP of *ybIs733 [myo-3::EGL-15BGAR]* reporter with wild type (**c**), *fox-1 (e2643)* and *asd-1 (yb978)/+*; *fox-1* (**d**), *asd-1* and *asd-1; fox-1/+* (**e**) and *asd-1* and *asd-1; fox-1* (**f**) backgrounds. Scale bar, 100 μm. (**g**) RT-PCR analysis of mRNAs derived from the *BGAR* reporter mini-gene (top) and total RNA (bottom). Genetic backgrounds of *ybIs733* reporter are indicated. Each band corresponds to mRNAs schematically illustrated in **Figure 1b**.

These results indicate that *asd-1* and *fox-1* cooperatively regulate the *egl-15* reporter in body-wall muscles.

Regulation mechanisms of the *egl-15* reporter

We searched for a *cis* element corresponding to *asd-1* and *fox-1* function by modifying the reporter mini-gene. Because vertebrate Fox-1 has been shown to specifically bind to the (U)GCAUG sequence[22] and a UGCAUG sequence is conserved in the 3′ portion of *egl-15* intron 4 in nematodes (**Fig. 4a**), we examined whether this sequence is involved in the regulation of *egl-15* reporter. When we introduced a point mutation into this sequence in the reporter mini-gene (**Fig. 4b**), the expression of E5A-RFP was hardly detectable, and instead, the expression of E5B-GFP was enhanced in the body-wall muscles (**Fig. 4c**), and the exclusive expression of E5B-GFP was not affected in the hypodermis (**Supplementary Fig. 1** online). These profiles suggested that the presence of the UGCAUG sequence is required for the repression of E5B-GFP and/or the selection of E5A-RFP in the body-wall muscles. To discriminate these possibilities, we introduced mutations to inactivate the splicing acceptor site for E5B-GFP. In this case, expression of E5A-RFP was restored in the body-wall muscles (**Supplementary Fig. 2** online). In the hypodermis, inactivation of the splicing acceptor site for E5B-GFP also resulted in the selection of E5A-RFP (**Supplementary Fig. 3** online). In contrast, the deletion of the

