JmjC Domain-Encoding Genes Are Conserved in Highly Regenerative Metazoans and Are Associated with Planarian Whole-Body Regeneration

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Abstract

The capacity for regeneration varies greatly among metazoans, yet little is known about the evolutionary processes leading to such different regeneration abilities. In particular, highly regenerative species such as planarians and cnidarians can regenerate the whole body from an amputated fragment; however, a common molecular basis, if any, among these species remains unclear. Here, we show that genes encoding Jumonji C (JmjC) domain-containing proteins are associated with high regeneration ability. We classified 132 fully sequenced metazoans into two groups with high or low regeneration abilities and identified 118 genes conserved in the high regenerative group that were lost in species in the low regeneration group during evolution. Ninety-six percent of them were JmjC domain-encoding genes. We denoted the candidate genes as high regenerative species-specific JmjC domain-encoding genes (*HRJD*s). We observed losses of *HRJD*s in *Helobdella robusta*, which lost its high regeneration ability during evolution based on phylogenetic analysis. By RNA sequencing analyses, we observed that *HRJD* orthologs were differentially expressed during regeneration in two Cnidarians, as well as Platyhelminthes and Urochordata, which are highly regenerative species. Furthermore, >50% of the head and tail parts of amputated planarians (*Dugesia japonica*) died during regeneration after RNA interference of *HRJD* orthologs. These results indicate that *HRJD* are strongly associated with a high regeneration ability in metazoans. *HRJD* paralogs regulate gene expression by histone demethylation; thus, *HRJD* may be related to epigenetic regulation controlling stem cell renewal and stem cell differentiation during regeneration. We propose that *HRJD* play a central role in epigenetic regulation during regeneration.

Key words: regeneration, evolution, RNA-Seq, histone demethylation, JmjC domain.

Introduction

Regeneration is a process wherein lost body parts are replaced, and unveiling the complicated biological processes associated with regeneration remains a major challenge in biology. The regeneration abilities of metazoans have been classified into five levels, including regeneration of the whole body, structural elements (e.g. limbs, tail, and fins), internal organs (e.g. heart and liver), tissues (e.g. gut lining), and cells (e.g. axon and muscle fiber regeneration) (Bely and Nyberg 2010). Regeneration is an obvious beneficial trait; however, large differences in regeneration capability are found among metazoans (Bely and Nyberg 2010; Agata and Inoue 2012). For example, planarians and sea anemones can regenerate a new individual from a piece of the body, whereas some

animals such as birds and nematodes cannot regenerate any structures (Sánchez Alvarado 2000; Bely and Nyberg 2010; Agata and Inoue 2012).

It is critical to understand the molecular basis leading to the different regeneration abilities among metazoans, and some common genetic factors related to regeneration have already been reported. Wnt/β-catenin, fibroblast growth factor (FGF), and Bone morphogenetic proteins (BMPs) signaling are commonly associated with regeneration in metazoans and are important regulators of cell differentiation or self-renewal during regeneration (Holstein 2012). For example, *Wnt* genes are expressed in the regenerating heart of mammals (Ozhan and Weidinger 2015), fins of zebrafish (Poss et al. 2000), tail of amphibians

(Caubit et al. 1997), and head of hydra (Bode 2003). Furthermore, BMP cooperates with FGF signaling to induce nerve cells to regulate limb regeneration in amphibians (Makanae et al. 2016). BMP genes regulate the symmetric regeneration in the early stage of regeneration in planarians (Reddien et al. 2007). These genes are often shared in several metazoan regeneration processes including structure and whole-body regeneration; however, a shared genetic basis (if one exists) truly conferring a high regeneration ability, such as whole-body regeneration, is still unknown.

New techniques such as next-generation sequencing and high-performance supercomputing enable high-throughput, genome-wide data analysis for large numbers of species. A recent report revealed a common set of differentially expressed genes (DEGs) responding to the regeneration process among distantly related species (Fumagalli et al. 2018). It may be possible to identify conserved genes that are associated with regeneration capacity in highly regenerative metazoans by performing comparative-genome and whole-transcriptome analyses. Basal lineages including Cnidaria, Placozoa, Ctenophora, and Porifera can regenerate their whole body, although the evolution of genetic background conferring the ability has been poorly understood yet (Sánchez Alvarado 2000; Bely and Nyberg 2010; Agata and Inoue 2012; Bely et al. 2014). Based on the phylogenetic distribution of high regenerative species in metazoans, the highly regenerative ability seemed to originate from early animals after they acquired multicellularity as an epiphenomenon of development (Bely and Nyberg 2010). We hypothesized that a common ancestor of all metazoans had a high regeneration ability and that particular genes were lost independently in multiple phyla, resulting in some species in individual phyla showing low regeneration abilities. To test this hypothesis, we defined species that regenerate whole, anterior, or posterior body parts as highly regenerative species, and other species that regenerate only appendages such as limbs, tails, fins, or lower structures as low regenerative species. According to this definition, we classified metazoans with fully sequenced genomes as highly regenerative species (Urochordata, Cephalochordata, Echinodermata, Hemichordata, Annelida, Platyhelminthes, Cnidaria, Placozoa, Ctenophora, and Porifera) or low regenerative species (Vertebrata, Arthropoda, Mollusca, and Nematoda) (supplementary table S1, Supplementary Material online). The purpose of this study was to identify genes that are differentially expressed during regeneration in four highly regenerative metazoans (*Schmidtea mediterranea*, *Nematostella vectensis*, *Hydra vulgaris*, and *Ciona intestinalis*) whose orthologs were conserved only in the genomes of species in the highly regenerative group during evolution. We provide evidence that the identified candidate genes are associated with regeneration in planarians by RNA interference (RNAi) knockdown experiments (Rouhana et al. 2013).

Materials and Methods

Identification of Genes Specific to Highly Regenerative Species

We downloaded the complete protein sequences of 126 metazoan species with fully sequenced genomes deposited in Ensembl (release 87) and Ensembl Metazoa (release 33). We also downloaded the protein sequences for highly regenerative species, *Acanthaster planci* v1.0 (Hall et al. 2017) and *Ptychodera flava* v3.0 (Simakov et al. 2015), from OIST (http://marinegenomics.oist.jp), *H. vulgaris* from NCBI (Chapman et al. 2010), *Branchiostoma floridae* v1.0 (Putnam et al. 2008) from JGI Genome Portal (https://genome.jgi.doe.gov), *S. mediterranea* (Robb et al. 2015) from SmedGD (http://smedgd.neuro.utah.edu), and *Exaiptasia pallida* v1.0 (Baumgarten et al. 2015) from REEFGENOMICS (http://aiptasia.reefgenomics.org). To identify genes conserved only among highly regenerative species, we classified the species into two groups (low and highly regenerative species), based on their regenerative abilities according to the work of Bely and Nyberg (2010) and related papers (supplementary table S1, Supplementary Material online). We did not use species for which a high regeneration ability has not been documented (Bely and Nyberg 2010), even though their genomic sequences were available. In most phyla, the representative regenerative ability of a phylum reported by Bely and Nyberg (2010) is consistent with the regenerative abilities of species in the phylum, but the degree of the regeneration ability does not match between *C. teleta* (high regenerative species) and *H. robusta* (low regenerative species) in Annelida. We found that 116 species belonging to 4 phyla and 14 species belonging to 10 phyla were in the low and highly regenerative groups, respectively (supplementary table S1, Supplementary Material online). Cephalochordata was not classified in the highly regenerative group by Bely and Nyberg (2010), although a recent study reported the extensive regeneration ability of *Branchiostoma lanceolatum* (Somorjai et al. 2012); therefore, we classified Cephalochordata into the highly regenerative group. We conducted BlastP searches using protein sequences of all potential highly regenerative species as queries against protein sequences from each species used in this study to obtain homologs of potential highly regenerative species (threshold: BlastP score >100). Genes conserved among all species in the highly regenerative group that were lost in all species of the low regenerative group were regarded as candidate genes possibly associated with highly regeneration ability. We also identified genes for which a phylum of the low regenerative group had an ortholog as candidate genes (table 1).

