Insights into the Involvement of Spliceosomal Mutations in Myelodysplastic Disorders from Analysis of SACY-1/DDX41 in Caenorhabditis elegans

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ABSTRACT Mutations affecting spliceosomal proteins are frequently found in hematological malignancies, including myelodysplastic syndromes and acute myeloid leukemia (AML). DDX41/Abstrakt is a metazoan-specific spliceosomal DEAD-box RNA helicase that is recurrently mutated in inherited myelodysplastic syndromes and in relapsing cases of AML. The genetic properties and genomic impacts of disease-causing missense mutations in DDX41 and other spliceosomal proteins have been uncertain. Here, we conduct a comprehensive analysis of the Caenorhabditis elegans DDX41 ortholog, SACY-1. Biochemical analyses defined SACY-1 as a component of the C. elegans spliceosome, and genetic analyses revealed synthetic lethal interactions with spliceosomal components. We used the auxin-inducible degradation system to analyze the consequence of SACY-1 depletion on the transcriptome using RNA sequencing. SACY-1 depletion impacts the transcriptome through splicing-dependent and splicing-independent mechanisms. Altered 3’ splice site usage represents the predominant splicing defect observed upon SACY-1 depletion, consistent with a role for SACY-1 in the second step of splicing. Missplicing events appear more prevalent in the soma than the germline, suggesting that surveillance mechanisms protect the germline from aberrant splicing. The transcriptome changes observed after SACY-1 depletion suggest that disruption of the spliceosome induces a stress response, which could contribute to the cellular phenotypes conferred by sacy-1 mutant alleles. Multiple sacy-1/ddx41 missense mutations, including the R525H human oncogenic variant, confer antimorphic activity, suggesting that their incorporation into the spliceosome is detrimental. Antagonistic variants that perturb the function of the spliceosome may be relevant to the disease-causing mutations, including DDX41, affecting highly conserved components of the spliceosome in humans.

KEYWORDS SACY-1/DDX-41; spliceosome; myelodysplastic disorders; C. elegans

Mutations affecting components of the spliceosome are frequently found in hematological malignancies, including myelodysplastic syndromes (MDS; Yoshida et al. 2011; reviewed by Yoshida and Ogawa 2014; Coltri et al. 2019), which comprise a heterogeneous set of myeloid neoplasms characterized by anemia and cytopenia that progress to acute myeloid leukemia (AML) to varying degrees (Tefferi and Vardiman 2009). The genetic properties and genomic impacts of disease-causing missense mutations in spliceosomal proteins have been uncertain. Nonetheless, mutations affecting spliceosomal components are predictive of poor clinical outcomes in AML patients (Papaemmanuil et al. 2016). Exactly how mutations in spliceosomal components contribute to malignancy is uncertain. An attractive model, but one that has not been firmly established, is that aberrant splicing might interfere with tumor suppressor activity. This model suggests that malignancy-associated spliceosomal mutations are either loss-of-function or confer antimorphic (i.e., antagonistic) activity. Importantly, genome sequencing data...
in patients is currently being used in the clinic to generate personalized prognoses, with the idea of optimally targeting existing therapies and generating new treatment strategies (Grinfeld et al. 2018). One potential therapeutic approach under development is the discovery of splicing inhibitors (Effenberger et al. 2017; Kim and Abdel-Wahab 2017; DeNicola and T’ang 2019). Although mutations affecting several spliceosomal proteins appear to be beneficial to tumor cells, excessive splicing abnormalities are likely to be lethal to all cells. Splicing inhibitors have been demonstrated to target tumor cells with splicing mutations by inducing excessive splicing abnormalities, but cells with intact splicing machinery appear to be resistant to these agents (Seiler et al. 2018). In fact, several new splicing inhibitors are currently in clinical trials.

The spliceosomal components frequently affected in MDS, occurring in ~60–70% of patients, include the biochemically well-defined factors SF3B1, SRSF2, and U2AF1 (Yoshida et al. 2011; reviewed by Yoshioka and Ogawa 2014). More recent studies have implicated DDX41, a DEAD-box RNA helicase highly conserved in metazoans, whose precise biochemical function in the spliceosome is less well understood (Ding et al. 2012; Polprasert et al. 2015; Cardoso et al. 2016; Lewinsohn et al. 2016; Li et al. 2016; Diness et al. 2018; reviewed by Maciejewski et al. 2017). DDX41 appears to be specifically recruited to the catalytically active C complex (Jurica et al. 2002; Bessonov et al. 2008), which performs the second step of splicing, in which the 5’ and 3’ exons are ligated and an intronic lariat is released. DDX41 is one of many spliceosomal proteins specific to metazoans and not found in budding yeast (Bessonov et al. 2008).

Whole genome sequencing studies suggest that DDX41 mutations are associated with hematological malignancies that are considered to be different clinical entities. For example, examination of clonal evolution of relapsed AML cases identified DDX41 as one of several genes found to be mutated in secondary, but not primary, tumors, suggesting that de novo DDX41 mutations might contribute to disease progression (Ding et al. 2012). By contrast, studies of familial AML syndromes suggest that preexisting germline DDX41 mutations in trans to newly arising somatic mutations cause the development of hematological malignancies (Polprasert et al. 2015; Cardoso et al. 2016; Lewinsohn et al. 2016; Li et al. 2016). Germline biallelic DDX41 missense mutations were recently reported in two siblings that exhibited intellectual disability, psychomotor delays, and facial and skeletal dysmorphologies, with one sibling presenting with childhood leukemia (Diness et al. 2018). Other work suggests that DDX41 might be a multifunctional protein; in addition to its nuclear function in RNA splicing, it has been suggested to function as a cytoplasmic DNA sensor in a signaling pathway that detects infecting double-stranded DNA and initiates an antiviral interferon response (Zhang et al. 2011; Parvatiyar et al. 2012; Stavrou et al. 2015, 2018; reviewed by Jiang et al. 2017). However, more recent work suggests that cyclic GMP-AMP synthase (cGAS) functions as the major DNA sensor and is several orders of magnitude more effective in inducing interferon beta synthesis than DDX41 (Sun et al. 2013). Two studies, one of DDX41 and another of its Drosophila ortholog, Abstrakt, suggested a role in regulating translation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 (Peters et al. 2017) and the Inscuteable protein (Irion et al. 2004), respectively, though the exact mechanism of these activities has not been elucidated, and indirect effects acting at the level of splicing were not addressed in these studies.

To better understand the highly conserved functions of DDX41, we undertook a comprehensive molecular genetic analysis of its ortholog, sacy-1, in the nematode Caenorhabditis elegans. The cGAS and STING-mediated cytoplasmic DNA sensing pathway is absent in C. elegans (Wu et al. 2014), suggesting that this model may be useful for addressing spliceosomal functions of DDX41/SACY-1. Our prior work identified the DEAD-box helicase SACY-1 as a negative regulator of oocyte meiotic maturation functioning in the germline upstream of the TIS11 CCCH zinc-finger RNA-binding proteins OMA-1 and OMA-2 (Kim et al. 2012). Genetic analysis also established roles for SACY-1 in regulating the hermaphrodite sperm-to-oocyte switch, and in preventing necrotic cell death of gametes. Genetic experiments further suggested an essential role for sacy-1(+?) in early embryos and larvae that appeared to be maternally rescued. At the time of our original study, searchable databases of the scientific literature had not yet annotated DDX41 (or its Drosophila ortholog, Abstrakt) as spliceosomal components identified by proteomics. We therefore did not recognize that SACY-1 was likely involved in splicing.

In this study, we undertook a comprehensive molecular genetic analysis of the functions of SACY-1 in C. elegans. Our results demonstrate that SACY-1 is a component of the C. elegans spliceosome that exhibits genetic interactions with other spliceosomal components. Depletion of SACY-1 in the germine or soma was found to have major impacts on the transcriptome through splicing-dependent and splicing-independent mechanisms. Alterations in 3’ splice site selection represent the most prevalent changes in splicing patterns observed following SACY-1 depletion, consistent with its function as a component of the spliceosomal C complex, which carries out the second step in splicing. Missplicing events are more prevalent upon SACY-1 depletion in the soma than in the germline, leading us to suggest that surveillance mechanisms protect the germline from aberrant splicing events. The gene expression changes observed after SACY-1 depletion suggest that perturbations of spliceosomal function might induce a stress response, which could be relevant to the cellular phenotypes conferred by sacy-1 mutant alleles. Further, our genetic results reveal that multiple sacy-1 missense mutations confer a dosage-sensitive antimorphic activity, most consistent with the possibility that they compromise the function of the spliceosome by perturbing the action of other spliceosomal proteins. Moreover, the human oncogenic DDX41 R525H mutation was introduced into the
**C. elegans** genome using CRISPR-Cas9 genome editing and found to exhibit weak antagonistic activity. Antagonistic activities that perturb the function of the spliceosome, as defined by genetic analysis in *C. elegans*, may be relevant to the disease-causing mutations affecting highly conserved components of the spliceosome in humans, including DDX41.

**Materials and Methods**

**C. elegans strains and genetic analysis**

The genotypes of strains used in this study are reported in Supplemental Material, Table S1. Genes and mutations are described in WormBase (www.wormbase.org; Harris et al. 2014) or in the indicated references. Culture and genetic manipulations were conducted at 20°C unless specified otherwise.