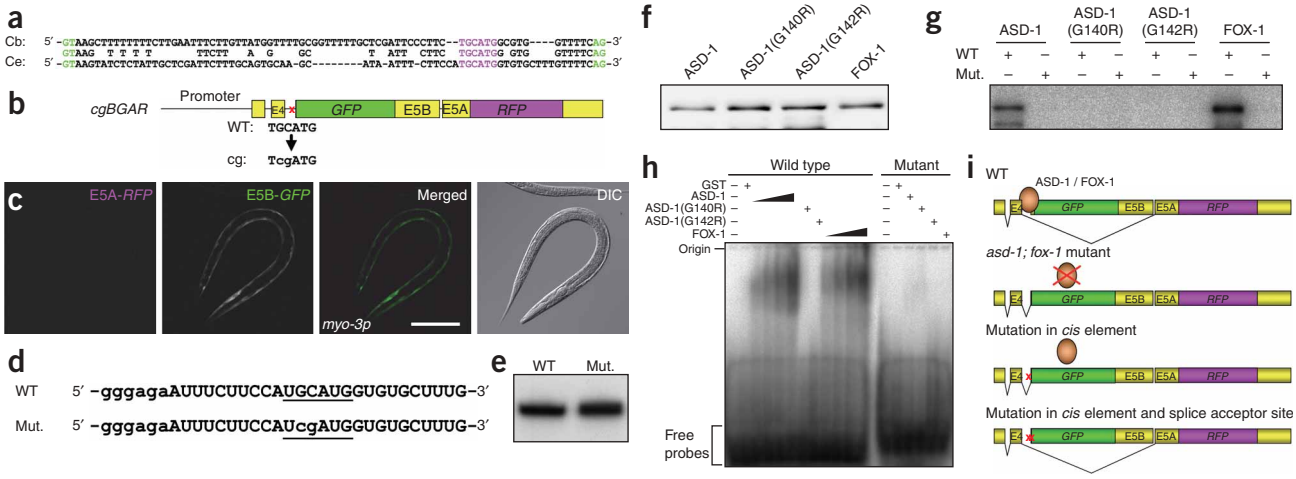

Figure 4 | Regulation of *egl-15* reporter in body-wall muscles. (**a**) Nucleotide sequence alignment of *egl-15* intron 4 from *C. briggsae* (Cb) and *C. elegans* (Ce). Conserved nucleotides are indicated between the two sequences. The UGCAUG stretch is in magenta. Conserved dinucleotides in splice donor and acceptor sites are in green. Red 'x' indicates the site of mutation introduced in the reporter mini-gene. (**b**) Schematic representation of *cgBGAR* mini-genes. TGCATG stretch in intron 4 was mutagenized to TcgATG. (**c**) Transgenic worm expressing *cgBGAR* reporter under *myo-3* promoter. Scale bar, 100 μm. (**d**) Sequences of wild-type (WT) and mutant (Mut.) RNA probes used in the *in vitro* RNA-binding assays. Uppercase letters indicate sequences derived from *egl-15* intron 4. The UGCAUG stretch is underlined. (**e**) Radioisotope-labeled RNA probes used in the assays. RNA probes were electrophoresed under denaturing conditions and autoradiographed. (**f**) Recombinant GST-fusion proteins used in the assays were electrophoresed and detected with anti-GST. (**g**) Cross-linking assay. Recombinant proteins and RNA probes used are indicated. (**h**) Electophoretic mobility shift assay. RNA probes and recombinant proteins used are indicated. Twofold dilution series of ASD-1 and FOX-1 proteins were used. (**i**) Schematic model of tissue-specific regulation of *egl-15* reporters in body-wall muscles.

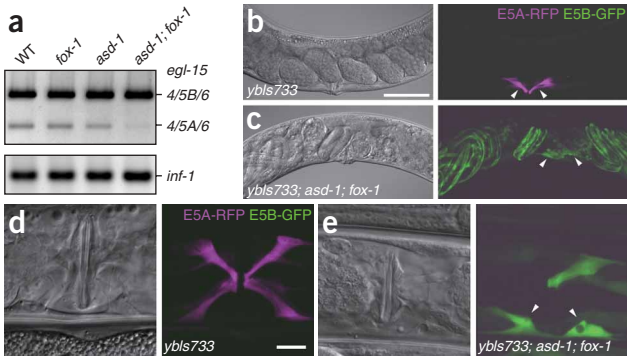

Figure 5 | Regulation of the endogenous *egl-15* exon 5s by *asd-1* and *fox-1*. (**a**) RT-PCR analyses of endogenous *egl-15* (top); *inf-1* was used as a reaction control (bottom). Genetic backgrounds of the *ybIs733* reporter are indicated. (**b**–**e**) DIC images and merged confocal images of *ybIs733* reporter worms (**b**,**d**) and *asd-1; fox-1; ybIs733* (**c**,**e**). Lateral views of gravid adult hermaphrodites (**b**,**c**) and magnified ventral views of the vulva (**d**,**e**). Arrowheads indicate vulval muscles. Anterior is to the left. Scale bar in **b**, 50 μm; in **d**, 10 μm.

authentic E5B sequences did not affect the inclusion or repression of E5B-GFP (**Supplementary Note 2** online). These results indicated that the UGCAUG stretch was required to repress the selection of E5B-GFP, and that E5A-RFP could be selected when upstream E5B-GFP was unavailable.