RNA Sequencing Analyses of the Regeneration Process

We used published RNA sequencing (RNA-Seq) data sets for *N. vectensis* (Schaffer et al. 2016), *H. vulgaris*, *C. intestinalis*

Table 1

Number of Genes Specifically Conserved in High Regenerative Metazoans

Phyla	Species	Regeneration Group	Number of Genes Conserved in High Regenerative Species	
			With JmjC Domain	Without JmjC Domain
Porifera	*A. queenslandica*	High	1	0
Placozoa	*Trichoplax adhaerens*	High	16	1
Ctenophora	*Mnemiopsis leidyi*	High	3	0
Cnidaria	*N. vectensis*	High	9	1
Cnidaria	*H. vulgaris*	High	2	0
Cnidaria	*E. pallida*	High	8	0
Annelida	*H. robusta*	Low[a]	1	0
Annelida	*C. teleta*	High	3	0
Mollusca	*Lottia gigantea*	Low[b]	2	0
Mollusca	*Octopus bimaculoides*	Low[b]	1	0
Mollusca	*Crassostrea gigas*	Low[b]	3	1
Platyhelminthes	*S. mediterranea*	High	2	0
Echinodermata	*A. planci*	High	5	0
Echinodermata	*Strongylocentrotus purpuratus*	High	3	0
Hemichordata	*P. flava*	High	7	1
Cephalochordata	*B. floridae*	High	21	0
Urochordata	*Ciona savignyi*	High	18	0
Urochordata	*C. intestinalis*	High	13	1
Total			118	5

[a]*Helobdella robusta* is a low regenerative species having a JmjC domain-encoding gene.
[b]Mollusca having JmjC domain-encoding genes is in the low regenerative group.

(Spina et al. 2017), and *S. mediterranea* (Zeng et al. 2018) for our analyses. During the regeneration process in *N. vectensis*, individuals were divided into two parts, and total RNA from oral and physa blastemas was extracted at 0, 8, 24, and 72 h post-amputation (hpa) (Schaffer et al. 2016). Total *H. vulgaris* blastema RNA was extracted from the head blastemas at 0, 2, 4, 6, 12, and 48 hpa. *Ciona intestinalis* RNA samples were also extracted from tissues of non-regenerated normal oral siphons immediately after amputation as controls and from tissues at different times during oral regeneration (1, 3, and 8 days post-amputation [dpa]). The RNA samples of *S. mediterranea* were extracted from regenerating individuals at various times (0, 1, 3, 6, 9, 12, 24, 36, 48, 60, 72 hpa and 4, 5, 6, 7, 10, 14 dpa) (Zeng et al. 2018). The single-end libraries from *H. vulgaris*, *N. vectensis*, *C. intestinalis*, and *S. mediterranea* samples were sequenced using the Illumina HiSeq 2500, HiSeq 2000, and Ion Torrent Proton platforms, respectively. We downloaded RNA-Seq data sets derived from both normal and regenerative conditions for three highly regenerative species from the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra), which were previously deposited under accession numbers PRJNA270225 (*H. vulgaris*), PRJNA330595 (*N. vectensis*), and PRJNA421768 (*S. mediterranea*). We did not use raw *C. intestinalis* reads from the SRA because the number of mapped short reads from *C. intestinalis* could be downloaded from the Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo). The numbers of RNA-Seq sample replicates for each stage were

two, two, four, and three for *H. vulgaris*, *N. vectensis*, *S. mediterranea*, and *C. intestinalis*, respectively.

We checked the quality of the downloaded RNA-Seq reads for *H. vulgaris* and *N. vectensis* by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Contaminating Illumina adapters in the reads and low-quality reads (quality value <30) were excluded using the FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Then, we aligned short RNA-Seq reads for *H. vulgaris*, *N. vectensis*, and *S. mediterranea* to their reference genomes from NCBI, Ensembl Metazoa (http://metazoa.ensembl.org), and SmedGD (http://smedgd.neuro.utah.edu), respectively, using Tophat2, version 2.1.1 (Kim et al. 2013). Next, we estimated the read counts using HTSeq, version 0.6.1 (Anders et al. 2015). Read counts for *C. intestinalis* were estimated according to our previously published procedure (Spina et al. 2017). Finally, we identified DEGs under normal and regenerative conditions in these three species using the "TCC" package, version 1.20.0 (Sun et al. 2013) in R.

Identification of Orthologous Gene Groups for Four Highly Regenerative Species

We conducted an all-against-all BlastP search (BlastP score >100) with the protein sequences for *N. vectensis*, *H. vulgaris*, *S. mediterranea*, and *C. intestinalis* and found the best hit (highest BlastP score) for each species. Genes sharing the same best hit were clustered for the four highly regenerative species. We considered clustered genes as genes in the same

orthologous gene group. We determined the number of orthologous gene groups depending on the presence of genes for all four species in an orthologous gene group.

Phylogenetic Relationships of Jumonji C Domain-Encoding Genes in Metazoans

To understand the phylogenetic relationships of high regenerative species-specific Jumonji C (JmjC) domain-encoding genes (*HRJD*s) in metazoans, we analyzed all *HRJD*s in the highly regenerative species shown in table 1 against outgroup genes. The inferred *HRJD* orthologs identified by BlastP were included in the highly regenerative species and the Mollusca species. When searching for outgroup genes, we found a human gene (ENSG00000155666, *KDM8*) that was the best hit with respect to the protein sequences of our candidate genes in *N. vectensis* by BlastP searching (BlastP score >100). Then, we collected orthologs found in highly regenerative species and in several model organisms (human, mouse, chicken, zebrafish, fruit fly, and worm) that were the best hits with respect to the human KDM8 protein sequence by BlastP searching. We aligned the protein sequences using MAFFT v7.310 (Katoh et al. 2002) and generated a maximum-likelihood phylogenetic tree using RAxML, v8.2.10 (Stamatakis 2014).

Whole-Mount In Situ Hybridization

The planarians (*Dugesia japonica*, strain SSP) were cultured in an incubator at 22–24 °C. The planarians used in this study (5–7 mm in length) were starved for approximately 1 week before conducting the experiments. We conducted whole-mount in situ hybridization for two homologs (*DjHRJDa* and *DjHRJDb*) of our candidate genes in the planarian *D. japonica*, which is one of most well-characterized highly regenerative species (table 1). Amplified DNA fragments of *DjpiwiA* (positive control) were inserted into plasmids according to methods described in a previous study (Shibata et al. 2016). *DjHRJDa* and *DjHRJDb* were cloned into the pCR II-TOPO and pBluescript SK (+/–) vectors, respectively. The *DjpiwiA* plasmid was linearized by digestion with *SamI*, and the *DjHRJDa* and *DjHRJDb* plasmids were linearized by digestion with *BamHI*. T7 RNA polymerase was used to prepare digoxigenin-labelled antisense RNA probes using linearized plasmids as the templates. The expression signals were detected using antisense RNA probes. The method followed was described in detail previously (Shibata et al. 2016). The sequences of primers used are shown in supplementary table S4, Supplementary Material online.

Synthesis of *DjHRJDa*, *DjHRJDb*, *EGFP*, and *DjpiwiB* dsRNA

We prepared dsRNA as described previously (Alvarado and Newmark 1999; Rouhana et al. 2013). *EGFP* (negative control) and *DjpiwiB* (positive control) (Hayashi et al. 2010; Shibata et al. 2016) were inserted into the plasmid

pBluescript SK, *DjHRJDa* was cloned into vector pCR II-TOPO, and *DjHRJDb* was cloned into pBluescript SK (+/–). Each gene was amplified using Ex Taq polymerase to add a T7 promoter site on both ends of the target sequences. The PCR products were gel-purified using the Gel/PCR Extraction Kit (Genetics) and used as a template for synthesizing dsRNA. The primer and dsRNA sequences are shown in supplementary table S4, Supplementary Material online.

RNAi Knockdown by the Feeding Method

Twenty-five microliters of chicken liver solution, 6 μl of 2% agarose, and 6 μl of dsRNA (32 μg/μl) were mixed and fed to a group of 15 planarians. This food mixture was prepared in small aliquots (~6 μl each) and frozen at −30 °C for 30 min. Then, we conducted three successive feedings at 2-d intervals. siRNA against *EGFP* was used as a negative control and *DjpiwiB* siRNA was used as a positive control.

RNAi Knockdown by Microinjection

Planarians (*D. japonica*, strain SSP) were cultured in an incubator at 22–24 °C. The individuals used in this study (5–7 mm in length) were starved for approximately 1 week before microinjection. The dsRNA was injected 3–5 times into planarians (32 nl/injection) with two successive treatments given 2 days apart using a Drummond Scientific Nanoject injector (Broomall, PA, USA). Control planarians were injected with *EGFP* dsRNA, which did not target any mRNA encoded in the planarian genome. At 6 h following the last set of injections, the planarians were divided into three parts (the head, trunk, and tail) along the anteroposterior axis using sterile surgical blades. We incubated the fragments at 24 °C in the dark for regeneration. After 2 weeks of regeneration. We counted the surviving fragments in each group to calculate the survival rate.

