RESEARCH ARTICLE

JMJD-1.2/PHF8 controls axon guidance by regulating Hedgehog-like signaling

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ABSTRACT

Components of the KDM7 family of histone demethylases are implicated in neuronal development and one member, PHF8, is often found to be mutated in cases of X-linked mental retardation. However, how PHF8 regulates neurodevelopmental processes and contributes to the disease is still largely unknown. Here, we show that the catalytic activity of a PHF8 homolog in Caenorhabditis elegans, JMJD-1.2, is required non-cell-autonomously for proper axon guidance. Loss of JMJD-1.2 dysregulates transcription of the Hedgehog-related genes wrt-8 and grd-16, the overexpression of which is sufficient to induce the axonal defects. Deficiency of either wrt-8 or grd-16, or reduced expression of homologs of genes promoting Hedgehog signaling, restores correct axon guidance in jmjd-1.2 mutants. Genetic and overexpression data indicate that Hedgehog-related genes act on axon guidance through actin remodelers. Thus, our study highlights a novel function of jmjd-1.2 in axon guidance that might be relevant for the onset of X-linked mental retardation and provides compelling evidence of a conserved function of the Hedgehog pathway in C. elegans axon migration.

KEY WORDS: Epigenetics, Histone demethylase, Neuronal development, Axon guidance, Hedgehog signaling, C. elegans

INTRODUCTION

The KDM7 family of histone demethylases, which consists of three members, KDM7A (KIAA1718), KDM7B (PHF8) and KDM7C (PHF2), is characterized by the presence of a C-terminal JmjC domain and a PHD finger domain in the N-terminal portion. The members of this family have been associated with neurodevelopmental processes (Kleine-Kohlbrecher et al., 2010; Qiu et al., 2010; Qi et al., 2010; Tsukada et al., 2010; Huang et al., 2010) and deletions or mutations of PHF8 are often identified in cases of X-linked mental retardation (XLMR) (Siderius et al., 1999; 2005; Koivisto et al., 2007; Abidi et al., 2007). Several studies have shown that PHF8 is required to control the expression of neuronal genes (Kleine-Kohlbrecher et al., 2010; Qiu et al., 2010; Fortscherger et al., 2010; Fortscherger and Shiekhattar, 2011); however, how PHF8 controls specific aspects of neuronal development and functions that may be relevant for the onset of the cognitive disorders remains poorly comprehended.

In mammals, the understanding of how specific factors orchestrate neurodevelopmental processes is challenged by the complexity of the nervous system, which consists of heterogeneous cellular populations comprising billions of neurons. The nematode Caenorhabditis elegans presents an excellent model to investigate these processes on a simpler and more tractable scale: its nervous system consists of 302 cells, whose morphology, function and connectivity have been extensively characterized (Sulston, 1983; White et al., 1986). Many chromatin regulators are conserved in C. elegans and important information regarding the role of these proteins in neuronal development has recently been obtained using this model system (Weinberg et al., 2013; Zheng et al., 2013; Mariani et al., 2016). PHF8 shares high homology with the C. elegans JMJD-1.2 protein, which retains both the JmJC and the PHD finger domains. However, the catalytic activity of JMJD-1.2 appears to differ from that of the mammalian counterpart, as several studies have shown its ability to demethylate not only H3K9me2, but also H3K27me2 and H3K23me2. Both in vitro and in vivo (Kleine-Kohlbrecher et al., 2010; Lin et al., 2010; Vandamme et al., 2015). In agreement with the role of PHF8 in neuronal processes, JMJD-1.2 is required for normal locomotion in C. elegans, highlighting the importance of this protein in the establishment of neuronal functionalities (Kleine-Kohlbrecher et al., 2010).