We then asked whether ASD-1 and FOX-1 proteins could directly bind to the UGCAUG sequence identified in the *egl-15* intron 4 *in vitro*. As expected, both ASD-1 and FOX-1 proteins bound to an RNA probe derived from wild-type intron 4, but not to an RNA probe containing a mutation within this sequence (**Fig. 4d–h**). Mutant ASD-1 proteins ASD-1(G140R) and ASD-1(G142R), derived from *asd-1* mutant alleles, did not bind to the RNA probes (**Fig. 4f–h**), indicating that the binding was essential for ASD-1 function. From these results, we propose a model of regulation of the *egl-15* alternative splicing reporter in body-wall muscles (**Fig. 4i**). In the wild-type background, ASD-1 and FOX-1 bind to the UGCAUG stretch in intron 4 to repress the selection of E5B-GFP, thereby enhancing the selection of E5A-RFP. When ASD-1 and FOX-1 are absent, or the UGCAUG sequence is mutagenized, the *cis* element is not occupied and upstream E5B-GFP is preferably selected. When E5B-GFP is unavailable because of a mutation in the splicing acceptor site, downstream E5A-RFP can be selected.

asd-1 and *fox-1* regulate endogenous *egl-15* gene

Lastly, we examined whether our model, that the selection of *egl-15* exon 5A depends on *asd-1* and *fox-1* function, could be applied to the endogenous *egl-15* gene. RT-PCR analysis revealed that the amount of endogenous *egl-15* mRNA isoforms including exon 5A was indeed reduced in the double mutant (**Fig. 5a**). Mutations within *egl-15* exon 5A have been shown to cause the aberrant migration of the sex myoblasts leading to the Egg laying–defective (Egl) phenotype[19]. We found that the *asd-1; fox-1* double mutant was also Egl with a much smaller number of embryos on the culture plates (data not shown) and the presence of late stage embryos in the uterus (**Fig. 5b,c**). Further, the vulval muscles were off the vulva in the double mutant (**Fig. 5b–e**). These results indicated that *asd-1; fox-1* double mutants phenocopied the specific loss of function of EGL-15 (5A) isoforms, and that *asd-1* and *fox-1* were redundantly required for the selection of endogenous *egl-15* exon 5A in sex myoblasts (**Supplementary Fig. 4** online). The transgenic reporters can thus be used to explain regulation mechanisms of alternative splicing of endogenous genes *in vivo*.

DISCUSSION

In this study we established an alternative splicing reporter system and showed that the *egl-15* reporter was differentially expressed in various tissues, presumably reflecting the intrinsic nature of each cell or 'cellular codes'. Complete penetrance of expression patterns of the *egl-15* reporters in wild-type and mutant worms suggests the presence of strict regulation mechanisms *in vivo*. It should be noted that the reporter expression profile was dependent on the dosage of the *asd-1* and *fox-1* genes (**Fig. 3c–f**), whereas *asd-1* and *fox-1* single mutants were phenotypically normal. These facts emphasize that this transgenic reporter system allowed identification of *asd-1* and *fox-1* as redundant regulators of endogenous *egl-15*, which would not be achieved by conventional genetic screening of Egl mutants. The high sensitivity of the reporter, however, may lead to identification of *trans*-acting factors or signaling molecules that are not necessarily involved in the regulation of endogenous genes. It is critical to confirm that the endogenous genes are also regulated in the same manner. Use of the splicing mutants may facilitate the experimental validation of the regulator-target relation *in vivo*. The mutants will also allow global search for candidate target genes by using whole-genome tiling arrays. Further collection and profiling of reporters based on other genes may lead to comprehensive spatiotemporal characterization of alternative splicing events in the organism.

In generating transgenic alternative-splicing reporters, we assumed that *C. elegans* is an appropriate model organism to generate an *in vivo* reporter system. Most of the worm genes have multiple exons[17]. The average size of worm introns (267 base pairs; bp) is comparable to that of internal exons (218 bp), which is in great contrast to those in human (>3,300 bp versus 145 bp)[24]. Furthermore, larger internal exons of several hundred base pairs are frequently found in *C. elegans*[24]. These facts lead us to assume that exonic elements would be less essential for the splicing machinery to define exons in pre-mRNAs and insertion of *GFP* or *RFP* cDNA in internal exons would have less effect on the authentic splicing in *C. elegans* than in vertebrates. We investigated various forms of the transgenic reporters for the *egl-15* exons 5A and 5B—with the separate mini-genes (**Supplementary Note 1**) and with the unified mini-genes (**Fig. 1** and **Supplementary Note 2**). These reporters showed essentially the same tissue-specific expression profiles of GFP and RFP proteins, and the experimentally confirmed splicing patterns of mRNAs corresponded to the alternative selection of E5s. These results verified our assumption that the insertion of the *GFP* or *RFP* cDNA into internal E5B or the deletion of the authentic exon 5B sequence did not affect the inclusion or repression of E5B in the *egl-15* reporters. Further analyses lead us to the model that binding of *trans*-acting factors to the intron 4 determines whether to include or repress the exon 5B (**Supplementary Fig. 4**). The position of the essential *cis* elements, however, cannot always be applied to other mutually exclusive alternative splicing events, as we encountered a case in which a downstream intron was essential for the regulated expression of mutually exclusive exons (H.K.;