Quantitative Real-Time PCR Analysis to Determine the RNAi Efficiency

We examined the relative expression levels of target genes in four groups (*EGFP* RNAi, *DjHRJDa* RNAi, *DjHRJDb* RNAi, and double knockdown [DKD]) by real-time PCR. We extracted total RNA from the whole bodies of 15 individual *D. japonica* samples in each group, and cDNA was synthesized using a QuantiTect Transcription Kit (Qiagen). We carried out quantitative analysis as previously described in Nishimura et al. (2012). The sequences of primers used for all experiments are shown in supplementary table S2, Supplementary Material online.

Results

Regeneration-Related Genes Conserved in Highly Regenerative Species

To identify genes conserved only in the genomes of species with a high regeneration ability, we conducted a BlastP search

against a database of protein sequences from 132 metazoans (supplementary table S1, Supplementary Material online) using the protein sequences of 14 species from 10 phyla with the potential for high regeneration as queries. No genes were specifically found in the genomes of species in the highly regenerative group (BlastP score >100; table 1). Then, we used a less stringent condition in which the candidate genes included not only those in highly regenerative phyla, but also those in a phylum with low regeneration, to screen for genes that are largely specific to highly regenerative species. In this manner, we found 123 genes conserved in all species of the highly regenerative phyla and one phylum of species with low regeneration (Mollusca) (table 1). As discussed below, we found the ortholog from the low regenerative species *Helobdella robusta* in the highly regenerative phylum Annelida (Bely and Nyberg 2010).

JmjC Domain-Encoding Genes Conserved in Highly Regenerative Species

To investigate the protein domains found in genes conserved in highly regenerative species, we analyzed their protein sequences using SMART (http://smart.embl-heidelberg.de/). Remarkably, 96% of the conserved genes encoded JmjC domain-containing proteins (table 1). Only two (EDO46114 and ENSCINT00000030781) of the genes conserved in highly regenerative species did not have any known protein domains. We designated the JmjC domain-encoding genes as highly regenerative species-specific JmjC domain-encoding genes (*HRJD*s). In general, JmjC domain-containing proteins are related to epigenetic factors and belong to a large gene family (Klose et al. 2006). Although many JmjC domain-containing proteins have other domains such as an FBOX domain or PHD domain (Klose et al. 2006), *HRJD*s are in a group that contains only a JmjC domain. It has been reported that JmjC domain-containing proteins play an essential role in regulating stem cell renewal (Loh et al. 2007; Shen et al. 2009; Xiao et al. 2017). JmjC domain-containing proteins have conserved residues within the predicted cofactor-binding sites (Klose et al. 2006; Xiao et al. 2017). Three amino acid residues bind to the Fe(II) cofactor, and two additional residues bind to αKG within the JmjC domain. There are amino acid variations in the conserved residues representing active or abrogated enzymatic activities. Each of the identified *HRJD*s has at least one ortholog with active amino acid residues in highly regenerative species, except for *HrHRJD1* in *H. robusta*, which only has a different amino acid variation in the conserved residue, which may abolish protein function (fig. 1). We examined whether the substituted amino acid in *HrHRJD1* was deleterious by Provean (Choi et al. 2015) (http://provean.jcvi.org/). The Provean score was −6.350, which was deleterious. The result indicates that *HrHRJD1* in *H. robusta* may have a divergent function compared with other *HRJD* orthologs.

Phylogenetic Relationship of Genes in the *HRJD* Family

To examine the phylogenetic relationships of *HRJD*s in the fully sequenced genomes of metazoans, we analyzed all *HRJD*s shown in table 1. We also used the sequence for the human KDM8 protein, which is the closest human paralog for *HRJD*s, as well as *KDM8* orthologs in highly regenerative species and several model species (chicken, mouse, zebrafish, fruit fly, *Caenorhabditis elegans*, and *Xenopus tropicalis*) as outgroup genes. The phylogenetic tree showed that *HRJD* orthologs in the highly regenerative species clustered together and were completely separated from the *KDM8* outgroup genes, as supported by high bootstrap values (100; fig. 2A). Note that we did not find any *HRJD* orthologs in low regenerative species except for the leech *H. robusta* and the Mollusca. *Helobdella robusta* had only one *HRJD*s HelroP165856 (*HrHRJD1*), and another Annelida (*Capitella teleta*) had three *HRJD*s (*CtHRJD1*, *CtHRJD2*, and *CtHRJD3*), as shown in figure 2A. These results suggest that *H. robusta* lost *HRJD*s during evolution. We propose that low regenerative species lost *HRJD*s independently during metazoan evolution. The tree topology of *HRJD*s does not follow that of the species tree (fig. 2B) because multiple duplication (and/or loss) events of *HRJD*s are ancient and their protein sequences are quite divergent. Thus, it is hard to detect duplication or loss events in *HRJD*s by comparing the gene tree to the species tree.

Changes in Expression Levels for *HRJD*s during Regeneration

To identify genes expressed in response to the regeneration process, we used RNA-Seq data sets from four highly regenerative metazoans (*S. mediterranea*, *N. vectensis*, *H. vulgaris*, and *C. intestinalis*). Before analyzing the RNA-Seq data sets, we identified 3,070 orthologous gene groups shared by the four high regenerative species (see Materials and Methods section). RNA was extracted from oral and physa blastemas at 0, 8, 24, or 72 h post-amputation (hpa) in *N. vectensis* (Schaffer et al. 2016); from the head blastemas of *H. vulgaris* (0, 2, 4, 6, 12, and 48 hpa); and from the blastemas of *C. intestinalis* oral siphons (Spina et al. 2017) (0, 1, 3, and 8 dpa). RNA samples from *S. mediterranea* were extracted from regenerating planarians at 0, 1, 3, 6, 9, 12, 24, 36, 48, 60, and 72 hpa, and 4, 5, 6, 7, 10, and 14 dpa (Zeng et al. 2018). We identified DEGs in *N. vectensis*, *H. vulgaris*, *S. mediterranea*, and *C. intestinalis*, respectively, by comparing their gene expression levels with those observed under normal conditions (supplementary table S2, Supplementary Material online).

Although not all *HRJD*s responded to the regeneration process, we found that four of nine, one of two, one of two, and four of 13 *HRJD*s overlapped with DEGs specific to *N. vectensis*, *H. vulgaris*, *S. mediterranea*, and *C. intestinalis*, respectively. The expression levels of 10 DEGs are shown in
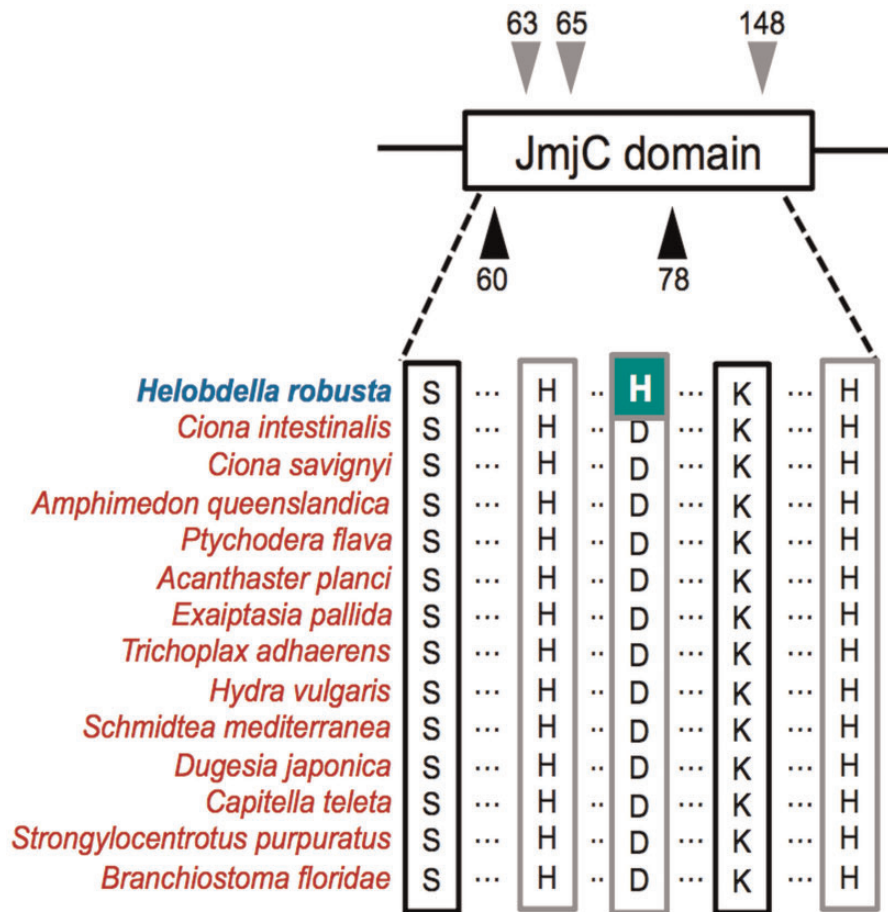
FIG. 1.—Amino acid alignments in highly conserved sites of the JmjC domain for high regeneration species and *H. robusta*. Gray and black arrows/boxes represent amino acid residues associated with Fe(II)-binding and αKG-binding sites, respectively. The amino acid of *HrHRJD1* marked by a solid white character in a green box is different compared with other highly regenerative species.

supplementary figure S1, Supplementary Material online. To test the enrichment of DEGs in *HRJDs*, we identified 238 orthologous gene groups (containing 6,304 genes) in which all four species had at least one DEG, out of 3,070 orthologous gene groups (containing 39,510 genes) shared by the four highly regenerative species (supplementary fig. S2, Supplementary Material online). DEGs were significantly enriched in genes of the *HRJD* orthologous group (10/26 vs. 6,304/39,510; $P = 0.0042$, χ^2 test) during the regeneration of the four highly regenerative species.