The following mutations were used: LGI–fog-1(q253ts), dpy-5(e61), glp-1(tm1478), unc-13(e51), unc-13(e1091), lin-41(tm2914), lin-41(tm1541[gfp::tev-s-tag::lin-41]), sacy-1(tm1487), sacy-1(tm1479), sacy-1(tm1480), sacy-1(tm1481), sacy-1(tm1482), sacy-1(tm1483), sacy-1(tm1484), sacy-1(tm1485), sacy-1(tm1486), sacy-1(tm1487), sacy-1(tm1488), sacy-1(tm1489), sacy-1(tm1490), sacy-1(tm1491), sacy-1(tm1492), sacy-1(tm1493), sacy-1(tm1494), sacy-1(tm1495), sacy-1(tm1496), sacy-1(tm1497), sacy-1(tm1498), sacy-1(tm1499), sacy-1(tm1500), sacy-1(tm1501), sacy-1(tm1502), sacy-1(tm1503), sacy-1(tm1504), sacy-1(tm1505), sacy-1(tm1506), sacy-1(tm1507), sacy-1(tm1508), sacy-1(tm1509), sacy-1(tm1510), sacy-1(tm1511), sacy-1(tm1512), sacy-1(tm1513), sacy-1(tm1514), sacy-1(tm1515), sacy-1(tm1516), sacy-1(tm1517), sacy-1(tm1532[3xFLAG::PreScission protease site::gfp::tev-s-tag::sacy-1]), sacy-1(tm1880[aid::gfp::tev::mrc::sacy-1]), and sacy-1(tm1887); LGII–tra-2(e2020), iel57[et-3::TIR1:mRuby::unc-54 3′UTR + Cb unc-119(+)], iel64[gd-1::TIR1:mRuby::gd-1 3′UTR + Cb unc-119(+)]; LGIII–unc-119(ed3); LGIV–unc-24(e138), fem-3(e1996), and dpy-20(e1282); LGV–y-4(ok1806), her-1(hv1y101), emb-4(sa44), unc-51(e369), and fog-2(oz40). The following rearrangements were used: h22[bli-4(e937) let-7(q782) qIs483]; I(III), tmCl9[dpy-5(mls1236) + pmyo-2::mCherry] I (Dejima et al. 2018), mln1[dpy-10(e128) mls14] II, and tmCl12[ecl-9 (mls194) + pmyo-2::Venus] V (Dejima et al. 2018). The following transgenics were used: tnEx37[sacy-1(+)] + sur-5::gfp, tnEx159[::gfp::sacy-1 + pDPMM0168B(unc-119(+)]) and tns102[::gfp::tev-s-tag::sacy-1 + Cb unc-119(+)] III.

For the analysis of genetic interactions between *sacy-1(tm1481)* and *fem-3(e1996)*, non-Unc non-Dpy non-GFP animals from *sacy-1(tm1481)/h22[bli-4(e937) let-7(q782) qIs483]; fem-3(e1996)/unc-24(e138) dpy-20(e1282)] were individually cultured and scored for germline phenotypes. Following scoring, the *fem-3* genotype of each animal was scored by conducting PCR with primers *fem-3* F2 and *fem-3* R2 and sequencing the products.

To map the cold-sensitive (15°C) and temperature-sensitive (25°C) phenotypes of *sacy-1(tm1480)*, 34 Unc non-Dpy recombinants were obtained from *sacy-1(tm1480)/dpy-5(e61) unc-13(e1091)* heterozygotes. The recombinant chromosomes were bred to homozygosity and scored for the presence or absence of the *sacy-1(tm1480)* mutation by conducting PCR with primers H27M09.1F1 and H27M09.1R4, and sequencing purified PCR products with primer H27M09.1F2. We found that 7 of the 34 recombinants contained *sacy-1(tm1480)* and were cold-sensitive and temperature-sensitive. By contrast, 27 recombinants were *sacy-1(+) and grew at 15 and 25°C. These data indicate that *sacy-1(tm1480)* mutation is inseparable from the cold-sensitive and temperature-sensitive phenotypes (e.g., within ~0.06 map units). In addition, 32 Dpy non-Unc recombinants were selected. Interestingly, all the homozygous recombinants were fertile at both 15 and 25°C, including the 22 recombinants that contained the *sacy-1(tm1480)* mutation. Although these *dpy-5(e61) sacy-1(tm1480)* recombinants grew at 15 and 25°C, they produced appreciable numbers of dead embryos and grew more slowly than their *sacy-1(+) counterparts. This result suggests that the *dpy-5(e61)* mutation suppresses the cold-sensitive and temperature-sensitive phenotypes of *sacy-1(tm1480)*. Previous work has shown that mutant alleles of collagen genes can suppress temperature-sensitive mutations in other gene products, possibly by triggering a stress response (Levy et al. 1993; Maine and Kimble 1993; Nishiwaki and Miwa 1998). That *dpy-5(e61)* suppresses *sacy-1(tm1480)* was further shown by constructing *dpy-5(e61) sacy-1(tm1480)sacy-1(tm1480) unc-13(e1091)* heterozygotes (n = 30), of which 20 exhibited the *sacy-1(tm1480)* sperm-defective phenotype at 25°C and 10 were fertile. Thus, *dpy-5(e61)* exhibits semidominance for its effects on body morphology and for suppression of *sacy-1(tm1480)*. To examine the dominant high incidence of males (Him) phenotype of *sacy-1(tm1480)* and its interaction with *sacy-1(tm1887)*, we compared the percentage of males produced at 25°C by *dpy-5(e61)sacy-1(tm1480) unc-13(e1091)* and *dpy-5(e61)sacy-1(tm1887)sacy-1(tm1480) unc-13(e1091)* heterozygotes.

**RNA interference**

Genome-wide RNA interference (RNAi) screening employed the Ahringer feeding library (Kamath et al. 2003) using the RNAi culture media described by Govindan et al. (2006) at 22°C. The empty vector L4440 was used as a control. The identity of RNAi clones was verified by DNA sequencing. Gene-specific RNAi was performed by placing wild-type or *sacy-1(tm1385)* gravid hermaphrodites on RNAi medium seeded with double-stranded RNA (dsRNA)-expressing *Escherichia coli* (Timmons and Fire 1998). The gravid hermaphrodites were immediately treated with 20% bleach to release the F1 embryos. Phenotypes were assessed 3–4 days later. In scoring RNAi clones for enhanced defects specific to the *sacy-1(tm1385)* mutant background, we could not reliably score clones that produced high levels of embryonic lethality in the wild-type genetic background. For quantification of phenotypes, sterility and gamete degeneration were scored in the F1 generation, and embryonic lethality was scored in the F2 generation produced by the RNAi-treated F1 animals.

**Immunofluorescence, fluorescent labeling, and microscopy**

Dissected gonads were fixed in 3% paraformaldehyde as described (Rose et al. 1997). Fixed gonads were stained with rabbit anti-RME-2 antibody (Grant and Hirsh 1999; kindly

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provided by B. Grant, Rutgers University, 1:50), a mixture of two purified mouse monoclonal anti-MSP antibodies (Kosinski et al. 2005, each at 1:300), rabbit anti-phospho-histone H3 (Ser10) antibody (1:400; Millipore). Secondary antibodies were Alexa 488-conjugated donkey anti-rabbit antibodies (1:500; Jackson ImmunoResearch) and Cy3-conjugated goat anti-mouse antibodies (1:500; Jackson ImmunoResearch). 4',6-diamidino-2-phenylindole (DAP1) was used to detect DNA. DIC and fluorescent images were acquired on a Zeiss motorized Axioplan 2 microscope with either a 40× Plan-Neofluor (numerical aperture 1.3) or a 63× Plan-Apochromat (numerical aperture 1.4) objective lens using an Axiocam MRm camera and AxioVision software (Zeiss). DIC and GFP fluorescent images of sacy-1(tm5503);tnEx159 and sacy-1(tm1632) adults were acquired on a Nikon A1R resonant scanning confocal microscope using a Plan Fluor 40× Oil DIC (numerical aperture 1.3) objective lens. Image acquisition utilized the large image function of NIS-Elements AR (v. 5.11.00) with an image overlap setting of 15%

**Genome editing and generation of transgenics**

CRISPR-Cas9 genome editing used pRB1017 to express single guide RNA (sgRNA) under control of the C. elegans U6 promoter (Arribere et al. 2014). The sequences of all oligonucleotides used are listed in Table S2. To generate sgRNA clones, annealed oligonucleotides were ligated to Bsal-digested pRB1017 plasmid vector, and the resulting plasmids were verified by Sanger sequencing. pDD162 served as the source of Cas9 expressed under control of the eef-1A.1/eft-3 promoter (Dickinson et al. 2013). Indels were targeted to exon 2 of sacy-1 using sacy-1 sgRNA7 (pCS20). The injection mix contained pCSS20 (25 ng/μl), pDD162 (50 ng/μl), and Pmyo-2::tdTomato (4 ng/μl). sacy-1(tn1602–tn1612) were recovered from injections into DG3913 lin-41 (tn1541 [gfp::tev::s-tag::lin-41]) and sacy-1(tn1615–tn1617) were recovered from injections into the wild type (strain N2).

An N-terminal gfp fusion to endogenous sacy-1, sacy-1 (tn1632 [3xFlag::PreScission protease site::gfp::tev::s-tag::sacy-1]), was constructed using sacy-1 sgRNA1 (pCS486) and a repair template generated by conducting the PCR with oligonucleotide primers sacy-1 5HAF and sacy-1 3HAR, using a gfp::tev::s-tag::sacy-1 recombobrinated fosmid (SK212; Kim et al. 2012) as template. Genome editing employed the dpy-10 coconversion method (Arribere et al. 2014). The injection mix contained pJA58 (7.5 ng/μl), AF-ZF-827 (500 nM), pCS486 (50 ng/μl), repair template (50 ng/μl), and pDD162 (50 ng/μl) and was injected into wild-type worms. Correct targeting was verified by conducting PCR with primer pairs GFP_7215 and H27M09.1_R5 and GFP_1094R and H27M09.1_seqF1 followed by DNA sequencing. DG3768 was constructed using microparticle bombardment with SK212 as described (Pratiss et al. 2001).

An N-terminal auxin-inducible degron (aid) fusion to sacy-1, sacy-1 (tn1880[aid::gfp::myc::sacy-1]), was constructed using sacy-1 sgRNA1 and a repair template generated by conducting the PCR with oligonucleotide primers sacy-1 AID5F and sacy-1 AID3R using a wee-1.3::aid::gfp::myc clone (pCS75, C.A. Spike, unpublished results) as template. The injection mix, prepared as described above, was injected into C1A322 worms. sacy-1 (m1880[aid::gfp::myc::sacy-1]) was identified by screening the progeny of 414 F1 Roller animals for GFP fluorescence. Correct targeting was verified by conducting PCR with primer pairs GFP R1 and H27M09.1_F5 and GFP_F1 and H27M09.1_R5 followed by DNA sequencing.

The R525H mutation in DDX41 was imported into C. elegans (e.g., SACY-1[R534H]) using genome editing (Paix et al. 2014) with sacy-1 sgRNA11 and sacy-1 sgRNA12 and a single-stranded repair oligonucleotide (sacy-1 GM1), which introduces the R534H mutation and two synonymous changes to alter the protospacer adjacent motif and to facilitate screening using an introduced AvaI restriction site. The injection mix contained pJA58 (7.5 ng/μl), AF-ZF-827 (500 nM), sacy-1 sgRNA11 (25 ng/μl), sacy-1 sgRNA12 (25 ng/μl), sacy-1 GM1 (500 nM), and pDD162 (50 ng/μl) and was injected into wild-type worms. Edited loci were verified by PCR and DNA sequencing using primers sacy-1 seq F1 and sacy-1 seq R1.