unpublished observation). Therefore, it is critical for constructing splicing reporters that the expression of GFP and RFP proteins directly reflects either of the alternative splicing events and that expression profiles of the reporter correspond to those of the endogenous genes.

The Fox-1 families regulate alternative splicing by binding to the UGCAUG sequence with extraordinarily high sequence specificity compared to other *trans*-acting RNA-binding proteins. We demonstrated the first genetic evidence that the Fox-1 family regulates tissue-specific mutually exclusive alternative splicing of an endogenous gene *in vivo* via specific binding to the UGCAUG sequence in a dose-dependent manner. *egl-15* encodes FGFR and alternate usage of exon 5s determines ligand specificity[19]. One of the mammalian counterparts of *egl-15*, *FGFR2*, also has tissue-specific mutually exclusive exons IIIb and IIIc, and these exons ultimately confer ligand specificity[25]. A recent report showed that the expression of Fox-2 in cultured cells regulated the transition from FGFR2(IIIc) to FGFR2(IIIb) by binding to UGCAUG sequences[26]. These correlations between *C. elegans* and mammals suggest that the regulation mediated by the Fox-1–UGCAUG interaction is an evolutionarily conserved mechanism to determine tissue-specific alternative splicing patterns in metazoans. The consequences of Fox-1–UGCAUG interaction, however, seem to be more complicated. In the case of *egl-15* exon 5A and 5B, the binding of FOX-1 or ASD-1 to a single UGCAUG stretch in intron 4 represses the selection of exon 5B, leading to the selection of downstream exon 5A. In the same way, in cultured cells, vertebrate Fox-1 repressed the inclusion of mitochondrial ATP synthase F1γ exon 9 and nonmuscle exon of α-actinin gene by binding to UGCAUG sequences within the upstream intron[22]. In contrast, vertebrate Fox-1 or Fox-2 can induce the inclusion of fibronectin EIIIB exon[22], FGFR2 exon IIIb[26] and neuron-specific c-*src* N1 exon[27,28] by binding to UGCAUG stretches within the downstream intron. The differential effect of Fox-1–UGCAUG interaction may depend on relative positions of the sequences[28]. Furthermore, A2bp1/Fox-1 and Rbm9/Fox-2 genes have multiple promoters and multiple alternative exons, and these isoforms showed differential subcellular localization and effects on alternative splicing events[27,28], suggesting complicated regulation of Fox-1–UGCAUG–mediated alternative splicing. Existence of mutants with various types of *egl-15* reporter expression profiles shown in **Figure 2d–i** suggests that several unidentified genes are involved in the regulation of the *egl-15* reporter. Identification of these genes would lead to further understanding of selection mechanisms of *egl-15* exon 5s and/or regulation of *asd-1* and *fox-1* function *in vivo*. Because *C. elegans* has relatively small introns and the regulation mechanisms are expected to be simple, this transgenic reporter worm system may help to understand basic rules of alternative splicing regulation in higher eukaryotes.