During the development of the nervous system, a multitude of attractive and repulsive cues orchestrate the migration of neuronal cells and the direction of their processes (Ayala et al., 2007; Robichaux and Cowan, 2014). In addition to well-characterized ligands (such as netrins, slits, ephrins and semaphorins), a component of the Hedgehog family of morphogens, Sonic hedgehog, has been shown to regulate neural cell migration and axon guidance in vertebrates (Jarov et al., 2003; Bourikas et al., 2005; Sánchez-Camacho and Bovolenta, 2008; Hammond et al., 2009; Yam and Charron, 2013). Following synthesis and intracellular processing, Hedgehog (Hh) ligands are secreted by the combined action of receptors [i.e. Dispatched (DISP)], diffuse in the extracellular matrix through the interaction with lipoproteins and proteoglycans (LRP2 and Glypican 6) and target cells expressing Patched receptors (PTCH1/2). The binding of Hh to Patched activates the signaling pathway in receiving cells by releasing Smoothened (SMO) inhibition and triggering a signal transduction...
cascade through the regulation of Fused (FU) and Suppressor of fused (SUFU). Ultimately, this leads to the activation of Gli transcription factors, which control the expression of Hh target genes (Guerrero and Kornberg, 2014). Hh can also activate an alternative pathway (Jenkins, 2009) that is Gli- and, in some cases, SMO-independent, that leads to transcription-independent responses. Interestingly, this non-canonical Hh pathway regulates neural cell migration and axon guidance by modulating actin cytoskeleton reorganization (Bijlsma et al., 2007; Yam et al., 2009; Sasaki et al., 2010).

In the nematode, clear homologs of key components of the Hh pathway, including Hh, SMO, FU and SUFU have not been identified. Instead, the C. elegans genome encodes an abundance of what are collectively known as Hh-related proteins (WRT, GRL, GRD, QUA, HOG) based on their partial homology to domains found in the Hh proteins (Bürglin and Kuwabara, 2006; Kolotuev et al., 2009). Other molecules of this signaling pathway appear conserved in C. elegans, and homologs of factors required for Hh secretion (CHE-14, PTD-2/DISP), trafficking (GPIN-1/Glypicans 6, RIB-1/EXT1, RIB-2/EXTL3, LRP-1/LRP2, PHG-1/Gas1), Hh receptors (PTC-1, PTC-3/Patched) and transcription factors responsible for mediating Hh transcriptional activities (TRA-1/Gli1/3) have been identified. Ablation of some components of the Hh pathway results in defects in molting and body morphology (Zugasti et al., 2005; Hao et al., 2006a), suggesting that despite a considerable divergence some Hh functions are maintained in C. elegans.

RESULTS

jmjd-1.2 mutants display neuronal defects

To gain insight into the role of JMJD-1.2 in the nervous system, we analyzed different axon guidance and neuronal cell migration events in the jmjd-1.2 mutant allele tm3713, which carries an in-frame deletion that removes the region encoding the PHD finger domain (Kleine-Kohlbrecher et al., 2010; Lin et al., 2010). Although transcribed (Kleine-Kohlbrecher et al., 2010), the deleted locus results in loss of the JMJD-1.2 protein, as we and others could not detect JMJD-1.2 by western blot or immunofluorescence, suggesting that tm3713 is probably a null mutant (Fig. 1A,B) (Lin et al., 2010). Using a specific antibody, JMJD-1.2 appears expressed in many, if not all, nuclei (Fig. 1B, Fig. S1A), confirming the expression pattern identified analyzing the expression of the transgene oyIs14.

Here, we show that the catalytic activity of JMJD-1.2 is required in the developing nervous system and hypodermis of C. elegans embryos to ensure correct axon guidance of specific neurons. Genome-wide analyses, in combination with genetic and overexpression studies, indicate that the defective axon guidance observed in jmjd-1.2 mutants stems from the dysregulated transcription of a set of genes that includes two Hh-related molecules. Genetic analyses indicate that the axon guidance defects in jmjd-1.2 mutant animals or that are caused by ectopic expression of Hh-related genes depend on actin remodelers, suggesting that the Hh-like pathway controls axon guidance by regulating actin dynamics.
expression of a GFP-tagged JMJD-1.2 construct (Kleine-Kohlbrecher et al., 2010).

Using transgenic animals carrying fluorescent markers for specific neurons, we found that loss of JMJD-1.2 led to the aberrant migration of axons projected by specific pairs of neurons, namely PVQs and HSNs. In wild-type animals, PVQ cellular bodies are located in the lumbar ganglia and their projections run along two distinct bundles of the ventral nerve cord. In *jmjd-1.2(tm3713)* mutant animals, PVQ neurons are born and positioned correctly, but their axons fail to maintain the correct trajectory and aberrantly cross over the ventral midline in 22% of the population (Fig. 1C,D). A similar phenotype was identified in a null mutant generated by the CRISPR/Cas9 system (Fig. S1B). Axonal defects were also observed in the HSN neurons (Table 1, Fig. S1C,D). Occasionally, the HSN cellular bodies, which are normally located in the vulva region, undermigrated, suggesting an additional role for *jmjd-1.2* in neuronal cell body positioning. Moreover, in *jmjd-1.2(tm3713)* mutants, the expression of the *evls82h* transgene, which is specifically expressed in DA/DB motoneurons, was often reduced in the body and projection of DB5 (Table 1, Fig. S1E,F). Transgenic animals carrying constructs expressed in other neurons, however, showed normal patterning of axon migration (Table 1), suggesting that loss of *jmjd-1.2* does not affect the whole architecture of the nervous system.