**Antibody production, purification, and western blotting**

sacy-1 cDNA sequences were cloned into the E. coli expression vector pMal-c2 to create an inducible fusion protein wherein maltose binding protein was fused to amino acids 411–578 of SACY-1 (MBP::SACY-1(411–578)). MBP::SACY-1(411–578) was column- and gel-purified and used to immunize rabbits. Immunizations and sera collection were performed using standard protocols (Cocalico Biologicals, Reamstown, PA). Rabbit antibody (R217) was affinity purified, and was suitable for use in western blots with partially purified SACY-1 preparations. Hybridoma cell lines producing anti-GFP monoclonal antibodies 12A6 and 4C9 (Sanchez et al. 2014) were obtained from the Developmental Studies Hybridoma Bank and prepared as described (Tsukamoto et al. 2017). Proteins were separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and visualized after western blotting. Blots were blocked with 5% nonfat dried milk. Primary antibodies used to detect proteins were affinity-purified rabbit anti-SACY-1(411–578) R217 antibody (100 ng/ml) and rabbit anti-GFP NB600-308 antibody (250 ng/ml; Novus Biologicals). The secondary antibody used for western blots was peroxidase-conjugated donkey anti-rabbit antibody (1:30,000; Jackson ImmunoResearch). Detection was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**Identification of SACY-1-associated proteins**

Tandem affinity purification of SACY-1 was conducted using strains DG4068 and DG4070 using modifications of a previously described protocol (Tsukamoto et al. 2017). Immunopurified proteins were precipitated with 16.7% trichloroacetic acid (TCA), washed with acetone at −20°, and briefly separated on a 12% NuPAGE Bis-Tris gel, stained with Colloidal Blue Staining Kit (Invitrogen). Lanes were
subdivided into eight gel slices and mass spectrometry was performed at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School) using an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific). Protein identification used the Sequest software program (Thermo Fisher Scientific) to match the fragmentation pattern of tryptic peptides to the *C. elegans* proteome. The data were filtered to a 1–2% peptide false discovery rate. File S1 reports the mass spectrometry results and the additional filtering criteria for identifying nonspecific interactions.

Single-step immunopurifications were also conducted on a smaller scale using 80 mg of protein lysate from DG3768 *sacy-1(tm5503) I; unc-119(ed3) tnl1012[sacy-1;p::gfp::tev::s-tag::sacy-1 + Cb unc-119(+)] III. GFP::TEV::S-tag::SACY-1 and associated proteins were isolated using monoclonal anti-GFP antibody 12A6 and TEV protease cleavage as described (Tsukamoto et al. 2017). In addition to our standard conditions (300 mM KCl) higher stringency washes were conducted using 1 M KCl or 300 mM KCl with 5 μg/ml RNase A.

**RNA sequencing**

The auxin-inducible degradation system (Zhang et al. 2015) was used to deplete *SACY-1* using strain backgrounds in which TIR1 was expressed in the germline (CA1352) or soma (CA1200). Experimental (DG4700 and DG4703) and control strains (CA1352 and CA1200) were grown on peptone-enriched nematode growth medium with NA22 as a food source. Embryos were isolated by alkaline hypochlorite treatment (20% bleach and 0.5 N NaOH), washed in M9 buffer and allowed to hatch overnight in the absence of food. For each of three biological replicates, 60,000 L1-stage larvae were cultured on two 150 × 15 mm Petri dishes containing peptone-enriched medium with OP50. The worms were grown to the young adult stage and harvested by washing off the plates with M9, then placed on fresh plates containing peptone-enriched medium and 2 mM auxin seeded with *E. coli*. Total RNA was isolated using TRIzol LS Reagent (Invitrogen) and the RNeasy Micro Kit (Qiagen, Valencia, CA). Poly(A)^+ RNA was selected from 1 μg of total RNA using the NEBNext Ultra Kit (New England Biolabs, Ipswich, MA). Libraries were prepared and sequenced by Genewiz (South Plainfield, NJ). Paired-end reads of 150 bp were obtained on an Illumina HiSeq 4000 instrument with an average depth >31 million reads per sample.

**Bioinformatics**

After trimming adapters with Trim Galore (v0.6.0) and cutadapt (v1.18), reads were assessed for quality with FastQC (v0.11.8), mapped to the *C. elegans* reference genome with STAR (v2.7.2a) guided by gene annotations defined in Ensembl (release 97) and sorted and indexed with samtools (v1.7). Gene-level abundance was estimated for Ensembl defined annotations using the featureCounts function in the Bioconductor package Rsubread (v1.28.1). An average of 28 million high-quality (MAPQ > 55) reads mapped to annotated genes within each sample. Principal component analysis and inspection of 5' vs. 3' read coverage indicated that one soma control sample (CA1200-2) contained degraded RNA and was excluded from further analysis. Differential gene expression of Ensembl defined genes was determined using DESeq2 (v1.26.0). P values were adjusted for multiple test correction using the Benjamini–Hochberg procedure. The fold change, adjusted P values, the mean number of counts across samples, and the number of complementary DNA fragments per kilobase of transcript per million mapped reads (FPKM) were used to define differentially expressed genes. Gene ontology (GO) data were obtained from WormBase release WS273, and analyzed taking length bias into account using the Goseq (v1.38.0) package. Novel transcripts in each of the high-quality samples and in the previously published GSE57109 (Ortiz et al. 2014) dataset were identified using StringTie (v2.0.4) and merged together with the Ensembl annotations to generate a comprehensive annotation set. These annotations were used with RMATS (v4.0.2 turbo) to determine statistically significant differences, expressed as false discovery rates (FDRs), for splicing events between conditions. Coverage data were visualized with Gviz (v 1.30.0). A custom R script with details for the analysis and figure generation is available at https://github.com/micahgearhart/sacy1.

**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains and reagents are available upon request. RNA sequencing data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through accession number GSE144003. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11825673.

**Results**

**SACY-1 is a component of the *C. elegans* spliceosome**

To characterize *SACY-1*-associated proteins, we conducted tandem affinity purifications using strains in which we used CRISPR-Cas9 genome editing to insert 3xFLAG and eGFP tags at the *SACY-1* N-terminus, separated by a PreScission protease recognition sequence (Figure 1 and Figure S1). The resulting *sacy-1(tm1632)[3x flag::PreScission::gfp::tev::s-tag::sacy-1]*) strain was viable and fertile and exhibited no apparent germline or somatic defects. Although 3xFLAG::GFP::SACY-1 is expressed in all cells except sperm, it appears most abundant in the female germline (Figure 1, A–D). Thus, we conducted purifications from protein lysates prepared from adult animals in which the germline was feminized (experiment I) and also from adult hermaphrodites (experiment II). This approach was taken to utilize the most
abundant source of SACY-1, and also to potentially gain insights of how it might function as a negative regulator of meiotic maturation in the absence of sperm, though our data were not informative from this standpoint. In both experiments, we found that 3xFLAG::GFP::SACY-1 associated with 52 proteins defined as spliceosomal proteins in other systems (Figure 1F and Table S3; Jurica et al. 2002; Bessonov et al. 2008). We also detected nine additional spliceosomal proteins in the purification from the female, but not the hermaphrodite, genetic background (Table S4), but this might be a consequence of the fact that more protein extract was used in that experiment. We also detected 29 other proteins in our tandem affinity purifications (Table S5). Homologs of several of these factors have been implicated in the regulation of RNA splicing, including NRDE-2 (Jiao et al. 2019), CIR-1 (Maita et al. 2005; Kasturi et al. 2010), and CDK-12 (Rodrigues et al. 2012). These biochemical studies, taken together with the results from the genome-wide RNAi screen described below, are consistent with the possibility that both specific and pleiotropic defects conferred by sacy-1 mutant alleles result from spliceosomal defects.

Biochemical studies established that DDX41/Abstrakt is a component of the C complex, which requires the presence of an RNA splicing substrate to form, and is sufficient to catalyze exon ligation in the absence of additional factors (Jurica et al. 2002; Bessonov et al. 2008). Treatment of isolated C complexes with high salt was observed to remove DDX41/Abstrakt and dozens of other spliceosomal proteins from a C complex RNP core (Bessonov et al. 2008). To begin to identify spliceosomal proteins that might associate with SACY-1 at high stringency, we conducted purifications in the presence of 1 M KCl or RNase A. We identified eight spliceosomal proteins that maintained high levels of association with SACY-1 (recovery of at least 50% of peptides) under both stringent conditions (RBM-39, RNP-6, DDX-35, ACIN-1, CYN-13, PRPF-4, CYN-12, and C16C10.4; Table S6). Interestingly, DDX35/PPWD1 is a C complex protein, which is removed from the C complex RNP together with DDX41/Abstrakt after high salt treatment (Bessonov et al. 2008).

Additional studies will be needed to determine whether SACY-1 and DDX-35 (or the other spliceosomal proteins) might interact directly.

**Genetic interactions between sacy-1 and several genes encoding spliceosomal proteins**

To better understand the function of sacy-1, we conducted a genome-wide RNAi screen for loci that resulted in more severe phenotypes when knocked down by RNAi in a sacy-1 reduction-of-function (rf) genetic background [sacy-1(tm1385rf)] than in the wild type. We screened 18,101 RNAi clones from the Ahringer RNAi library, and identified five clones that produced increased levels of sterility, gamete degeneration, or embryonic lethality when knocked down in the sacy-1(tm1385rf) genetic background (Table 1). Because these three phenotypes are observed in a variety of sacy-1 mutant alleles (Kim et al. 2012 and as described below), we consider these genetic interactions to reflect an enhancement of sacy-1 mutant phenotypes or to be synthetic lethal interactions with the rf allele. The five RNAi clones target the transcripts of three genes (Table 1): mog-2 (one clone), Y111B2A.25 (one clone), and emb-4 (three clones). To test whether sacy-1 expression and/or localization is affected by RNAi of mog-2, Y111B2A.25, or emb-4, we conducted RNAi of these genes in sacy-1(tm5503) mutant animals expressing the rescuing gfp::sacy-1 transgene (tnEx159). In no case did we observe that an RNAi treatment altered the expression or localization of the GFP::SACY-1 transgene; the expression level and predominant nuclear localization of GFP::SACY-1 after RNAi was similar to that of the control animals (S. Kim and D. Greenstein, unpublished results). This result suggests that the defects caused by the RNAi treatments in the sacy-1(tm1385rf) mutant background are not caused by changes in the expression or localization of SACY-1.