METHODS

Plasmid construction. We constructed reporter mini-genes as described in **Figure 1a**. We constructed various reporter cassettes in Gateway Entry vectors (Invitrogen). We introduced mutations using the QuickChange method (Stratagene). Sequences of primers used in the construction are available in **Supplementary Methods** online. We constructed various promoter vectors in Gateway Destination vectors (Invitrogen) modified from pPD49.26 (gift of A. Fire). Nucleotide sequences of promoter vectors are available on the *C. elegans* promoter database (http://www.shigen.nig.ac.jp/c.elegans/promoter/index.jsp). We constructed expression vectors by homologous recombination between reporter cassettes and promoter vectors. *GFP* cDNA was derived from pEGFP-N2 (Clontech), and *RFP* cDNA was derived from *mRFP1* (gift of R.Y. Tsien).

Worm culture and microscopy. We cultured worms following standard methods. We prepared transgenic lines using *lin-15 (n765)* as a host. We generated integrant lines by ultraviolet light irradiation[29]. We mutagenized worms by treating gravid worms with 47 mM ethyl methanesulfonate (EMS) for 4 h. We screened for mutants using a fluorescence-assisted worm sorter (COPAS BIOSORT; Union Biometrica). We performed single nucleotide polymorphism (SNP)-based mapping as described[30]. We used a confocal microscope (Fluoview FV500; Olympus), for image scanning and processed the acquired images with Metamorph (Molecular Devices).

RT-PCR. We extracted total RNAs from mixed-stage worm culture with RNeasy Mini column (Qiagen). We synthesized the first-strand cDNAs with oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Sequences of primers used in the RT-PCR assays are available in **Supplementary Methods**.

***In vitro* binding assays.** We produced recombinant proteins as glutathione-*S* transferase (GST) fusion proteins by induction with L-arabinose in *Escherichia coli* strain BL21-AI (Invitrogen). We purified GST fusion proteins with glutathione-Sepharose 4B (Pharmacia) and dialyzed them against RNA-binding buffer (200 mM KCl in HEPES-KOH (pH 7.9) with 1 mM DTT and 10 mM PMSF). We synthesized RNA probes by *in vitro* transcription with [α^{32}P]UTP. We performed *in vitro* binding experiments in the presence of 130 ng/μl *E. coli* tRNA in 25 μl of RNA binding buffer for 30 min at 20 °C. We performed cross-linking analysis by irradiating 18,000 J/m^2 of ultraviolet light with a cross-linker CL-1000 (UVP). We performed the electrophoretic mobility shift assay essentially as described[22] with 5% polyacrylamide gel and 0.5 × TBE.

Gene nomenclature and database accession numbers. The gene name class *asd-* and gene assignment *asd-1* have been approved by the CGC Genetic Map and Nomenclature Curator. The nucleotide sequence of *asd-1* cDNA has been deposited in the GenBank/EMBL/DDBJ databases with the accession number DQ485531.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