**JMJD-1.2 acts during embryogenesis in nervous system and hypodermis to ensure correct axon guidance**

To study the function of JMJD-1.2 in axon guidance, we focused on the analysis of the PVQs, well-studied interneurons that fully develop during embryogenesis. To test whether the PVQ axonal defects observed in *jmjd-1.2* mutants occur in embryogenesis or during larval development, we analyzed the PVQ neurons of freshly hatched L1 mutant larvae. As shown in Fig. 2A, the penetrance of the phenotype at this stage was comparable to that of L4 animals, suggesting that the defects arise during embryonic development and are not related to defective maintenance of axon pathfinding (Aurelio et al., 2002; Pocock et al., 2008; Bénard et al., 2012).

Transgenic expression of a translational fusion between the *jmjd-1.2* genetic locus and GFP could rescue the PVQ defects observed in *jmjd-1.2* mutants at L1 and L4 stages. (A) Quantification of PVQ axonal crossover defects in *jmjd-1.2(tm3713)* mutants at L1 and L4 stages. *n > 100. (A-C) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant (one-way ANOVA followed by Tukey’s multiple-comparison test). Three independent lines for each transgene (indicated by #) were analyzed.

### Table 1. Summary of phenotypes in *jmjd-1.2(tm3713)* mutants

<table>
<thead>
<tr>
<th>Neurons examined (marker used)</th>
<th>Defective animals (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphid neurons (Dil)</td>
<td>WT</td>
<td>0</td>
</tr>
<tr>
<td>VNC neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interneurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVK interneurons (bwts2)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>AVG interneurons (ots182)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PVQ interneurons (hdls26)</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>PVQ interneurons (oyls14)</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Motoneurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSN motoneurons (zdls13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axon guidance</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>Cell migration</td>
<td>5</td>
<td>34</td>
</tr>
<tr>
<td>D-type motoneurons (ots12)</td>
<td>31</td>
<td>50</td>
</tr>
<tr>
<td>Midline left/right choice</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DA/DB motoneurons (evls82b)</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>Sensory neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE neurons (lqls2)</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Tail neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phasmid neurons (Dil)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Neurons were examined using the indicated transgenic markers and the diffusible dye DiI. Statistical significance of the difference between wild type and mutants was assessed with Fisher’s exact test. *n > 100. Animals were considered defective if: *neurons were not stained with Dil; †axons crossed over the VNC; ‡neuronal cells were misplaced; §the commissure of at least one motoneuron extended on the wrong side of the body; ¶transgene expression was reduced in DB5; **transgene expression was absent in DVB; ††axons failed to reach the VNC.
in tm3713, confirming that the axon guidance phenotype is due to loss of JMJD-1.2 (Fig. 2B, Table S1). Previous studies have shown that jmjd-1.2 is expressed at all developmental stages and in multiple tissues, including neurons, muscles, intestine and hypodermis (Kleine-Kohlbrecher et al., 2010), a spatiotemporal expression confirmed by immunostaining with a specific antibody (Fig. 1B).

To uncover the focus of action of JMJD-1.2 in the context of PVQ axon guidance, we re-expressed GFP-tagged JMJD-1.2 in the tm3713 background using a panel of tissue-specific promoters (Fig. 2C, Fig. S2, Table S1). We drove jmjd-1.2 expression under the control of either rab-3 (nervous system) or dpy-7 (hypodermis) promoters and found significant rescue of PVQ defects in each case, although the rescue effect was slightly enhanced by re-expressing JMJD-1.2 in both tissues simultaneously (Fig. 2C). By contrast, we could not rescue the phenotype by expressing jmjd-1.2 under the myo-3 (body wall muscle) promoter, showing that the presence of JMJD-1.2 in muscle cells is not required for axon guidance (Fig. 2C). In agreement with the requirement of JMJD-1.2 in multiple tissues, driving its expression in the PVQ or in the pioneer PVP neurons was not sufficient to restore the normal guidance of PVQs, suggesting that JMJD-1.2 does not work cell-autonomously nor in PVPs (Fig. S3). Altogether, these data strongly suggest that JMJD-1.2 controls the guidance of PVQs in both nervous system and hypodermis.