**mog-2(RNAi)** induces a higher penetrance of sterility, gamete degeneration, and embryonic lethality in the sacy-1(tm1385) mutant genetic background in comparison to the wild type (Figure S2 and Table 1). Y111B2A.25 is annotated as a pseudogene (Agarwal et al. 2010; www.wormbase.org). Y111B2A.25 is part of an operon, and the expressed sequence tag (EST) data show that the Y111B2A.25 locus is transcribed, but the transcript lacks protein-coding ability. In C. elegans, 240 bp of sequence identity is sufficient to induce off-target RNAi effects (Rual et al. 2007). Use of the Basic Local Alignment Search Tool (BLAST) indicates that Y111B2A.25(RNAi) might target the cacn-1 locus, which encodes a spliceosomal protein and shares ~200 bp of sequence identity with Y111B2A.25. To test whether the enhanced sterility induced by Y111B2A.25(RNAi) in the sacy-1(tm1385) genetic background might be explained by an off-target effect to cacn-1, we conducted cacn-1(RNAi) and found that the cacn-1(RNAi) induces complete sterility in both the sacy-1(tm1385) and wild-type animals (Figure S2 and Table 1). Interestingly, under the cacn-1(RNAi) condition, the sacy-1(tm1385) animals show additional phenotypes, such as high penetrance of a protruding vulva (Pv1) phenotype, and slow growth compared to the wild type, suggesting a genetic interaction between cacn-1 and sacy-1 that might be partially masked by the strong phenotypes induced by cacn-1(RNAi).

Thus, we reasoned that the short identity shared between cacn-1 (RNAi) dsRNA might induce strong sterility and Pb phenotypes. We tested this possibility by serially diluting the cacn-1(RNAi) response by serially diluting the cacn-1(RNAi)-inducing bacteria with bacteria containing the empty vector control (L4440). Consistent with the possibility
Figure 1 Tandem affinity purification of SACY-1 to identify associated proteins. DIC (A and C) and fluorescence (B and D) photomicrographs of an adult hermaphrodite generated by CRISPR-Cas9 genome editing to express an epitope-tagged version of SACY-1 suitable for tandem affinity purification (strain DG4070 sacy-1(tn1632[3x3FLAG::PreScission protease site::gfp::tev::s-tag::sacy-1])1). The regions within the dotted rectangles are magnified in (C and D). Oocytes (1–3), the spermatheca (sp), and the vulva (vu) are indicated. Bars, 100 μm in (A and B); 50 μm in (C and D). (E) Strategy for tandem affinity purification. Cleavage with PreScission protease releases GFP::SACY-1 and associated proteins from the affinity matrix, whereas the fragment with the 3xFLAG epitope and nonspecifically bound proteins are retained on the matrix. The second purification step utilized an anti-GFP affinity resin and a low pH elution. Western blot analysis of fractions from the tandem affinity purification procedure analyzed with anti-GFP or R217 anti-SACY-1(411–578) antibodies are shown in Figure S1. (F) Spliceosomal proteins reproducibly associated with SACY-1 by tandem affinity purification. Spliceosomal proteins are organized into spliceosomal subcomplexes according to Bessonov et al. (2008) or the supplemental references in Table S3. The primary data on which figure is based are presented in File S1 and Table S3. The area of each circle represents the maximal percentage coverage of each protein in a single gel slice from the purification from DG4068 (see Table S3). For example, the percent coverage of SACY-1 was 78.9% and the percent coverage of MOG-2 was 56.1%. The names of the human orthologs of the C. elegans proteins are listed in Table S3.
that \textit{Y111B2A.25} (RNAi) targets \textit{cacn-1}, limiting the efficacy of the \textit{cacn-1} (RNAi) response revealed specific enhancement of sterility, gamete degeneration, and embryonic lethality in the \textit{sacy-1(tm1385)} genetic background (Table S7). Notably, the response to limited \textit{cacn-1} (RNAi) exhibited by \textit{sacy-1(tm1385)} animals was remarkably similar to their response to \textit{Y111B2A.25} (RNAi) (Table S7). The human and \textit{Drosophila} orthologs of \textit{CACN-1} have been identified as components of spliceosomal C complexes (Jurica \textit{et al.} 2002; Bessonov \textit{et al.} 2008, 2010; Herold \textit{et al.} 2009; Fica \textit{et al.} 2019). Like DDX41/\textit{Abstrakt}, Cactin is recruited to the C complex of the spliceosome.

In addition to \textit{meg-2} and \textit{Y111B2A.25}, we identified three different RNAi clones targeting the \textit{emb-4} locus as strong enhancers of the \textit{sacy-1(tm1385)} sterility and gamete degeneration phenotypes (Figure S2 and Table 1). \textit{emb-4} encodes a nuclear protein orthologous to human Aquarius/IBP160/\textit{KIAA0560}/\textit{FASAP164}, an intron-binding spliceosomal protein with a helicase-like domain (Sam \textit{et al.} 1998; Jurica \textit{et al.} 2002; Bessonov \textit{et al.} 2008; Herold \textit{et al.} 2009; De \textit{et al.} 2015; Bertram \textit{et al.} 2017; Haselbach \textit{et al.} 2018). To extend these RNAi results, we examined genetic interactions between \textit{sacy-1} and \textit{emb-4}, employing the \textit{emb-4(sa44)} reduction-of-function allele. When combined with the \textit{sacy-1(tm5503)} null allele, we observed enhancement of lethal vulval rupture and protruding vulva (Pvl) phenotypes in \textit{emb-4(sa44)} double mutants (Table 2). We also observed enhancement of these phenotypes in \textit{emb-4(sa44)} double mutants, which were derived from \textit{sacy-1(tm1385)}/+; \textit{emb-4(sa44)} parents (Table 2). Interestingly, the F1 progeny of fertile \textit{sacy-1(tm1385)}; \textit{emb-4(sa44)} homozygous adults exclusively produced dead embryos or arrested L1-stage larvae, unlike each of the single mutants, which were viable and fertile (Table 3).

The genetic interactions between \textit{sacy-1} and the spliceosomal proteins identified in our genome-wide RNAi screen for \textit{sacy-1} enhancers (\textit{MOG-2}, \textit{EMB-4}, and \textit{CACN-1}) likely reflect biochemical interactions because these three proteins were very well represented in our tandem affinity purifications of \textit{SACY-1}-associated proteins (~35–56% peptide coverage; Table S3). Taken together, these genetic and biochemical interactions are consistent with the possibility that the multiple \textit{sacy-1} mutant phenotypes result from compromised functions of the spliceosome.

\textbf{Impact of \textit{sacy-1} on the transcriptome}

\textit{Depletion of SACY-1 using the auxin-inducible degradation system}: RNA sequencing studies using human patient samples suggested a role for DDX41 in splice site selection for a small number of human genes (Polprasert \textit{et al.} 2015). Thus, we sought to address the impact of \textit{SACY-1} on the transcriptome by exploiting the power of the \textit{C. elegans} system for transcriptomics under well-controlled conditions. We chose to use the auxin-inducible degradation system (Zhang \textit{et al.} 2015) to acutely deplete \textit{SACY-1} in the adult stage, and, thus, avoid indirect impacts on the transcriptome arising as a developmental consequence of strong loss-of-function phenotypes (e.g., germline degeneration and cell fate changes). Because \textit{SACY-1} is expressed in the germline and soma, we used strains bearing germline (CA1352 \textit{iCIS64}) or somatically expressed (CA1200 \textit{iCIS57}) TIR1 F-box proteins to deplete AID::GFP::\textit{SACY-1} in each tissue individually (Figure S3 and Figure S4). Depletion of AID::GFP::\textit{SACY-1} in the germline starting at approximately the L3 stage phenocopied the
gamete degeneration phenotype observed in *sacy-1* null mutants (see below) in a small proportion of the animals (3 of 270; Figure S3). This result is consistent with genetic mosaic analysis showing that the gamete degeneration phenotype is cell autonomous (Kim et al. 2012), and it also highlights the difficulty of recapitulating null phenotypes through auxin-inducible degradation. When AID::GFP::SACY-1 is depleted in the germline starting at the L4 stage, many F1 progeny arrest as embryos or larvae, consistent with the idea that maternally contributed *sacy-1(+)* activity is essential. Animals that escape the lethality and progress to adulthood often display the germline degeneration phenotype (40%; *n* = 139). When AID::GFP::SACY-1 is depleted in the soma beginning at the L4 stage, the resulting F1 progeny grew very slowly, taking ∼4–6 days to reach adulthood (instead of 2.5 days) and were sterile (Figure S3). Taken together, depletion of SACY-1 using the auxin-inducible degradation system resulted in a reducing condition less severe than the null phenotype, but more severe than conferred by rf missense alleles (see below).

For analysis of transcriptomes, we exposed young adult hermaphrodites to auxin for 24 hr before preparing total RNA for sequencing. Examination of the worms showed that AID::GFP::SACY-1 was depleted from the targeted tissues (Figure S4). Total RNA was prepared from each of three biological replicates and their respective controls, which were the parent strains expressing TIR1 in the germline (ieSi64 or soma (ieSi57) also treated with auxin. Poly(A)+ mRNA was sequenced using 150 bp paired-end reads, and the sequencing reads were aligned to the WB235/ce11 genome. Principal component analysis (PCA) revealed that the biological replicates clustered together (Figure 2A), which is indicative of experimental reproducibility. However, PCA indicated that the control strains for the germline (CA1352) and soma (CA1200) depleted samples did not cluster together, which indicates that, under these conditions, the two strain backgrounds exhibit marked differences in their transcriptomes (Figure 2A)—a finding that was further confirmed with a more granular assessment of mRNA expression level differences of individual genes (Figure S5A). Thus, in our analysis, we compared the germline- and soma-depleted SACY-1 transcriptomes only to their respective controls.

**Table 2 Genetic interactions between sacy-1 and emb-4: enhancement of germline and somatic sacy-1 mutant defects**

| Genotype                          | Vulval rupture (%) | Sterile and PV+ (%) | Sterile* (%) | Fertile (%) |
|----------------------------------|--------------------|---------------------|--------------|-------------|
| sacy-1(tm5503) (n = 284)         | 3                  | 1                   | 96           | 0           |
| sacy-1(tm5503)/+; emb-4(sa44)b (n = 205) | 0                  | 0                   | 0            | 100         |
| sacy-1(tm5503); emb-4(sa44)b (n = 242) | 83                 | 16                  | 1            | 0           |
| sacy-1(tm1385) (n = 278)         | 0                  | 0                   | 0            | 100         |
| sacy-1(tm1385)/+; emb-4(sa44)b (n = 201) | 0                  | 0                   | 0            | 100         |
| sacy-1(tm1385); emb-4(sa44)b (n = 144) | 36                 | 4                   | 3            | 57*         |

*a* Sterile animals exhibit the sacy-1(+) gamete degeneration phenotype.