H.K. contributed to the overall experiments. T.K. and S.M. contributed to mutant screening and chromosome mapping. H.K. and M.H. organized this work. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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1. Black, D.L. Protein diversity from alternative splicing: a challenge for bioinformatics and post-genome biology. *Cell* **103**, 367–370 (2000).
2. Maniatis, T. & Tasic, B. Alternative pre-mRNA splicing and proteome expansion in metazoans. *Nature* **418**, 236–243 (2002).
3. Stamm, S. *et al.* Function of alternative splicing. *Gene* **344**, 1–20 (2005).
4. Eyras, E., Caccamo, M., Curwen, V. & Clamp, M. ESTGenes: alternative splicing from ESTs in Ensembl. *Genome Res.* **14**, 976–987 (2004).
5. Kampa, D. *et al.* Novel RNAs identified from an in-depth analysis of the transcriptome of human chromosomes 21 and 22. *Genome Res.* **14**, 331–342 (2004).
6. Johnson, J.M. *et al.* Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302**, 2141–2144 (2003).
7. Pan, Q. *et al.* Revealing global regulatory features of mammalian alternative splicing using a quantitative microarray platform. *Mol. Cell* **16**, 929–941 (2004).
8. Sharp, P.A. The discovery of split genes and RNA splicing. *Trends Biochem. Sci.* **30**, 279–281 (2005).
9. Shin, C. & Manley, J.L. Cell signalling and the control of pre-mRNA splicing. *Nat. Rev. Mol. Cell Biol.* **5**, 727–738 (2004).
10. Hagiwara, M. Alternative splicing: a new drug target of the post-genome era. *Biochim. Biophys. Acta* **1754**, 324–331 (2005).
11. Matlin, A.J., Clark, F. & Smith, C.W. Understanding alternative splicing: towards a cellular code. *Nat. Rev. Mol. Cell Biol.* **6**, 386–398 (2005).
12. Blencowe, B.J. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem. Sci.* **25**, 106–110 (2000).
13. Hastings, M.L. & Krainer, A.R. Pre-mRNA splicing in the new millennium. *Curr. Opin. Cell Biol.* **13**, 302–309 (2001).
14. Xu, X. *et al.* ASF/SF2-regulated CaMKIIdelta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* **120**, 59–72 (2005).
15. Lundquist, E.A. *et al.* The mec-8 gene of *C. elegans* encodes a protein with two RNA recognition motifs and regulates alternative splicing of unc-52 transcripts. *Development* **122**, 1601–1610 (1996).
16. Lisbin, M.J., Qiu, J. & White, K. The neuron-specific RNA-binding protein ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA. *Genes Dev.* **15**, 2546–2561 (2001).
17. Sakharkar, M.K. & Kangueane, P. Genome SEGE: a database for 'intronless' genes in eukaryotic genomes. *BMC Bioinformatics* **5**, 67 (2004).
18. Nagasaki, H., Arita, M., Nishizawa, T., Suwa, M. & Gotoh, O. Species-specific variation of alternative splicing and transcriptional initiation in six eukaryotes. *Gene* **364**, 53–62 (2005).
19. Goodman, S.J., Branda, C.S., Robinson, M.K., Burdine, R.D. & Stern, M.J. Alternative splicing affecting a novel domain in the *C. elegans* EGL-15 FGF receptor confers functional specificity. *Development* **130**, 3757–3766 (2003).
20. Skipper, M., Milne, C.A. & Hodgkin, J. Genetic and molecular analysis of fox-1, a numerator element involved in *Caenorhabditis elegans* primary sex determination. *Genetics* **151**, 617–631 (1999).
21. Gomes, J.E. *et al.* The maternal gene spn-4 encodes a predicted RRM protein required for mitotic spindle orientation and cell fate patterning in early *C. elegans* embryos. *Development* **128**, 4301–4314 (2001).
22. Jin, Y. *et al.* A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing via the pentanucleotide GCAUG. *EMBO J.* **22**, 905–912 (2003).
23. Auweter, S.D. *et al.* Molecular basis of RNA recognition by the human alternative splicing factor Fox-1. *EMBO J.* **25**, 163–173 (2006).
24. Lander, E.S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921 (2001).
25. Dell, K.R. & Williams, L.T. A novel form of fibroblast growth factor receptor 2. Alternative splicing of the third immunoglobulin-like domain confers ligand binding specificity. *J. Biol. Chem.* **267**, 21225–21229 (1992).
26. Baraniak, A.P., Chen, J.R. & Garcia-Blanco, M.A. Fox-2 mediates epithelial cell-specific fibroblast growth factor receptor 2 exon choice. *Mol. Cell. Biol.* **26**, 1209–1222 (2006).
27. Nakahata, S. & Kawamoto, S. Tissue-dependent isoforms of mammalian Fox-1 homologs are associated with tissue-specific splicing activities. *Nucleic Acids Res.* **33**, 2078–2089 (2005).
28. Underwood, J.G., Boutz, P.L., Dougherty, J.D., Stoilov, P. & Black, D.L. Homologues of the *Caenorhabditis elegans* Fox-1 protein are neuronal splicing regulators in mammals. *Mol. Cell. Biol.* **25**, 10005–10016 (2005).
29. Mitani, S. Genetic regulation of *mec-3* gene expression implicated in the specification of the mechanosensory neuron cell types in *Caenorhaabditis elegans*. *Dev. Growth Differ.* **37**, 551–557 (1995).
30. Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H. & Plasterk, R.H. Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28**, 160–164 (2001).