The JmjC and PHD finger domains of JMJD-1.2 are essential for correct axon guidance

JMJD-1.2 is structured around two evolutionarily conserved domains: the JmjC domain and the H3K4me3-binding PHD finger (Kleine-Kohlbrecher et al., 2010; Lin et al., 2010). The JmjC domain of JMJD-1.2 has been shown to demethylate H3K9me2, H3K27me2 and H3K23me2 in vitro and in vivo and, accordingly, increased signal of these post-translational modifications is found in the tm3713 mutant strain (Kleine-Kohlbrecher et al., 2010; Lin et al., 2010; Vandamme et al., 2015). To test the relevance of JMJD-1.2 enzymatic activity in axon guidance, we mutated the JmjC domain (Fig. 3A) by substituting two conserved amino acids required for the catalytic activity (Klose et al., 2006; Kleine-Kohlbrecher et al., 2010), and performed rescue experiments by re-expressing the inactive form of JMJD-1.2 (JMJD-1.2_JmjCmut) in the tm3713 background. Whereas wild-type JMJD-1.2 could restore normal PVQ guidance, the mutated protein could not (Fig. 3B, Table S1), revealing the essential role of JMJD-1.2 catalytic activity in axon guidance. Interestingly, mutations affecting the demethylase activity of the human homolog PHF8 are linked to neurological disorders (Koivisto et al., 2007; Loenarz et al., 2010; Qiu et al., 2010). Among others, the missense mutation c.836C>T encodes an F279S variant of the protein that is associated with mild XLMR and dysmorphic features (Koivisto et al., 2007). This mutation resides in the JmjC domain and has been shown to disrupt the catalytic activity of the protein (Loenarz et al., 2010; Qiu et al., 2010). As the region is highly conserved in C. elegans, we were able to generate a version of JMJD-1.2 bearing a similar mutation (JMJD-1.2_XLMR, Fig. 3A). When re-expressed in the tm3713 background, this mutant protein did not rescue PVQ axon guidance defects (Fig. 3B, Table S1).