*b* The hT2(qIs48) balancer chromosome, which is dominantly marked with GFP, was used to differentiate between sacy-1(tm5503) heterozygotes and homozygotes.

*c* The progeny of sacy-1(tm1385)/hT2(qIs48); emb-4(sa44) hermaphrodites; the balancer chromosome provides maternal sacy-1(+) function. The fertile F1 progeny of these animals are maternal-effect lethal, see Table 3.

*d* These adult hermaphrodites produce a majority of embryos that fail to hatch, see Table 3.

**Changes in transcript abundance following SACY-1 depletion:** We observed two classes of transcriptome alterations upon depletion of SACY-1 in the germline or soma: changes in transcript abundance and alterations in splicing patterns. In terms of transcript abundance, we observed 242 down-regulated genes (twofold down-regulation, adjusted *P* < 0.05, FPKM in soma control ≥ 2.5, mean counts across samples > 25) in the RNA samples depleted for somatic SACY-1 (Figure 2, B and C and File S2). Notably these down-regulated genes included many cuticle collagen genes and genes affecting cuticular morphology and body size (col-17, col-41, col-46, col-47, col-90, col-128, col-149, dpy-3, dpy-4, dpy-5, dpy-6, dpy-8, dpy-9, dpy-13, dpy-20, lon-3, mlt-7, qua-1, rol-6, rol-8, sqt-1, and sqt-2). Consistent with this observation, the top enriched GO term for transcripts reduced in abundance in the SACY-1 soma-depleted samples was “cuticle development involved in collagen and cuticulin-based cuticle molting cycle” (Figure S5B). We also observed 242 up-regulated genes (twofold up-regulation, adjusted *P* < 0.05, FPKM in SACY-1 soma-deplete ≥ 2.5, mean counts across samples > 25) in the SACY-1 soma-depleted samples (Figure 2, B and C and File S2). The top enriched GO term for transcripts with increased abundance in the SACY-1 soma-depleted samples related to cellular responses to heat stress, the unfolded protein response, and innate immune responses (Figure S5B), suggesting that the organism might perceive the reduction of *sacy-1(+) function* as a stressor, and then mount a response that then alters the transcriptome.

In the SACY-1 germline-depleted samples, we observed 126 down-regulated genes (twofold down-regulation, adjusted *P* < 0.05, FPKM in germline control ≥ 2.5, mean counts across samples > 25; Figure 2, B and D and File S2). The top enriched GO terms for transcripts with decreased abundance in the SACY-1 germline-depleted samples included the response to heat stress and the unfolded protein response (Figure S5B), suggesting the response to *sacy-1(+) depletion* differs between the soma and germline. We observed 311 transcripts to exhibit increased abundance in the SACY-1 germline-depleted sample (twofold up-regulation, adjusted *P* < 0.05, FPKM in SACY-1 germline-deplete ≥ 2.5, mean counts across samples > 25; Figure 2,
### Table 3 Genetic interactions between sacy-1 and emb-4: enhancement of embryonic lethality

| Genotype\(^a\) | Embryonic lethal (%) | Viable (%) |
|---------------|---------------------|------------|
| sacy-1(tn1385) (n = 949) | 1 | 99 |
| emb-4(sa44) (n = 1348) | 4 | 96 |
| sacy-1(tn1385); emb-4(sa44)\(^b\) (n = 620) | 97 | 0 |

\(^a\) The number of embryos examined.

\(^b\) The F1 progeny of fertile sacy-1(tn1385); emb-4(sa44) parents derived from the sacy-1(tn1385); emb-4(sa44) heterozygotes analyzed in Table 2.

B and D and File S2). The top enriched GO terms for transcripts with increased abundance in the SACY-1-germline depleted samples included SCF-dependent proteasomal ubiquitin-dependent protein catabolic processes (Figure S5B). As discussed below, this observation may be relevant to the role of SACY-1 in germline sex determination.

**Alteration of splicing patterns following SACY-1 depletion:**

In the SACY-1 soma-depleted samples, we observed significant (FDR < 0.05) alterations in splicing patterns for 1606 transcripts (Figure 3A). These splicing alterations fell into several broad classes: the use of alternative 5’ splice sites, the use of alternative 3’ splice sites, abnormal splicing within an exon, skipped exons, and retained introns. The largest class of splicing changes was the use of alternative 3’ splice sites, consistent with the fact that DDX41 was shown to be recruited to the C complex, which mediates the second step in splicing (Bessonov et al. 2008). Multiple splicing defects were sometimes observed within a single gene. For example, in the case of the RNA-binding protein ETR-1, which has multiple isoforms and is expressed in the soma and germline (Boateng et al. 2017), depletion of SACY-1 results in intron retention and multiple alterations in 3’-splice-site usage (Figure 3B). In the SACY-1 germline-depleted samples we observed significant (FDR < 0.05) alterations in splicing for 796 transcripts (Figure 3A). Thus, splicing defects appeared less prevalent in the SACY-1 germline-depleted samples than the SACY-1 soma-depleted samples. One possibility is that nonsense mediated decay or other surveillance pathways actively clear misspaced mRNAs from the germline. Some alternative splicing events were observed in both the RNA preparations depleted for SACY-1 in the germline and the soma (Figure 3A). For example, we observed retention of an intron in prdx-6 mRNA in both experiments (Figure 3C). Likewise, we observed alternative 3’ splice site selection for the heterochronic gene lin-28 in both SACY-1-depleted samples, which results in a change in reading frame (Figure 3D).

**Germline sex-specific splicing patterns and the involvement of SACY-1:** Alternative splicing events enriched in oogenic or spermatogenic germlines were previously identified (Ortiz et al. 2014). Consequently, we reanalyzed their dataset in order to directly compare with the alternative splicing events observed upon SACY-1 depletion. We observed 1600 genes for which there was a significant (FDR < 0.05) germline sex-specific splicing pattern (Figure S6 and File S3). We noted that upon SACY-1 depletion in either the soma or germline, oocyte-enriched splicing events were favored (Figure 3A and Figure S6). This result suggests that SACY-1 plays a role in the selection of 3’ splice sites for many genes, and raises the possibility that the appropriate balance of splice variants may play a role in cellular differentiation.

**sacy-1 reduction-of-function mutation enhances germline tumor formation**

Prior work showed that mutational or RNAi treatments affecting the function of multiple spliceosomal components enhance weak gain-of-function (gf) mutations in glp-1/Notch, resulting in the ectopic proliferation of undifferentiated germ cells in the proximal gonad arm (Mantina et al. 2009; Kerins et al. 2010; Wang et al. 2012). This phenotype, which is referred to as a proximal proliferation or Tumorous phenotype, can also be enhanced by mutations in genes that function in GLP-1/Notch signaling (reviewed by Hubbard and Schedl 2019). Since the preceding analysis suggested that SACY-1 is a functional component of the spliceosome, we predicted that the sacy-1(tn1385rf) mutation would enhance the Tumorous phenotype conferred by the glp-1 (ar202gf) mutation (Pepper et al. 2003). Thus, we constructed the sacy-1(tn1385rf); glp-1(ar202gf) double mutant and analyzed its phenotype at the permissive temperature of 15°C, which is the same temperature at which the sacy-1 reduction-of-function mutation enhances the glp-1/Notch phenotype. We expected that the double mutant should exhibit a more proximal proliferation phenotype than the single mutants. Under these conditions (40 hr post-L4 at 15°C), very few glp-1(ar202gf) young adult hermaphrodites were observed to exhibit a proximal proliferation phenotype with undifferentiated germ cells in the proximal gonad arm (~0.8%, Table 4). By contrast, many sacy-1(tn1385rf); glp-1(ar202gf) adults exhibited a Tumorous phenotype (~50%, Table 4). This phenotype was not observed in sacy-1(tn1385rf) single mutants (Table 4). To examine this phenotype further, we stained dissected and fixed gonads with the phosphohistone H3(Ser10) M-phase marker. Of 25 sacy-1(tn1385); glp-1(ar202) gonads scored, 16 (64%) contained phosphohistone H3-positive undifferentiated germ cells in the proximal gonad arm at 15°C. The average number of proximal phosphohistone H3-positive germ cells in the Tunorous gonadas was 22 ± 12. None of the sacy-1(tn1385) (n = 18) or glp-1(ar202) (n = 31) dissected gonads examined contained phosphohistone H3-positive undifferentiated germ cells in the proximal gonad arm at 15°C. These results are consistent with the idea that the sacy-1(tn1385rf) mutation, though homozygous viable and fertile (brood size ~350; Kim et al. 2012), compromises the function of the spliceosome, as assessed in a sensitized genetic background.

**sacy-1(tm5503) and sacy-1(tn1615) define the null phenotype**

The strong sacy-1 mutant allele, sacy-1(tm5503), deletes exons 2 and 3 and a portion of exon 4, and is homozygous sterile, displaying the gamete degeneration phenotype.
Consistent with the idea that \textit{sacy-1}(tm5503) defines the null phenotype, an antibody specific to a portion of the DEAD-box domain downstream of the tm5503 deletion (residues 411–578) fails to detect a protein product in extracts from \textit{sacy-1}(tm5503) adults (Figure S1C). To further define the \textit{sacy-1} null phenotype, we used CRISPR/Cas9 genome editing to generate indels upstream of the DEAD-box-encoding regions by targeting Cas9.