DjHRJDa and *DjHRJDb* Expression in Neoblasts, and Differentiated Cells of *D. japonica*

The planarian is a typical highly regenerative species and a model species for examining the function of regeneration-related genes (Nishimura et al. 2012; An et al. 2018). *Dugesia japonica* and *S. mediterranea* each have two *HRJD* orthologs, which are *DjHRJDa* and *DjHRJDb* in *D. japonica* and *SmHRJDa* and *SmHRJDb* in *S. mediterranea* (fig. 2 and supplementary

table S3, Supplementary Material online). Based on the RNA-Seq analysis in *S. mediterranea*, we found that *SmHRJDb* was a DEG during regeneration, whereas *SmHRJDa* was not (fig. 3A). The expression level of *SmHRJDb* was negatively regulated at the beginning of regeneration, but that of *SmHRJDb* was upregulated after 6 days of regeneration (fig. 3A). We observed that the expression level of *smedwi-1*, *smedwi-2*, and *smedwi-3* continuously increased after amputation, but they started to decrease after 72 h (fig. 3B). Interestingly, the expression level of *SmHRJDb* negatively correlated with that of *smedwi-1* (Pearson product-moment correlation, $r = -0.87$, $P = 0.0047$) and *smedwi-3* (Pearson product-moment correlation, $r = -0.79$, $P = 0.012$) after 72 h (fig. 3A and B). This result indicates that there is direct or indirect interaction between *piwi* and *HRJDs* during the regeneration process. Amino acid sequences similarity between DjHRJDb and SmHRJDb, particularly those encoding the JmjC domain, was higher than that between DjHRJDa and SmHRJDa (fig. 3C).

We speculated that the regeneration-related genes might be expressed specifically in neoblasts which are planarian
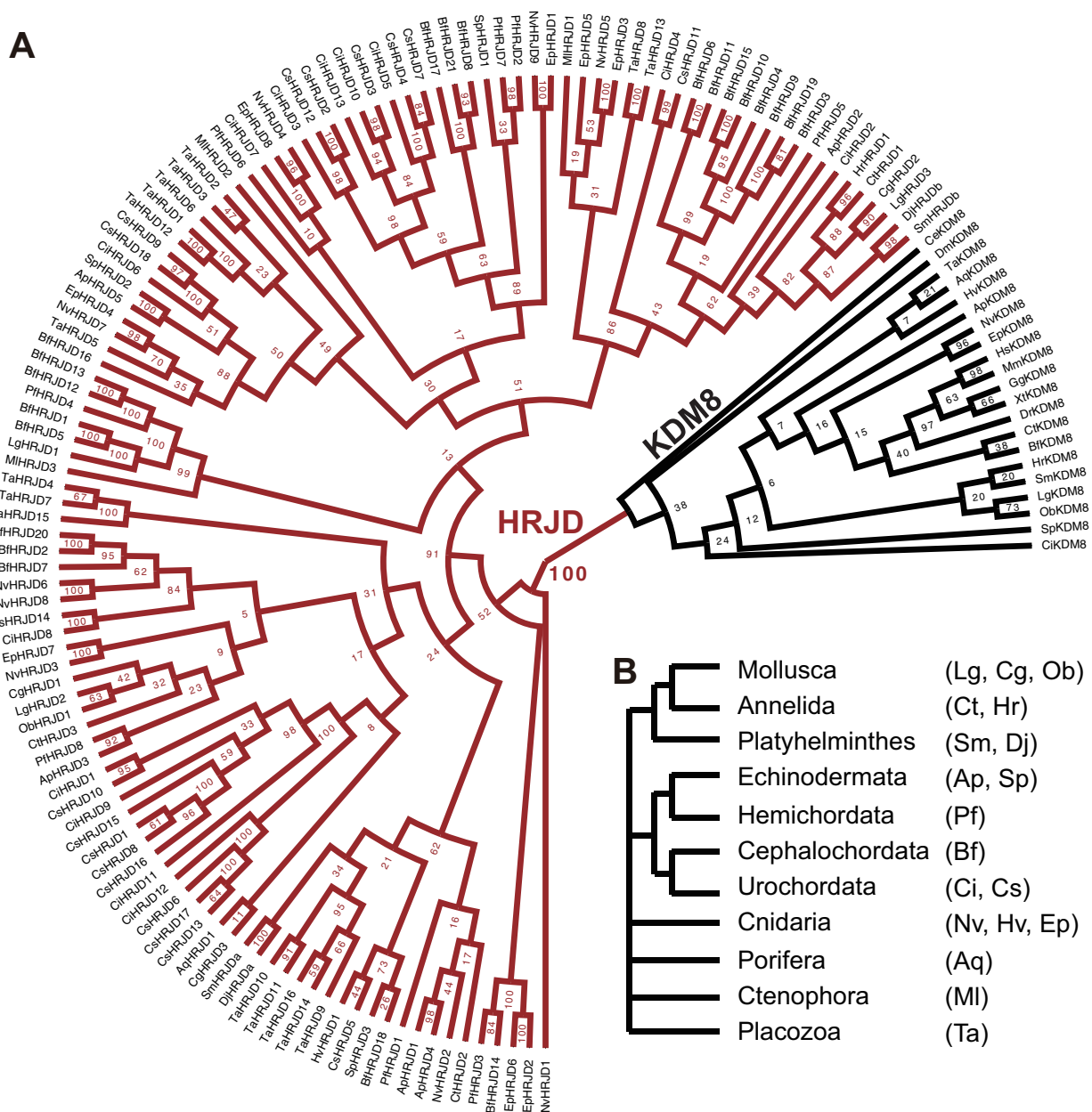
Fig. 2.—The phylogenetic relationships of metazoans in this study. (A) The phylogenetic relationships among *HRJDs* in highly regenerative species. Phylogenetic tree of *HRJDs* in the highly regenerative group aligned using MAFFT software and built using the maximum-likelihood algorithm of RAxML. The gene IDs corresponding to the names on the tree are shown in supplementary table S5, Supplementary Material online. Branch lines shown in red and black indicate *HRJD* and *KDM8* orthologs, respectively. The candidate genes of highly regenerative species were phylogenetically separated from the outgroup genes (*KDM8* orthologs). The solid and hollow arrows represent *HRJD* orthologs for *C. teleta* and *H. robusta*, respectively. This figure was made by FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). (B) The phylogenetic tree of highly regenerative groups and Mollusca conserved *HRJDs*. Abbreviations of species names (Ap, *Acanthaster planci*; Aq, *Amphimedon queenslandica*; Bf, *Branchiostoma floridae*; Ce, *Caenorhabditis elegans*; Ct, *Capitella teleta*; Ci, *Ciona intestinalis*; Cs, *Ciona savignyi*; Cg, *Crassostrea gigas*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Ep, *Exaiptasia pallida*; Gg, *Gallus gallus*; Hr, *Helobdella robusta*; Hs, *Homo sapiens*; Hv, *Hydra vulgaris*; Lg, *Lottia gigantea*; Ml, *Mnemiopsis leidyi*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*; Ob, *Octopus bimaculoides*; Pf, *Ptychodera flava*; Sm, *Schmidtea mediterranea*; Sp, *Strongylocentrotus purpuratus*; Ta, *Trichoplax adhaerens*; Xt, *Xenopus tropicalis*).