Besides the JmjC domain, JMJD-1.2 carries a PHD finger that, as in the mammalian counterpart, has been shown to mediate binding to H3K4me3 and to contribute to demethylase activity both in vitro and in vivo (Lin et al., 2010). To assess the role of this domain in axon guidance, we generated a mutation in a specific residue that is important for the binding to H3K4me3 and for the catalytic activity of JMJD-1.2 (Lin et al., 2010; Yang et al., 2010). As with the catalytically inactive protein, the enzyme bearing a mutated PHD finger (JMJD-1.2_PHDmut) could not rescue PVQ axon guidance (Fig. 3B, Table S1). Previous studies have shown that loss of JMJD-1.2 is associated with dysregulated gene transcription (Lin et al., 2010). As jmjd-1.2 expression is important during embryogenesis, we analyzed the transcriptome of jmjd-1.2(tm3713) mutant embryos by RNA sequencing (RNA-seq). Notably, only 22 genes displayed expression changes of more than 1.5-fold between wild type and mutants, of which 16 could be further validated by qPCR (Table 2).
expression of developmental stages, for example in L1, providing an important effect was not observed when the expression was forced at later defects that were similar to those associated with loss of JMJD-1.2, transiently induced during embryogenesis, we observed PVQ axon heat shock-inducible reproduce the PVQ guidance defects observed in overexpression of similar to the vertebrate counterpart, might play a key role in axon correction (Fig. 4A, Table S2). Interestingly, both et al., 2006b), raising the possibility that grl-16 we generated double mutants with phenotype. As viable mutants for some of these genes are available, although the Hh pathway has undergone a prominent divergence axonal defects depend on Hh signaling Although the Hh pathway has undergone a prominent divergence during evolution, some key molecules of the canonical Hh signaling are conserved in C. elegans (Bürglin and Kuwabara, 2006; Kolotuev et al., 2009) (Table S3) and are involved in the trafficking of proteins and sterols, similar to the vertebrate counterparts (Kuwabara et al., 2000; Hao et al., 2006b; Soloviev et al., 2011). As the defects observed in jmjd-1.2 mutants are related to increased expression of Hh-related genes, we speculated that a negative regulation of the Hh pathway could ameliorate the jmjd-1.2 phenotype. Homologs of genes required for Hh release (DISP/cle-14) and propagation of the signal (Glypican 6/gpn-1, EXT1/rib-1 or EXTL3/rib-2) have been identified in C. elegans. We analyzed the effect of reduction by RNA interference (RNAi) or deletion (using mutants when available) of these conserved genes in the jmjd-1.2 background and invariably observed a significant amelioration of the jmjd-1.2 axon guidance phenotype (Table 3). Similarly, we predicted that reduction of Hh-like receptors and coreceptors (Patched/PTC-1/PTC-3, Megalin/LRP2/LRP-1, Gas1/PHG-1) could also influence the jmjd-1.2 phenotype. Strikingly, reduction of lrp-1, phg-1 and ptc-1, but not ptc-3, in jmjd-1.2 mutants also resulted in significant amelioration of the phenotype, further supporting a causal role of dysregulated Hh signaling in the defective axon guidance of the jmjd-1.2 mutant (Table 3). By contrast, reduction of homologs of skn/hhat-1/hhat-2, required for palmitoylation of Hh, failed to significantly ameliorate the phenotype, similar to the ablation of grl-7, another Hh-related protein that was upregulated in our RNA-seq data. Overall, the genetic ablation or reduction by RNAi of several conserved components of the Hh pathway from the tm3713 background is sufficient to rescue the PVQ defects associated with loss of jmjd-1.2. That the defects observed in jmjd-1.2 mutants can be rescued by its ectopic expression in hypodermis or neurons suggests that the components of the Hh pathway might be dysregulated in multiple tissues and that the re-expression of jmjd-1.2 in hypodermis or neuron is sufficient to restore a correct level of Hh signaling and therefore proper axon guidance.

All together, these findings strongly suggest that aberrant activation of Hh signaling is responsible for the defects observed in jmjd-1.2 mutants. Furthermore, our data provide the first evidence that some evolutionarily conserved proteins of the Hh signaling pathway act in axon guidance in C. elegans.

**Implication of actin dynamics in jmjd-1.2 defects**

Many studies in several model organisms, including C. elegans, indicate that axon growth and guidance are ultimately regulated by actin cytoskeleton remodeling at growth cones (Kalil and Dent, 2005; Quinn and Wadsworth, 2008; Dent et al., 2011; Chia et al., 2014; Gomez and Letourneau, 2014). We therefore postulated that JMD-1.2 might regulate actin dynamics and tested this by systematically ablating several conserved actin regulators in the jmjd-1.2 genetic background and further analyzing the axon migration of PVQ neurons. We generated double mutants carrying the jmjd-1.2 deletion together with mutations in well-established actin regulator genes such as wisp-1/WASP, wee-1/WAVE, unc-34/Ena/VASP, cdc-42/CD42, mig-2/RAC, nck-1/NCK and wip-1/WIP. Whereas the removal of wee-1, mig-2, unc-34 or cdc-42 was ineffective, loss of wisp-1 and its regulators wip-1 and nck-1 resulted in a significant rescue of the jmjd-1.2 phenotype (Fig. 4E), strongly suggesting that the jmjd-1.2 axonal defects depend on aberrant actin remodeling mediated by wisp-1. Of note, none of the actin regulators is apparently dysregulated at the transcriptional level in jmjd-1.2 mutant animals, as indicated by RNA-seq analysis, suggesting that Hh signaling in C. elegans might regulate axon migration in a transcription-independent manner. Importantly, overexpression of grl-16 or wrt-8 in a wisp-1 mutant background was unable to induce PVQ axon guidance defects, suggesting that the axonal defects of jmjd-1.2 mutants depend on