(Figure 4A; Kim et al. 2012).
Figure 3  Quantification of altered splicing patterns upon SACY-1 depletion. (A) Bar graphs showing the number of genes with statistically significant (FDR < 0.05) changes in splicing patterns. The legend at the right depicts the nature of the observed splicing changes: A5’ SS, Alternate 5’ Splice Site; A3’ SS, Alternate 3’ Splice Site; RI, Retained Intron; MXE, Mutually Exclusive Exons; SE, Skipped Exon (B–D) Examples of alterations in splicing patterns following SACY-1 depletion in the germline or soma as indicated. The *etr-1* gene shows pronounced intron retention and two alternatively spliced 3’ sites in the SACY-1 soma depleted (gold) sample (B). A subset of *etr-1* transcript annotations are shown. The *prdx-6* gene has a retained intron in the SACY-1 soma depleted (gold) and germline depleted (red) samples (C). The soma and germline depleted samples have an increase in the usage of alternate splice acceptor in the *lin-28* gene that results in an altered reading frame (D). The solid lines represent the mean of the biological replicates and shaded regions represent the corresponding confidence interval.
double-strand DNA breaks to exon 2 with an efficient sgRNA. We generated \textit{sacy-1} indels in both wild-type as well as \textit{lin-41}(\textit{tn1541}[gfp::lin-41]) hermaphrodites, the latter serving to provide a marker for oocyte development (Spikes et al. 2014a,b). In these experiments, we generated 14 new \textit{sacy-1} alleles (\textit{tn1602–tn1612} and \textit{tn1615–tn1617}). Of these, 13 displayed the gamete degeneration phenotype, consistent with this representing the null phenotype. Not surprisingly, GFP::\textit{LIN-41} levels declined and the protein became undetectable as oocytes degenerated (D. Greenstein, unpublished results). One new CRISPR-Cas9-induced allele, \textit{sacy-1}(\textit{tn1615}), was sequenced, and found to result from a 10-bp deletion at the end of exon 2, which is predicted to introduce a stop codon prior to the DEAD-box domain (Figure 4). \textit{sacy-1}(\textit{tn1615}) and \textit{sacy-1}(\textit{tm5503}) exhibited indistinguishable phenotypes (Table 5), consistent with the possibility that they define the null phenotype. Among the CRISPR-Cas9-induced alleles, \textit{sacy-1}(\textit{tn1617}) was exceptional in that it was homozygous viable and fertile, though slow growing, despite the fact that the deletion removes the initiation codon of \textit{sacy-1} (Figure 4A). This exceptional allele might utilize an alternative start codon just prior to the DEAD-box domain, although this possibility was not explored. Since null mutations in \textit{sacy-1} result in hermaphrodite sterility, there is the possibility that maternal \textit{sacy-1} activity contributes to the development of the germline and soma. Indeed, when the gamete degeneration phenotype is delayed through germline feminization, the mating of \textit{sacy-1(+) null females to wild-type males produces embryos that arrest prior to morphogenesis (Kim et al. 2012).

\textit{Reduction-of-function sacy-1 mutations in \textit{C. elegans} affect highly conserved residues in the DEAD-box and helicase domains}

To better understand the functions and activities of the highly conserved \textit{SACY-1}/DDX41 protein (Figure 4B), we conducted forward genetic screens for new \textit{sacy-1} mutations, taking advantage of the fact that reductions of \textit{sacy-1} function by mutation or RNAi can suppress the self-sterility of \textit{fog-2} null mutations (Kim et al. 2012), which is caused by a failure to produce sperm (Schedl and Kimble 1988). Thus, we conducted a noncomplementation screen for new mutations that enable fertility in trans to the \textit{sacy-1}(\textit{tn1385}) \textit{rf} allele in the \textit{fog-2(oz40)} genetic background (Figure S7). In a screen of 15,577 haploid genomes, we isolated five new \textit{sacy-1} missense alleles (\textit{tn1479–tn1483}; Figure 4; see Table 5 for a list of all \textit{sacy-1} alleles central to this work and their properties). Phenotypic analyses, which will be described in detail below (see Table 5 and Table 6), indicate that three of these missense alleles (\textit{tn1479, tn1480}, and \textit{tn1481}) appear to confer antagonistic activities. All the \textit{sacy-1} missense alleles thus far isolated alter highly conserved amino acids, and several of these mutations are nearby or in subdomains of the DEAD-box affected by DDX41 mutations found in human neoplasms (Figure 4B). These missense alleles were modeled onto the crystal structures of DEADc and the HELICc domains of DDX41 (Schütz et al. 2010; Omura et al. 2016) and found likely to be surface accessible, suggesting that some of these mutant alleles could interfere with the function of other protein components of the spliceosome.

\textit{Novel sacy-1 mutant alleles appear to antagonize essential functions of the spliceosome}

\textit{A strong loss-of-function sacy-1 mutation is more severe than a null allele:} The strongest newly isolated allele, \textit{sacy-1}(\textit{tn1479}[G504E]), confers a more severe phenotype than \textit{sacy-1} null alleles—the majority of \textit{sacy-1}(\textit{tn1479}) adult hermaphrodites die by vulval rupture at 20° (Figure S8 and Table 6). This phenotype is observed only in a small minority of \textit{sacy-1}(\textit{tm5503}) hermaphrodites at 20° (Table 6). This observation suggests that \textit{sacy-1}(\textit{tn1479}) is a strong loss-of-function allele that confers antagonistic activity. One possibility is that the SACY-1[G504E] product is non-functional but incorporates into the spliceosome and antagonizes its function.

\textit{A recessive gain-of-function sacy-1 mutation masculinizes the hermaphrodite germline:} A novel mutation isolated in the noncomplementation screen was \textit{tn1481}, which confers a masculinization of germline (Mog) phenotype (Figure 5 and Table 6). All \textit{sacy-1}(\textit{tm1481}) homozygous hermaphrodites produce excess numbers of sperm but no oocytes (\textit{n} = 125). Staining of dissected gonads from adults showed that whereas all wild-type hermaphrodite gonad arms examined (\textit{n} = 64) expressed both the major sperm protein (MSP) and the RME-2 oocyte yolk receptor, all \textit{sacy-1}(\textit{tm1481}) gonad arms (\textit{n} = 178) expressed only MSP but not RME-2 (Figure 5). In our noncomplementation screen, we also isolated a \textit{gld-1} Mog allele, \textit{tn1478}, as a dominant suppressor of \textit{fog-2(oz40)}. \textit{gld-1}(\textit{tm1478}) results from the same G248R amino acid substitution reported for the \textit{gld-1}(\textit{q93}) Mog allele (Francis et al. 1995a,b; Jones and Schedl 1995). Thus it was important to determine whether the \textit{sacy-1}(\textit{tm1481}[P222LJ]) mutation was the cause of the Mog phenotype. This was ascertained by crossing a GFP::\textit{SACY-1} extrachromosomal array (\textit{tmEx159}) into the \textit{sacy-1}(\textit{tm1481}) genetic background. The \textit{tmEx159} extrachromosomal array expresses GFP::\textit{SACY-1} in the germline and soma at wild-type levels and rescues the \textit{sacy-1}(\textit{tm5503}) null allele (Figure 5).
We found that all sacy-1(tn1481); tnEx159[gfp::sacy-1+ unc-119(+)] hermaphrodites (n = 30) produced oocytes and sperm and were self-fertile. This result established that the P222L mutation in SACY-1 causes the Mog phenotype.

The suppression of fog-2 sterility by rf sacy-1 mutations is consistent with sacy-1(+) possessing a function that promotes the oocyte fate; this function is nonessential, however, because the strongest loss-of-function sacy-1 alleles are able to produce oocytes, which nevertheless undergo necrotic
Although the SACY-1(P222L) protein confers an oocyte-promoting function in the germ line by promoting the function of GLD-1 binding sites within the tra-2 3’-UTR, it antagonizes oocyte-promoting function in opposition to this oocyte-promoting function in the context of a model in which sacy-1(RNAi) suppresses the sterility of the dominant Him phenotype of sacy-1(tn1480). Thus, we reevaluated the interaction between tra-2 and sacy-1 genetically. In the first approach, we combined the sacy-1(tm5503) null allele with tra-2(e2020). We analyzed the sexual fate of the germ line by staining dissected gonads from adult animals with oocyte (RME-2) and sperm (MSP) markers. Whereas all wild-type gonad arms examined (n = 30) expressed RME-2 and MSP, all gonad arms of sacy-1(tm5503); tra-2(e2020) animals (n = 26) expressed only RME-2 and not MSP (Figure 7). This result is consistent with a model in which sacy-1 promotes the oocyte fate by promoting the function of tra-2 (Figure 6). This possibility is consistent with the finding that sacy-1(rf) alleles and sacy-1(RNAi) suppress the sterility of fog-2 mutants (Kim et al. 2012; this work), and the observation that a decrease in tra-2 dosage can also partially suppress fog-2 sterility (Schedl and Kimble 1988). Although the germ lines of sacy-1(tm5503); tra-2(e2020) adults were feminized, oocytes underwent meiotic maturation constitutively, consistent

### Table 5: sacy-1 alleles relevant to this study

| Allele          | Alteration          | Phenotypes                                         | Inferred activity                                      |
|-----------------|---------------------|----------------------------------------------------|-------------------------------------------------------|
| sacy-1(tm5503)a | 619 bp deletion     | Sterile, gamete degeneration                       | Likely null                                           |
| sacy-1(tn1615)b | 10 bp deletion      | Sterile, gamete degeneration                       | Likely null                                           |
| sacy-1(tn1385)a | G533R               | Viable and fertile, suppresses acy-4 sterility, suppresses fog-2 sterility | Reduction of function                                  |
| sacy-1(tn1391)a | G473R               | Viable and fertile, suppresses acy-4 sterility, suppresses fog-2 sterility | Reduction of function                                  |
| sacy-1(tn1440)a | G331R               | Viable and fertile, suppresses acy-4 sterility, suppresses fog-2 sterility | Reduction of function                                  |
| sacy-1(tn1482)b | D506N               | Viable and fertile, suppresses fog-2 sterility      | Reduction of function                                  |
| sacy-1(tn1483)b | G269E               | Viable and fertile, suppresses fog-2 sterility      | Reduction of function                                  |
| sacy-1(tn1480)b | H527Y               | Viable and fertile at 20° and suppresses fog-2 sterility | Loss of function with antimorphic activity, dominant Him |
| sacy-1(tn1481)b | P222L               | Sterile, masculinization of germline                | Recessive gain-of-function                            |
| sacy-1(tn1887)b | RS34H               | Viable and fertile                                 | Weak antagonistic activity, enhances the dominant Him phenotype of sacy-1(tn1480) |

*a Kim et al. (2012).  
*b This work.  
*c Suppression of acy-4 sterility was not tested.  
*d sacy-1(tn1483) adult hermaphrodites have smaller gonad arms, suggesting effects on germline proliferation (T. Tsukamoto and D. Greenstein, unpublished results).
with the finding that \textit{sacy-1} is a negative regulator of meiotic maturation (Kim et al. 2012). \textit{sacy-1(tm5503); tra-2(e2020)} animals did, however, exhibit a highly penetrant ovulation defect, which caused endomitotic oocytes to accumulate in the gonad arm (Emo phenotype; Figure 7).