adult pluripotent stem cells, because neoblasts play a central role in regeneration (Agata and Watanabe 1999; Newmark and Alvarado 2001; Agata et al. 2006). Therefore, we examined the expression patterns of *DjHRJDa* and *DjHRJDb* in

D. japonica by in situ hybridization (fig. 3D–I). The positive control *DjpiwiA*, which is a reliable marker of neoblasts (Hayashi et al. 2010; Shibata et al. 2016), was expressed only in neoblasts, and thus we observed that *DjpiwiA*
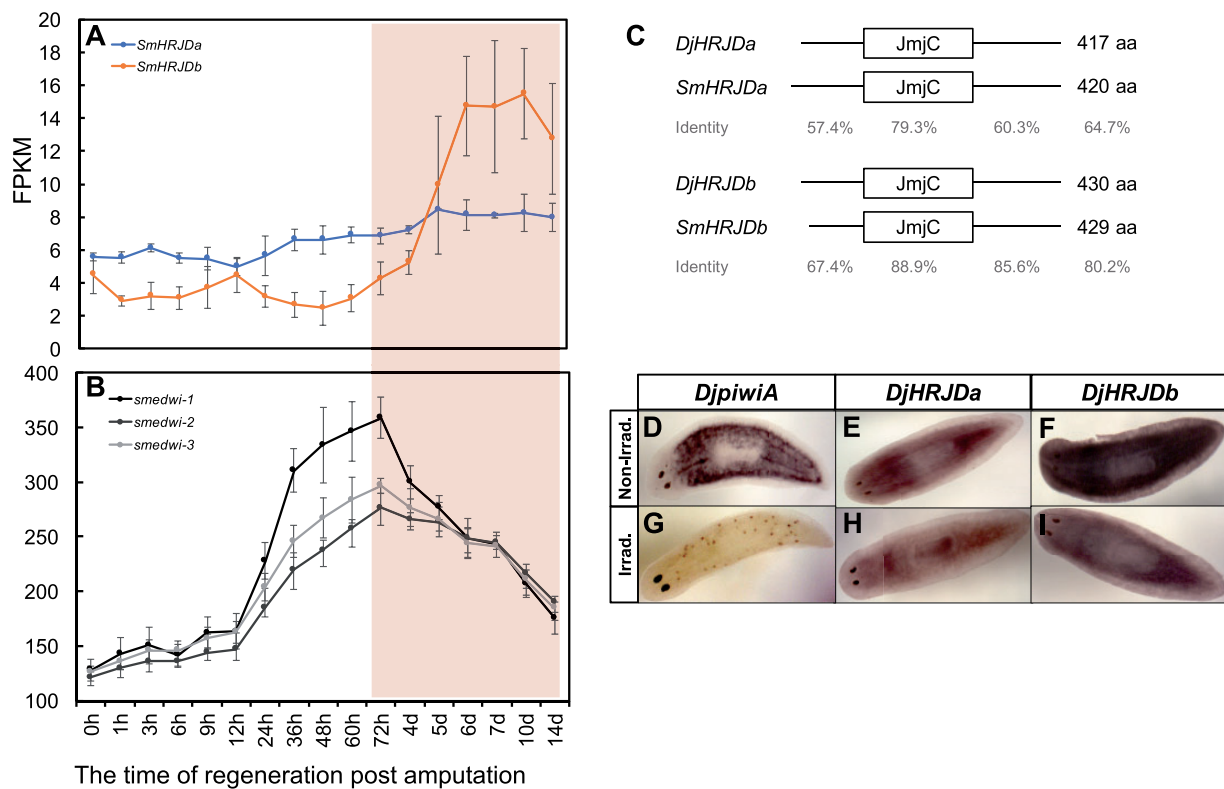
Fig. 3.—Gene expression of *HRJD* orthologs in planarians. (*A*) and (*B*) Expression changes of two *HRJDs* (*SmHRJDa* and *SmHRJDb*) and three *piwi* genes (*smedwi-1*, *smedwi-2*, and *smedwi-3*) during regeneration in *S. mediterranea*, respectively. The *x* axis and *y* axis indicate the time after amputation and FPKM (fragments per kilobase of exon per million mapped reads) values in *S. mediterranea*, respectively. The error bars indicate the standard error. The pink area represents the different expression pattern between *SmHRJDb* and *semdwi* genes, *SmHRJDa* was not a DEG during regeneration under any condition. (*C*) Schematic structures and the identity of amino acid sequences between *HRJD* orthologs in two planarians. The percentages represent the identity of amino acid sequences for each segmental region of a HRJD protein in two planarians. (*D–F*) The expression patterns of *DjpiwiA* (control), *DjHRJDa*, and *DjHRJDb* in nonirradiated planarians by in situ hybridization. (*G–I*) The expression patterns of *DjpiwiA* (control), *DjHRJDa*, and *DjHRJDb* in X-ray irradiated planarians by in situ hybridization.

expression disappeared after specifically killing neoblasts with X-ray irradiation (fig. 3D and G). In contrast, we observed high *DjHRJDa* and *DjHRJDb* expression in the whole body even after X-ray irradiation (fig. 3E, F, H, and I), although the signals for *DjHRJDa* and *DjHRJDb* expression without irradiation were higher than those in irradiated planarians. These results indicate that *DjHRJDa* and *DjHRJDb* were highly expressed in both neoblasts and differentiated cells of *D. japonica*.

Regenerative Failure after RNAi-Mediated Knockdown of Genes Encoding the JmjC-Domain Proteins DjHRJDa and DjHRJDb in *D. japonica*

To investigate whether *HRJDs* play an important role in high regeneration ability, we conducted RNAi-mediated knockdown of *DjHRJDa* and *DjHRJDb* in *D. japonica*. We used knockdown of *DjpiwiB* (a reliable marker of neoblasts) (Hayashi et al. 2010; Shibata et al. 2016) and *EGFP* as

RNAi positive and negative controls, respectively. It took approximately 5 days for planarians to digest the double-stranded RNA (dsRNA) after last feeding. We divided eight of 15 individuals into three pieces (the head, trunk, and tail) for each knockdown experiment, and the remaining seven individuals were in the intact group. Note that we did not distinguish among the amputated body parts (the head, trunk, and tail) in the experiment using the dsRNA feeding method. We did not observe abnormal phenotypes in intact individuals treated with dsRNA of *DjHRJDa*, *DjHRJDb*, or *EGFP* (negative control), even after 7 days. This result indicates that the *HRJDs* were not essential for survival. In contrast, following *DjpiwiB* knockdown (positive control), 85.7% of intact individuals and 87.5% of individuals with amputation died (fig. 4A). The effect of *DjpiwiB* knockdown was lethal to the planarians, whereas RNAi against *DjHRJDa*, *DjHRJDb*, and *EGFP* did not show any abnormal phenotypes after the first amputation. Then, we conducted a second amputation in which the target was the blastema of amputated individuals

(except for individuals with *DjpiwiB* knockdown). As a result, 28.6% and 46.4% of *DjHRJDa* and *DjHRJDb* RNAi knockdown planarians died (including one planarian showing an abnormal phenotype) during the 7-day regeneration process (fig. 4B–D).

Blastemas are important cells for regeneration in planarians (Tasaki et al. 2011). The distribution of blastemas is not uniform in the planarian body. In particular, neoblasts are enriched in the trunk compared with the head and tail (Orii et al. 2005). Thus, we examined the survival rate for each amputated part after RNAi knockdown. To observe more severe phenotypes, we conducted RNAi knockdown by injecting instead of feeding dsRNA. It took 6 h for the planarians to digest the dsRNA after the last microinjection, after which time we amputated all individuals into three pieces (e.g. the head, trunk, and tail) for each knockdown group. To estimate the efficiency of RNAi knockdown, we measured the relative expression levels of *DjHRJDa* and *DjHRJDb* after knockdown by quantitative PCR. We observed that the relative expression levels of *DjHRJDa* and *DjHRJDb* were reduced by 46.6% and 82.1%, respectively, when each gene was silenced individually (supplementary fig. S3, Supplementary Material online). In addition, DKD experiments (where both genes were targeted by RNAi) reduced the relative expression levels of *DjHRJDa* and *DjHRJDb* by 33.5% and 80.5%, respectively (supplementary fig. S3, Supplementary Material online). The knockdown planarians started to die 7 days after the amputation. Death following knockdown likely paralleled the observed expression increase in *SmHRJDb* (a *DjHRJDb* ortholog) in *S. mediterranea* (figs. 3A and 4E).