To extend these observations, we examined germ-line sexual fates in dissected gonads from \textit{sacy-1(tm1481Mog); tra-2(e2020)} adults. Whereas all wild-type gonad arms examined \((n = 21)\) expressed MSP and contained sperm, none of the \textit{sacy-1(tm1481Mog); tra-2(e2020)} gonad arms \((n = 37)\) expressed MSP or contained sperm. We noted that the Emo phenotype was less penetrant in \textit{sacy-1(tm1481Mog); tra-2(e2020)} gonad arms \((46\% \text{ penetrance})\). These results are consistent with the possibility that \textit{sacy-1(+)\textsuperscript{+}} promotes the oocyte fate by promoting the function of \textit{tra-2} in the germ-line and suggests that \textit{sacy-1(tm1481Mog)} may interfere with this function.

Interestingly, recessive loss-of-function mutations in six genes, \textit{mog-1--mog-6}, cause a Mog phenotype and encode spliceosomal components (Graham and Kimble 1993; Graham et al. 1993; Puoti and Kimble 1999, 2000; Belfiore et al. 2004; Zanetti et al. 2011). Mutation and RNAi depletion of many splicing factors have been observed to result in a Mog phenotype, suggesting that the germ-line splicing determination process is particularly sensitive to disruptions in RNA splicing (Konishi et al. 2008; Mantina et al. 2009; Kerins et al. 2010; Wang et al. 2012; Novak et al. 2015). Prior studies focusing on the \textit{C. elegans} soma were interpreted in the context of a model in which \textit{mog-1--mog-6} might function at the level of \textit{fem-3} through 3'UTR-dependent translational regulation (Gallegos et al. 1998); however, the experiments in that study did not address the regulation of \textit{fem-3} in the germ-line. We previously showed that \textit{sacy-1(tm5503); fem-3(e1996)} adult XX animals had feminized germlines (Kim et al. 2012). To examine the genetic relationship between \textit{sacy-1} and \textit{fem-3} further, we generated \textit{sacy-1(tm1481Mog); fem-3(e1996)} double mutants. We observed that 92\% \((n = 23)\) of \textit{sacy-1(tm1481Mog); fem-3(e1996)} animals were feminized. Mating of \textit{sacy-1(tm1481Mog); fem-3(e1996)} females \((n = 29)\) to wild-type males resulted in the production of embryos that failed to hatch \((n = 4140, 99.8\%)\) or arrested as larvae \((n = 7, 0.2\%)\). This result indicates that two copies of \textit{sacy-1(tm1481)} in the maternal germ-line, but not one \[e.g., \textit{sacy-1(tm1481)/sacy-1(tm5503)}\] heterozygotes are fertile] are incompatible with embryonic development. We found that 8\% of \textit{sacy-1(tm1481Mog); fem-3(e1996)} animals \((n = 25)\) produced oocytes and sperm and a few dead embryos (one of these animals produced sperm in one gonad arm but not the other). The production of sperm in a small percentage of \textit{sacy-1(tm1481Mog); fem-3(e1996)} hermaphrodites might depend on maternal \textit{fem-3(+)} activity. We did observe that a reduction in \textit{fem-3} dosage could suppress the \textit{sacy-1(tm1481Mog) phenotype} \((n = 60)\). Specifically, whereas 65\% \((n = 39)\) of \textit{sacy-1(tm1481); fem-3(e1996)/+} animals were Mog, exclusively producing sperm, 33\% \((n = 20)\) produced sperm and oocytes, and one animal \((2\%)\) was feminized. Taken together, our results seem most consistent with the possibility that \textit{sacy-1} functions at the level of \textit{tra-2} in the genetic epistasis pathway (Figure 6; Zanetti and Puoti 2013). However, since this analysis of genetic interactions involves the use of dominant and recessive gain-of-function mutations \[e.g., \textit{tra-2(e2020)} and \textit{sacy-1(tm1481)}\], we cannot rule out the possibility that \textit{sacy-1} affects other steps, including the regulation of the \textit{fem-1--fem-3} genes (Figure 6).

We noted that among the 311 transcripts increased in abundance in the \textit{SACY-1} germ-line-depleted sample was \textit{her-1} (Figure 2, B, D, and E and File S2). This might be due to an increase in X chromosome nondisjunction in embryos located in the uterus following germ-line depletion of \textit{sacy-1(+)\textsuperscript{+}}, but this possibility was not investigated. Because \textit{her-1} likely encodes an inhibitory ligand for the \textit{TRA-2} receptor in the sex-determination pathway (Perry et al. 1993; Figure 6), we tested whether the \textit{her-1(hv1y101)} null mutation could suppress the Mog phenotype of the recessive gain-of-function \textit{sacy-1(tm1481)} mutation, but this proved not to be the case \((n = 53 \text{ gonad arms})\). Since the genetic epistasis results suggest that \textit{SACY-1} might promote the expression of \textit{TRA-2} (Figure 6), we examined the effect of \textit{SACY-1} depletion in the germ-line on the levels of \textit{tra-2} mRNA and the fidelity of its splicing. We observed no statistically significant change in \textit{tra-2} mRNA levels or its splicing patterns (Figure 2F). We did not examine the expression of \textit{TRA-2} protein after \textit{SACY-1} depletion because a recent study suggested that

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**Table 6 sacy-1(tm1479), sacy-1(tm1480), and sacy-1(tm1481) confer antagonistic activity**

| Allele                        | Class                     | Gamete degeneration\(\%\) | Vulval rupture\(\%\) | Mog\(\%\) | T (%) |
|-------------------------------|---------------------------|-----------------------------|-----------------------|------------|------|
| sacy-1(tm5503) \((n = 143)\) | Strong loss-of-function   | 5.6                         | 94.4                  | 0          | 15\(\degree\) |
| sacy-1(tm5503) \((n = 96)\)  | Strong loss-of-function   | 96.9                        | 3.1                   | 0          | 20\(\degree\) |
| sacy-1(tm5503) \((n = 106)\) | Strong loss-of-function (from \textit{tm5503/+} parents) | 98.0                        | 2.0                   | 0          | 25\(\degree\) |
| sacy-1(tm5503) \((n = 231)\) | Strong loss-of-function (from \textit{tm5503/tm1480 parents}) | 14.7                        | 85.3                  | 0          | 25\(\degree\) |
| sacy-1(tm51615) \((n = 92)\) | Strong loss-of-function   | 97.8                        | 2.2                   | 0          | 20\(\degree\) |
| sacy-1(tm1479) \((n = 170)\) | Strong loss-of-function with antimorphic activity | 11.2                        | 88.8                  | 0          | 20\(\degree\) |
| sacy-1(tm1481) \((n = 125)\) | Recessive gain-of-function| 0                          | 0                     | 100        | 20\(\degree\) |

\(\text{Note:} \text{Allele Class} \text{ with the exception of sacy-1(tm5503) that have been exposed to X-rays.} \)
Figure 5 sacy-1(tn1481) adult hermaphrodites exhibit a masculinization of germline (Mog) sterile phenotype. DIC images of (A) wild-type and (B) sacy-1(tn1481) adult hermaphrodites. The wild-type animal contains oocytes and sperm and produces embryos but the sacy-1(tn1481) animal only produces sperm. (C–L) Dissected gonads stained for the RME-2 yolk receptor (C and G), the major sperm protein (D and H), or DNA (E, I, and K). Merged images are also shown (F, J, and L). The sacy-1(tn1481) mutant overproduces sperm to the exclusion of oocytes and is sterile. This phenotype is completely penetrant. Bar, 50 μm.
The recessive gain-of-function sacy-1(tn1480) allele confers multiple pleiotropic phenotypes in a temperature-dependent manner: Interestingly, sacy-1(tn1480) displayed both cold-sensitive (15 °C) and temperature-sensitive (25 °C) defects. At 15 °C, sacy-1(tn1480) homozygotes (n = 55), which were the offspring of heterozygous parents grown at 15 °C, laid dead eggs and produced arrested larvae (91%) or produced very few progeny (9%). At 25 °C, sacy-1(tn1480) hermaphrodites were sterile and produced abnormal and fertilization-defective sperm (Figure 8). sacy-1(tn1480) hermaphrodite sterility is rescued by mating with wild-type males at 25 °C. In addition to producing oocytes, some sacy-1(tn1480) adult hermaphrodites continued to produce sperm, as swollen germ cells specified in the male fate were detected distal to the loop region in 42% of gonad arms examined (Figure 8; n = 12). Thus, in addition to the other phenotypes it confers, the sacy-1(tn1480) mutation perturbs the sperm-to-oocyte switch at 25 °C. We also observed that sacy-1(tn1480) conferred a dominant high-incidence of males (Him) phenotype; sacy-1(tn1480)/+ heterozygous hermaphrodites produced 1.6% male progeny (n = 3759), as compared to 0.1% for the wild-type control (n = 6044; P < 0.01, Fisher’s exact test). Genetic mapping showed that the temperature-dependent pleiotropic defects were inseparable from the sacy-1(tn1480) mutation (see Materials and Methods). Like sacy-1(tn1481), the defects conferred by sacy-1(tn1480) were dosage sensitive. At 25 °C, nearly all sacy-1(tn1480)/sacy-1(tm5503) heterozygotes (99%, n = 88) were fertile (the average brood size was 132 ± 64, n = 34); however, the majority of their sacy-1(tm5503) homozygous progeny (85.3%, n = 231) undergo vulval rupture and burst as adults (Table 6). This is in contrast to sacy-1(tm5503) homozygotes derived from sacy-1(tn1480)/+ heterozygous parents, only 2% of which undergo vulval rupture (n = 106; Table 6). This result suggests that maternal sacy-1(tn1480) activity can antagonize the spliceosome in the absence of zygotic sacy-1(+) function. By these genetic criteria, sacy-1(tn1480) exhibits recessive and weakly dominant gain-of-function properties, depending on the phenotype examined.