The abnormal phenotypes observed in knockdown individuals occurred faster using the microinjection method than with the feeding method. We estimated the survival rates of each group at 14 days after treatment. Almost all amputated pieces in the negative control group (*EGFP* knockdown) survived (only one tail fragment died). Although the individual trunk fragments in knockdown individuals did not die, the amputated head and tail fragments died in the *DjHRJDa* (RNAi), *DjHRJDb* (RNAi), and the DKD groups (fig. 4E). Specifically, 50% of the head fragments and 58.8% of the tail fragments died after *DjHRJDa* and DKD RNAi knockdown, respectively (fig. 4E). This finding indicates that regeneration of the head and tail fragments, which include fewer neoblasts compared with the trunk, is likely to be influenced by RNAi knockdown of *DjHRJDa* and *DjHRJDb*. The death rate of DKD (*DjHRJDa* and *DjHRJDb*) individuals was higher than that for the groups where only *DjHRJDa* or *DjHRJDb* was knocked down. The expression level of the *DjHRJDb* ortholog in *S. mediterranea* significantly increased during the regeneration process, whereas that of the *DjHRJDa* ortholog did not change (fig. 3A). This result indicates that *DjHRJDb* expression is needed for regeneration.

Discussion

*HRJD*s Associated with the Regeneration Process

Common ancestors of metazoans likely had a high regeneration ability, and many species appear to have lost that ability during evolution (Bely and Nyberg 2010). We found that *HRJD*s were conserved specifically in highly regenerative species. The genes were lost in Vertebrata, Nematoda, and Arthropoda during evolution, although they were conserved in Mollusca. Bely and her colleagues pointed out that regeneration data for Mollusca are limited when compared with other phyla (Bely 2014). Although Mollusca species cannot regenerate many body parts, some of them can regenerate the head (Bely 2014). In addition, it has been reported that Echinoderm larvae can regenerate the whole body, whereas Echinoderm adults cannot (Vickery et al. 2001; Reinardy et al. 2015). Mollusca possessing *HRJD*s may have also a high potential for regeneration at some spatiotemporal stages (e.g. the larva stage). Further studies are needed to understand the regeneration capability of Mollusca. *HRJD*s may have multiple functions, with one function related to regeneration ability potentially being lost in Mollusca during evolution (fig. 5).

Members of the Annelida phylum are classified as highly regenerative species (Bely and Nyberg 2010; Bely et al. 2014), although some species (such as the leech) lost the ability to regenerate during evolution (Bely et al. 2014). We surveyed *HRJD*s of *C. teleta* and *H. robusta* in the Annelida phylum and identified three genes conserved in *C. teleta*, but only one gene conserved in *H. robusta* (table 1). Leeches lost two *HRJD*s and the regeneration ability during evolution (fig. 2). Highly regenerative species have multiple *HRJD*s, whereas *H. robusta* and the primitive multicellular aquatic metazoan *Amphimedon queenslandica* have only one *HRJD* (table 1). These findings suggest that the maintenance of multiple *HRJD*s may be associated with an increased capacity for regeneration.

Duplication of Ancestral Genes for *HRJD* and *KDM8*

There are seven groups (JHMD1, PHF2, JARID, JHDM3, UTX, JHDM2, and JmjC domain only) in the large family of JmjC domain-encoding genes (Klose et al. 2006). *HRJD*s and their closest paralogs *KDM8* are in the JmjC domain only group (fig. 2). It has been reported that KDM8 regulates pluripotency in human embryonic stem cells (Zhu et al. 2014). Amino acid sequences of *HRJD*s apparently diverged from those of KDM8 and thus they are distinguishable (fig. 2A). Although plants and fungi do not have HRJD orthologs, they have *KDM8* orthologs (Jones et al. 2010; Gacek-Matthews et al. 2015), metazoans have both HRJD and *KDM8* orthologs (fig. 2A). Thus, duplication of *KDM8* and *HRJD* would have occurred before the origin of metazoans and after metazoan-fungi split.
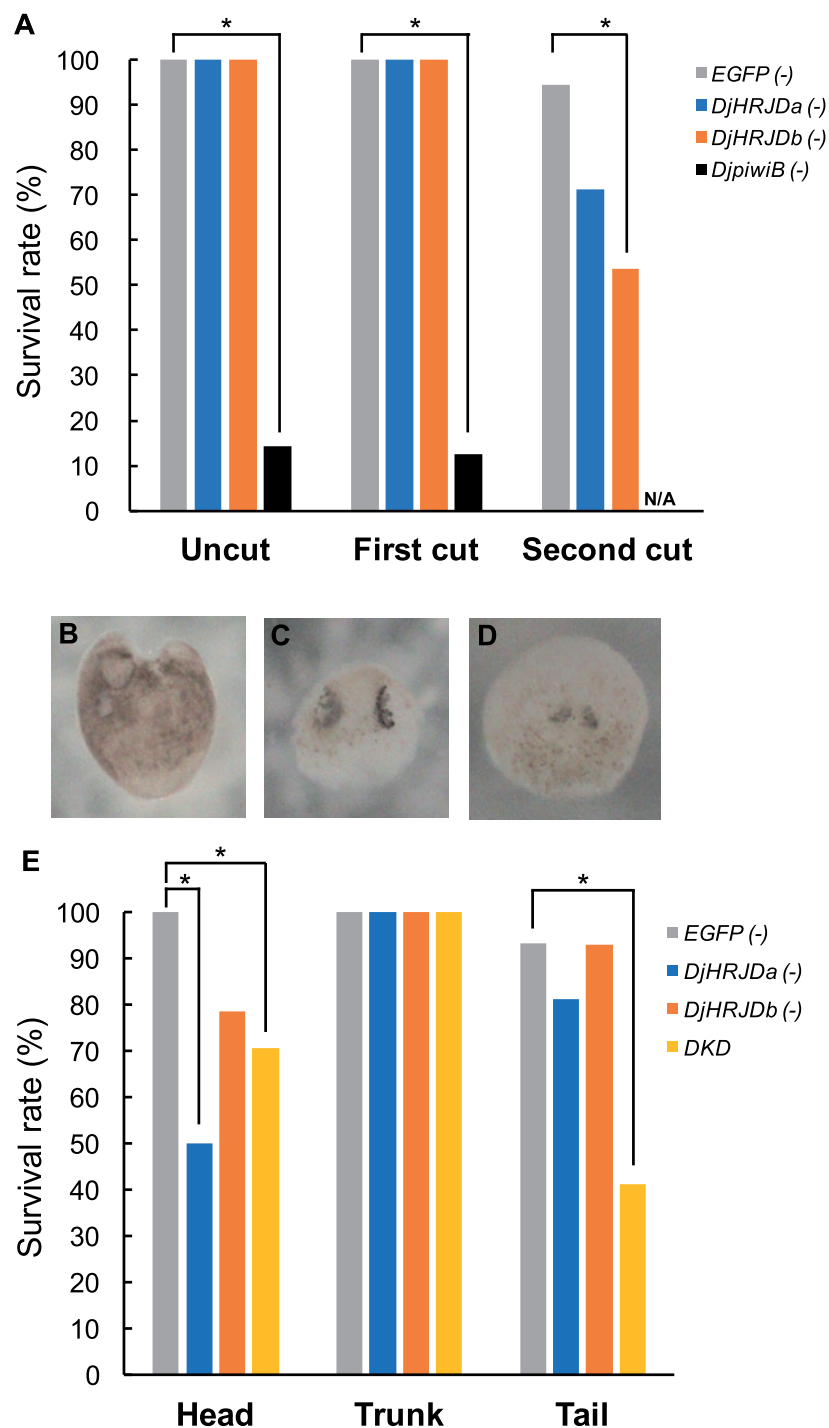
Fig. 4.—Survival rate in knockdown planarians after amputation. (A) The survival rate of amputated pieces during the planarian regeneration process by feeding dsRNA are shown. Individual planarians started to die after the second amputation ($n = 28$). (B–D) Pictures of dead head fragments after knocking down the expression of *DjHRJDa*, *DjHRJDb*, or *DjpiwiB* (positive control). (E) The survival rates of amputated pieces (head, trunk, and tail) during the planarian regeneration process by injecting dsRNA are shown. Individual planarians in the *DjHRJDa* RNAi ($n = 14$), *DjHRJDb* RNAi ($n = 16$), and DKD RNAi ($n = 17$) groups started to die at 1 week post-amputation, and we estimated their survival rates after 2 weeks. Asterisks represent a significant difference in survival rates between knockdown and control individuals (Fisher's exact test, *P* value <0.05).
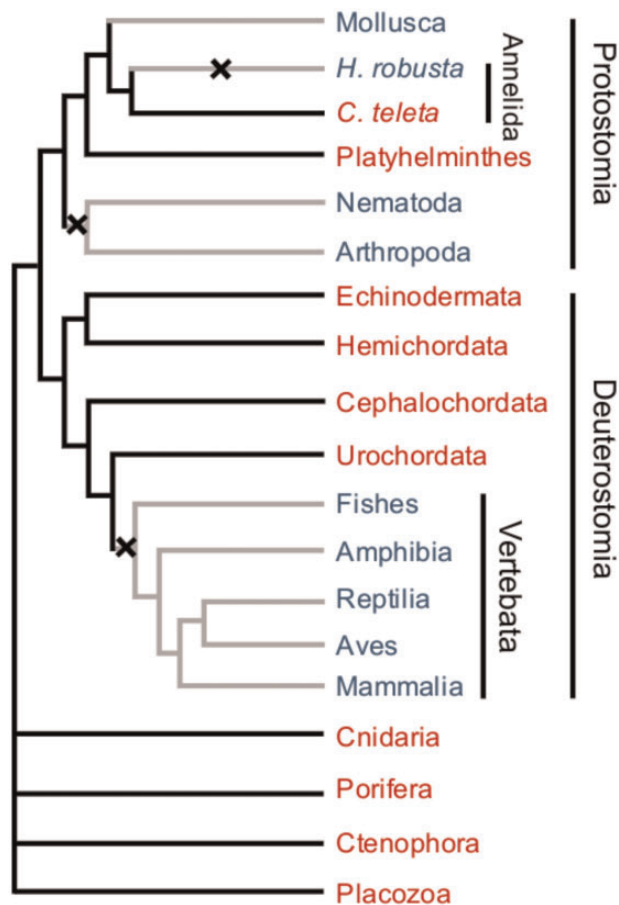
FIG. 5.—Overview of losses of high regeneration ability and *HRJDs* in metazoans during evolution. Phylogenetic relationships of metazoan phyla used in this study. Red and blue characters indicate highly and low regenerative phyla (or species), respectively. Black crosses and gray lines indicate losses of *HRJDs* and regeneration ability during evolution, respectively.

Functions of JmjC Domain-Containing Proteins

The JMJD1C protein contains a JmjC domain that is a paralog of *HRDJ* genes and regulates self-renewal for embryonic stem cells in mice (Peng et al. 2009). Thus, *HRJDs* may be involved in the regulation of stem cells during regeneration. We propose the following model regarding the molecular functions of *HRJDs*, based on the known functions of their paralogs that are conserved in metazoans. Members of the family of JmjC domain-containing proteins are epigenetic factors that remove methylation markers on histones to regulate target gene expression and that are part of the epigenetic memory system regulating cell fate and identity (Klose et al. 2006; Tsukada et al. 2006). It has been reported that lysine (K) and arginine (R) residues of histones can be methylated by paralogs of JmjC domain-containing proteins and that histone methylation correlates with transcriptional activation or repression based on the position of K, such as in H3K4 (Eissenberg and Shilatifard 2010), H3K9 (Park et al. 2011), H3K27 (Agger et al. 2007; Hamada et al. 2015; Wiles and

Selker 2017), H3K36 (Hsia et al. 2010; Suzuki et al. 2016), or based on the state of demethylation, as with mono- (me1), di- (me2), or tri-demethylation (me3) (Lachner et al. 2003; Martin and Zhang 2005; Chen et al. 2011).

JmjC domain-containing proteins also serve essential roles in regulating stem cell renewal. JMJD1C protein containing a JmjC domain in low regenerative vertebrate species, which is one of *HRJD* paralogs, demethylates H3K9 and thereby control ERK/MAPK signaling and the epithelial-to-mesenchymal transition, as well as regulate mouse embryonic stem cell self-renewal (Xiao et al. 2017). Other mouse paralogs (e.g. the Jmjd1a and Jmjd2c proteins) functioned as H3K9Me2 and H3K9Me3 demethylases to positively control stem cell renewal, and their depletion lead to stem cell differentiation (Loh et al. 2007). The mouse paralog JmjC domain-containing protein UTX, which is an H3K27 trimethylase, regulates pluripotency, as somatic cells lacking UTX fail to reprogram back to pluripotency induced by transcription factors (*Oct4*, *Sox2*, *Klf4*, and *Myc*) (Mansour et al. 2012). The human paralogs UTX and JMJD3 are H3K27 trimethylases that decrease the expression level of H3K27me3 associated with *HOX* genes and delocalized polycomb proteins *in vivo* during differentiation (Agger et al. 2007). Moreover, the cricket paralog UTX protein promotes joint formation through histone H3K27 methylation to regulate leg regeneration (Agger et al. 2007). We propose that *HRJDs* influence cellular processes through epigenetic regulation.

Although an ortholog of *HRJD* (*Mina53-like1*) in *C. intestinalis* (table 1) was proposed to promote mesenchymal cell differentiation during embryonic development (Tokuoka et al. 2004), a possible association of this gene with regeneration in *C. intestinalis* has not been examined. The expression level of *Mina53-like1* in *C. intestinalis* was upregulated by embryonic overexpression of the FGF genes *FGF9*, *FGF16*, and *FGF20* during development (Tokuoka et al. 2004). FGF genes are important for regeneration processes in many species, such as planarians, zebrafish, and *Xenopus laevis* (Lee et al. 2005; Fukui and Henry 2011; Shibata et al. 2016). Furthermore, previous data indicated that many genes expressed during embryogenesis are also expressed during regeneration (Christen et al. 2010; Hayashi et al. 2010). Therefore, we speculate that *Mina53-like1* in *C. intestinalis* also plays a role in regeneration.

HRJDs Associated with the High Regeneration Ability of Planarians

We identified two *HRJD* orthologs (*DjHRJDa* and *DjHRJDb*) in planarians and examined their biological functions by RNAi. We observed that many individuals with knocked down *DjHRJDa* and *DjHRJDb* expression did not regenerate their lost parts after 2 weeks. Reddien (2013) proposed two models for cell specification in planarian regeneration, namely the naïve-neoblast and specialized-neoblast models. Note that

an intermediate process of differentiation achieved by undifferentiated blastema cells was included in both models. The regenerative process in planarians involves two main processes: neoblast proliferation and neoblast differentiation (Agata et al. 2006). The knockdown planarians contained some progenitor cells even though they were not supplied by neoblasts. These progenitor cells normally became differentiated cells during regeneration after the first amputation in the *DjHRJD* knockdown group; thus, they could survive for 1 week. After 1 week, the progenitor cells were exhausted, which caused the planarians to die. This may explain why none of the planarians demonstrated abnormal phenotypes following RNAi knockdown of *DjHRJDs* during the first week, whereas many of them died later.

Conclusions

We have presented evidence of strong associations between *HRJDs* and a high regeneration ability in metazoans. Evolutionary conservation of the *HRJDs* appears to have been important for maintaining a high regenerative ability in metazoans, and species lacking this ability in Vertebrata, Arthropoda, and Nematoda have lost *HRJDs* during evolution. We propose that loss of the *HRJDs* during evolution is associated with the loss of a high ability for regeneration. These findings shed new insights into understanding the common molecular basis of high regeneration capability in metazoans.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

Literature Cited

Agata K, et al. 2006. Two different evolutionary origins of stem cell systems and their molecular basis. Semin Cell Dev Biol. 17(4):503–509.

Agata K, Inoue T. 2012. Survey of the differences between regenerative and non-regenerative animals. Dev Differ. 54(2):143–152.

Agata K, Watanabe K. 1999. Molecular and cellular aspects of planarian regeneration. Semin Cell Dev Biol. 10(4):377–383.

Agger K, et al. 2007. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449(7163):731–735.

Alvarado AS, Newmark PA. 1999. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proc Natl Acad Sci U S A. 96:5049–5054.

An Y, et al. 2018. The draft genome of *Dugesia japonica* provides insights into conserved regulatory elements of the brain restriction gene noudarake in planarians. Zool Lett. 4(24):1–12.

Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31(2):166–169.

Baumgarten S, et al. 2015. The genome of *Aiptasia*, a sea anemone model for coral symbiosis. Bioinformatics 31:2745–2747.

Bely AE. 2014. Early events in annelid regeneration: a cellular perspective. Integr Comp Biol. 54(4):688–699.

Bely AE, Nyberg KG. 2010. Evolution of animal regeneration: re-emergence of a field. Trends Ecol Evol. 25(3):161–170.

Bely AE, Zattara EE, Sikes JM. 2014. Regeneration in spiralians: evolutionary patterns and developmental processes. Int J Dev Biol. 58(6–8):623–634.

Bode HR. 2003. Head regeneration in Hydra. Dev Dyn. 226(2):225–236.