The human oncogenic DDX41 R525H mutation confers weak antagonistic activity in C. elegans

The R525H mutation in DDX41 has been reported in myeloid leukemias both as newly arising somatic mutations specific to the neoplastic cells, as well as inherited germline mutations (Polprasert et al. 2015; Lewinsohn et al. 2016; Sébert et al. 2019). Thus, it was of interest to examine the impact of the analogous mutation (R534H) on sacy-1 function. Interestingly, a substitution at the adjacent amino acid (G533R) results in the sacy-1(tn1385) reduction-of-function mutation (Figure 4). Thus, we used genome editing to introduce the R534H mutation in the C. elegans genome (see Materials and Methods). By several criteria, sacy-1(tm1887 [R534H]) homozygotes were indistinguishable from the wild type. Neither did sacy-1(tm1887) suppress acy-4 sterility (n = 140; sacy-1(tm1887); acy-4(ok1806) brood size was 1.5 ± 2.3) nor did it suppress fog-2 sterility (n = 48). All unmated sacy-1(tm1887); fog-2(oz40) gonad arms examined (n = 96) exhibited stacked oocytes, which indicates that the sacy-1(tm1887) mutation does not derepress meiotic maturation in the absence of sperm, like strong reduction-of-function mutations do. Further, the sacy-1(tm1887[R534H]) did not enhance the Tumorous phenotype of a gain-of-function glp-1/Notch allele (Table 4). The brood size of sacy-1(tm1887) (204 ± 37; n = 20) at 25 °C was similar to that of the wild type (202 ± 62, n = 30; P > 0.8, two-sample Z-test). Also, the incidence of males (0.2%, n = 4087) was similar to that observed in the wild type (0.1%, n = 6044). When placed in trans to the sacy-1(tm5503) null mutation, sacy-1(tm1887[sacy-1(tm5503) heterozygotes were found to be fertile at all temperatures examined (15, 20, and 25 °C; n > 100). However, we did observe that sacy-1(tm1887) significantly enhanced the dominant Him phenotype of sacy-1(tn1480).
sacy-1(tm5503); tra-2(e2020)

(P < 0.05, Fisher's exact test); sacy-1(tm1887)/sacy-1(tm1480) heterozygotes produced 5.7% males at 25°C (n = 1483) as compared to 1.6% for the +/sacy-1(tm1480) control (n = 3759). The brood size of sacy-1(tm1887)/sacy-1(tm1480) heterozygotes at 25°C (74 ± 65, n = 20) was also significantly lower than that of the +/sacy-1(tm1480) control (188 ± 68, n = 20; P < 0.001, two-sample Z-test). Because sacy-1(tm1480)/sacy-1(tm5503) heterozygotes do not exhibit an enhanced Him phenotype at 25°C (0.7% males, n = 4493), the sacy-1(tm1887[R534H]) mutation appears to possess a weak antagonistic activity.

Discussion

In this study, we report the results of a molecular genetic and biochemical analysis of SACY-1/DDX41 in C. elegans, conducted to gain potential insights into how DDX41 mutations might contribute to MDS and AML in humans. DDX41 is a
component of the spliceosomal C complex (Jurica et al. 2002; Bessonov et al. 2008), which carries out the second step in splicing. Pre-mRNA splicing is an essential process in eukaryotes (reviewed by Wahl et al. 2009). Thus, the finding that mutations in highly conserved genes encoding spliceosomal components are frequently found in hematological malignancies was unexpected (reviewed by Yoshida and Ogawa 2014). Unfortunately, the genetic properties of disease-causing mutations in spliceosomal proteins have been difficult to assess. Nonetheless, the observation that many of these

Figure 8  Sperm-defective phenotype of sacy-1(tn1480) at 25°C. Dissected gonads of wild-type (A–E) and sacy-1(tn1480) (F–J) stained with anti-MSP antibodies (B and G) and DAPI to detect DNA (C and H). Merged images are also shown (A, D, E, I, and J). At 25°C, sacy-1(tn1480) hermaphrodites produce swollen and abnormal sperm, which are incapable of fertilization. Note that defective sperm are also found near the bend region in sacy-1(tn1480) adults (F) indicating that there is a defect in the sperm-to-oocyte switch. Bar, 50 μm.
missense mutations map to highly conserved amino acids, within biochemically defined structural and functional domains, might suggest that they reduce but not eliminate gene function. An alternative possibility is that some of the oncogenic missense mutations might confer antagonistic gain-of-function properties. Indeed, oncogenic mutations in several splicing factors (e.g., SF3B1, U2AF1, SRSDF2, and SRSR2) present in the heterozygous condition (Yoshida and Ogawa 2014); however, it is probable that additional newly arising mutations in hematopoietic lineages contribute to neoplastic development.

In this regard, the DDX41 mutations inherited and arising in familial cases of AML are particularly informative (Polprasert et al. 2015). Affected individuals often inherit one copy containing a mutation, which is likely to be a null mutation (e.g., a frameshift mutation, pD1406s). In addition to this DDX41 germline mutation, the neoplasms in these patients invariably contain a second copy with a somatic mutation that arose in the hematopoietic lineage. Several observations are consistent with the possibility that sacy-1(+) function, potentially by compromising the function of the spliceosome. Most notable among these mutations is sacy-1(tm1481), which confers a masculinization of the germline phenotype resulting from the overproduction of sperm to the exclusion of the oocyte fate. Since multiple reduction-of-function sacy-1 alleles suppress the feminization of the germline phenotype caused by null mutations in fog-2 (Kim et al. 2012; this work), sacy-1(+) must possess a function to promote the oocyte fate. This oocyte-promoting function of sacy-1(+) might not be essential because sacy-1 null mutants produce oocytes, which nonetheless undergo necrotic degeneration. The caveat here is that sacy-1 null mutants only develop to adulthood because of maternally provided sacy-1(+), which might be sufficient to promote oogenesis. In any case, the sacy-1(tm1481) mutation might disrupt an oocyte-promoting function, either by interfering with maternally provided SACY-1 activity or the proteins with which it associates.

Several observations are consistent with the possibility that sacy-1(tm1481) interferes with, or compromises, the function of the spliceosome in germline sex determination in a dosage-sensitive manner. This view is also supported by our tandem affinity purification results showing that SACY-1 is a component of the C. elegans spliceosome, and interacts genetically with spliceosomal components. Significantly, multiple C. elegans spliceosomal components, functioning at different steps of the splicing reaction, can mutate to a masculinization of germline phenotype (reviewed by Zanetti and Puoti 2013). Interestingly, when placed in trans to a sacy-1 null mutation (e.g., tm5503), the resulting sacy-1(tm1481)/sacy-1(tm5503) heterozygous animals are viable and fertile, and can be maintained indefinitely as a heterozygous strain. This highly informative genetic result suggests that a single dose of sacy-1(tm1481) can mediate all its essential functions, but that two doses of the mutant protein interfere with the normal mechanisms of germline sex determination [i.e., sacy-1(tm1481) is a recessive gain-of-function antimorphic mutation]. Although sacy-1(tm1481)/sacy-1(tm5503) heterozygous adult hermaphrodites are fertile, when sacy-1(tm1481); fem-3(e1996) females are mated to wild-type males, they produce embryos that invariably fail to hatch. Similar results are obtained with the six other mog genes (Graham and Kimble 1993; Graham et al. 1993), which encode spliceosomal proteins (Puoti and Kimble 1999, 2000; Belfiore et al. 2004; Kasturi et al. 2010; Zanetti et al. 2011). Interestingly, the P222L amino acid substitution found in sacy-1(tm1481) is adjacent to the Q motif, which participates in nucleotide binding and hydrolysis (Schütz et al. 2010). It is tempting to speculate that SACY-1/DDX41 might promote remodeling of spliceosomes during the splicing reaction, and that two doses of SACY-1 P222L might disrupt these rearrangements. In a similar vein, sacy-1(tm1479)(G504E) exhibits a phenotype more severe than a null allele, suggesting that this mutation in the helicase domain too might possess a dosage-sensitive antimorphic activity. Whether missense
alleles in human DDX41 also possess antimorphic activity will require additional work, including biochemical analyses.

We imported the human oncogenic DDX41[R525H] mutation into C. elegans using CRISPR-Cas9 genome editing (R534H in SACY-1). Our analysis revealed that, in C. elegans, this mutation possesses very weak antagonistic activity. While it is possible that the genetic properties of this mutation might differ between C. elegans and mammalian systems, it is worthwhile noting that the human oncogenic variant must support the high levels of proliferation characteristic of neoplastic cells. While all the sacy-1 mutations we isolated in forward genetic screens occur at conserved amino acids, none of them match the human oncogenic mutations thus far isolated (Polprasert et al. 2015; Cardoso et al. 2016; Lewinsohn et al. 2016; Li et al. 2016; Dinesh et al. 2018; Sébert et al. 2019). This observation is consistent with the idea that the human oncogenic mutations are, at most, weakly antagonizing or weak rf mutations. Our forward genetic screens in C. elegans may have required a substantial reduction of function and thus might have missed very weak mutations like SACY-1(R534H) or other human oncogenic variants.

An attractive idea is that human oncogenic mutations affecting the spliceosome contribute to neoplastic development through effects on gene expression occurring through alterations in RNA splicing, as well as effects on the transcriptional machinery or RNA stability (Yoshimi et al. 2019). The very specific defects observed in individual sacy-1/ddx41 mutant alleles in C. elegans could be due to a failure to properly splicing key genes, as has been documented for several splicing-related mutations in Drosophila melanogaster (Van Buskirk and Schüpbach 2002; Roignant and Treisman 2010). In this study, we examined the effects of SACY-1 depletion at the adult stage on the transcriptome. We observed many instances of missplicing following SACY-1 depletion, though missplicing events were more prevalent when SACY-1 was depleted from the soma as compared with the germline, suggesting the possibility that surveillance mechanisms might serve to protect the germline by removing the products of aberrant splicing events. Although we observed missplicing events in the germline and soma upon SACY-1 depletion, no clear candidates emerged that might account for its role in germline sex determination. Genetic evidence suggests that sacy-1 promotes the activity of tra-2, but this appears to be independent of splicing because the expression level and splicing pattern of tra-2 mRNA was unchanged following depletion of sacy-1 in the germline. Whether sacy-1 mutations might affect the localization of tra-2 mRNA or its translation remains to be determined. In both the soma and the germline, we observed splicing-independent impacts on the abundance of many transcripts. The gene expression changes observed suggest that depletion of SACY-1 might elicit a stress response. One possibility is that defective proteins produced via missplicing contribute to the induction of stress responses. Alternatively, there might be surveillance pathways that respond to dysfunctional splicing by evoking homeostatic mechanisms. If analogous processes occur after perturbations of the spliceosome in humans, such stress responses might contribute to the vitality of tumor cells and could represent therapeutic targets. The extent to which gene expression changes and splicing alterations contribute to the various sacy-1 mutant phenotypes will require further study but will likely provide insights relevant to spliceosomal perturbations in humans.

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