Caubit X, Nicolas S, Parco YLE. 1997. Possible roles for Wnt genes in growth and axial patterning during regeneration of the tail in urodele amphibians. Dev Dyn. 210(1):1–10.

Chapman JA, et al. 2010. The dynamic genome of Hydra. Nature 464(7288):592–596.

Chen X, Hu Y, Zhou DX. 2011. Epigenetic gene regulation by plant Jumonji group of histone demethylase. Biochim Biophys Acta. 1809(8):421–426.

Choi Y, Chan AP, Craig TJ. 2015. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. BMC Biol. 8(5):1–14.

Christen B, et al. 2010. Regeneration and reprogramming compared. BMC Biol. 8:5.

Eissenberg JCA, Shilatifard A. 2010. Histone H3 lysine 4 (H3K4) methylation in development and differentiation. Dev Biol. 339(2):240–249.

Fukui L, Henry JJ. 2011. FGF signaling is required for lens regeneration in *Xenopus laevis*. Biol Bull. 221(1):137–145.

Fumagalli MR, Zapperi S, Porta CAM L. 2018. Regeneration in distantly related species: common strategies and pathways. NPJ Syst Biol Appl. 4:1–7.

Gacek-Matthews A, et al. 2015. KdmA, a histone H3 demethylase with bipartite function, differentially regulates primary and secondary metabolism in *Aspergillus nidulans*. Mol Microbiol. 96(4):839–860.

Hall MR, et al. 2017. The crown-of-thorns starfish genome as a guide for biocontrol of this coral reef pest. Nature 544(7649):231–234.

Hamada Y, et al. 2015. Leg regeneration is epigenetically regulated by histone H3K27 methylation in the cricket *Gryllus bimaculatus*. Development. 3:2916–2927.

Hayashi T, et al. 2010. Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its "index sorting" function for stem cell research. Dev Growth Differ. 52(1):131–144.

Holstein TW. 2012. The evolution of the Wnt pathway. Cold Spring Harb Perspect Biol. 4:1–17.

Hsia DA, et al. 2010. KDM8, a H3K36me2 histone demethylase that acts in the cyclin A1 coding region to regulate cancer cell proliferation. Proc Natl Acad Sci U S A. 107(21):9671–9676.

Jones MA, et al. 2010. Jumonji domain protein JMJD5 functions in both the plant and human circadian systems. Proc Natl Acad Sci U S A. 107(50):21623–21628.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30(14):3059–3066.

Kim D, et al. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14(4):R36.

Klose RJ, Kallin EM, Zhang Y. 2006. JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet. 7(9):715–727.

Lachner M, Roderick J, Sullivan O. 2003. Cell science at a glance an epigenetic road map for histone lysine methylation. J Cell Sci. 116(11):2117–2124.

Lee Y, Grill S, Sanchez A, Murphy-ryan M, Poss KD. 2005. FGF signaling instructs position-dependent growth rate during zebrafish fin regeneration. Development 132(23):5173–5183.

Loh Y, Zhang W, Chen X, George J, Ng H. 2007. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev. 21(20):2545–2557.

Makanae A, Mitogawa K, Satoh A. 2016. Cooperative inputs of BMP and FGF signaling induce tail regeneration in urodele amphibians. Dev Biol. 410(1):45–55.

Mansour AA, et al. 2012. The H3K27 demethylase UTX regulates somatic and germ cell epigenetic reprogramming. Nature 488(7411):409–413.

Martin C, Zhang Y. 2005. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 6(11):838–849.

Newmark PA, Alvarado AS. 2001. Regeneration in planaria. Encycl Life Sci. 1–7.

Nishimura O, Hirao Y, Tarui H, Agata K. 2012. Comparative transcriptome analysis between planarian *Dugesia japonica* and other platyhelminth species. BMC Genomics 13:1–17.

Orii H, Sakurai T, Watanabe K. 2005. Distribution of the stem cells (neoblasts) in the planarian *Dugesia japonica*. Dev Genes Evol. 215(3):143–157.

Ozhan G, Weidinger G. 2015. Wnt/β-catenin signaling in heart regeneration. Cell Regen. 4:1–12.

Park J, et al. 2011. Deacetylation and methylation at histone H3 lysine 9 (H3K9) coordinate chromosome condensation during cell cycle progression. Mol Cells 31(4):343–349.

Peng JC, et al. 2009. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell 139(7):1290–1302.

Poss KD, Shen J, Keating MT. 2000. Induction of lef1 during zebrafish fin regeneration. Dev Dyn. 286:282–286.

Putnam NH, et al. 2008. The amphioxus genome and the evolution of the chordate karyotype. Nature 453(7198):1064–1071.

Reddien PW. 2013. Specialized progenitors and regeneration. Development 140(5):951–957.

Reddien PW, Bermange AL, Kicza AM, Alvarado AS. 2007. BMP signaling regulates the dorsal planarian midline and is needed for asymmetric regeneration. Development 134(22):4043–4051.

Reinardy HC, Emerson CE, Manley JM, Bodnar AG. 2015. Tissue regeneration and biomineralization in sea urchins: role of Notch signaling and presence of stem cell markers. PLoS One 10:1–15.

Robb SMC, Gotting K, Ross E, Alejandro S. 2015. SmedGD 2. 0: the *Schmidtea mediterranea* genome database. Genesis 53(8):535–546.

Rouhana L, et al. 2013. RNA interference by feeding in vitro—synthesized double-stranded RNA to planarians. Methodol Dyn. 242(6):718–730.

Sánchez Alvarado A. 2000. Regeneration in the metazoans: why does it happen? BioEssays 22(6):578–590.

Schaffer AA, Bazarsky M, Levy K, Chalifa-Caspi V, Gat U. 2016. A transcriptional time-course analysis of oral vs. aboral whole-body regeneration in the Sea anemone *Nematostella vectensis*. BMC Genomics 17:1–22.

Shen X, et al. 2009. Jumonji modulates polycomb activity and self-renewal versus differentiation of stem cells. Cell 139(7):1303–1314.

Shibata E, et al. 2016. FGF signalling controls diverse aspects of fin regeneration. Development 143(16):2920–2929.

Shibata N, et al. 2016. Inheritance of a Nuclear PIWI from Pluripotent Stem Cells by Somatic Descendants Ensures Differentiation by Silencing Transposons in Planarian. Dev Cell 37(3):226–237.

Simakov O, et al. 2015. Hemichordate genomes and deuterostome origins. Nature 527(7579):459–465.

Somorjai IML, Somorjai RL, Garcia-Fernandez J, Escriva H. 2012. Vertebrate-like regeneration in the invertebrate chordate amphioxus. Proc Natl Acad Sci U S A. 109(2):517–522.

Spina EJ, Guzman E, Zhou H, Kosik KS, Smith WC. 2017. A microRNA–mRNA expression network during oral siphon regeneration in *Ciona*. Development 144(10):1787–1797.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9):1312–1313.

Sun J, Nishiyama T, Shimizu K, Kadota K. 2013. TCC: an R package for comparing tag count data with robust normalization strategies. BMC Bioinform.14:1–13.

Suzuki S, et al. 2016. Histone H3K36 trimethylation is essential for multiple silencing mechanisms in fission yeast. Nucleic Acids Res. 44(9):4147–4162.

Tasaki J, et al. 2011. ERK signaling controls blastema cell differentiation during planarian regeneration. Development 138(12):2417–2427.

Tokuoka M, Imai KS, Satou Y, Satoh N. 2004. Three distinct lineages of mesenchymal cells in *Ciona intestinalis* embryos demonstrated by specific gene expression. Dev Biol. 274(1):211–224.

Tsukada Y, et al. 2006. Histone demethylation by a family of JmjC domain-containing proteins. Nature 439(7078):811–816.

Vickery MCL, Vickery MS, Amsler CD, Mcclintock JB. 2001. Regeneration in Echinoderm larvae. Microsc Res Tech. 55(6):464–473.

Wiles ET, Selker EU. 2017. ScienceDirect H3K27 methylation: a promiscuous repressive chromatin mark. Curr Opin Genet Dev. 43:31–37.

Xiao F, et al. 2017. JMJD1C ensures mouse embryonic stem cell self-renewal and somatic cell reprogramming through controlling microRNA expression. Stem Cell Rep. 9(3):927–942.

Zeng A, et al. 2018. Prospectively isolated tetraspanin+neoblasts are adult pluripotent stem cells underlying planaria regeneration. Cell 173(7):1593–1608.

Zhu H, Hu S, Baker J. 2014. JMJD5 regulates cell cycle and pluripotency in human embryonic stem cells. Stem Cells 32(8):2098–2110.

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