**Table 2. Genes with dysregulated expression in jmjd-1.2(tm3713) mutants**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>qPCR</th>
<th>Allele</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C38D9.2</td>
<td>5.81</td>
<td>No</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>wrt-8</td>
<td>4.08</td>
<td>Yes</td>
<td>tm1585</td>
<td>Hh-like protein</td>
</tr>
<tr>
<td>F15E6.10</td>
<td>3.68</td>
<td>Yes</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>clec-230</td>
<td>3.13</td>
<td>Yes</td>
<td>ok3131</td>
<td>C-type lectin</td>
</tr>
<tr>
<td>asp-6</td>
<td>2.05</td>
<td>Yes</td>
<td>tm2213</td>
<td>Predicted protease</td>
</tr>
<tr>
<td>grl-7</td>
<td>2.05</td>
<td>Yes</td>
<td>ok2644</td>
<td>Hh-like protein</td>
</tr>
<tr>
<td>C02E7.7</td>
<td>1.99</td>
<td>No</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>C08B8.4</td>
<td>1.91</td>
<td>Yes</td>
<td></td>
<td>Predicted chitinase activity</td>
</tr>
<tr>
<td>F10D11.6</td>
<td>1.90</td>
<td>Yes</td>
<td></td>
<td>Predicted lipid binding</td>
</tr>
<tr>
<td>grl-16</td>
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<td>Yes</td>
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<td>asp-1</td>
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<td>Yes</td>
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<td>Predicted protease</td>
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<tr>
<td>lgc-22</td>
<td>1.83</td>
<td>Yes</td>
<td></td>
<td>Ligand-gated ion channel</td>
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<tr>
<td>ZK180.5</td>
<td>1.82</td>
<td>Yes</td>
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<tr>
<td>bcl-11</td>
<td>1.81</td>
<td>Yes</td>
<td></td>
<td>Homology to BCL11A</td>
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<tr>
<td>Y47D7A.13</td>
<td>1.79</td>
<td>n.t.</td>
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<tr>
<td>nep-17</td>
<td>1.77</td>
<td>Yes</td>
<td>ok3251</td>
<td>Predicted metallopeptidase</td>
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<tr>
<td>cut-3</td>
<td>1.72</td>
<td>Yes</td>
<td>ok1819</td>
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<td>F41F3.3</td>
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<td>cut-2</td>
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<td>Heat shock protein</td>
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</table>

List of genes found to be dysregulated by RNA-seq in jmjd-1.2(tm3713) compared with wild-type embryos. A filter of 1.5-fold difference and FDR correction (P<0.05) were applied. Validation by qPCR, available mutant alleles and gene functions are indicated; n.d., not determined; n.t., not tested.

jmjd-1.2 axonal defects depend on Hh signaling

Although the Hh pathway has undergone a prominent divergence during evolution, some key molecules of the canonical Hh signaling are conserved in C. elegans (Bürglin and Kuwabara, 2006; Kolotuev et al., 2009) (Table S3) and are involved in the trafficking of proteins and sterols, similar to the vertebrate
Hh-mediated actin regulation (Fig. 4F). This hypothesis is in agreement with previous studies reporting that Hh triggers a transcription-independent signaling cascade that acts rapidly and locally at the growth cone to regulate actin-based structure dynamics and axon migration (Charron and Tessier-Lavigne, 2005; Bijlsma et al., 2007; Yam et al., 2009; Sasaki et al., 2010). Altogether, these experiments suggest that jmjd-1.2 regulates Hh signaling and actin reorganization at the growth cone of migrating neurons.

DISCUSSION
In this study, we have found that jmjd-1.2 regulates some aspects of neuronal development, including axon guidance (PVQs and HSNs) and neuronal cell body migration (HSNs). As the axon migration in PVQs and HSNs occurs during embryonic and larval stages, respectively, these results suggest a regulatory role for jmjd-1.2 across development. We have also observed reduced expression of the transgene evlsb2b, carrying the terminal selector gene unc-129, in the DB5 motoneuron, supporting a function of jmjd-1.2 in the process of neuronal differentiation. Interestingly, this deficiency might contribute to the locomotion defects previously described in jmjd-1.2 mutants (Kleine-Kohlbrecher et al., 2010).

Notably, the catalytic activity of JMJD-1.2 is fundamental to ensure correct axon guidance. Given that this demethylase is able to remove the dimethyl mark from different lysine residues on histone H3, we cannot, at the moment, link the observed neuronal defects to a specific activity of JMJD-1.2 on one or more residues. In addition, our results indicate that the correct recruitment of JMJD-1.2 to
PVQ defects in *+RNAi control* (Hh-related genes) changes in the transcriptome of Fortscheberger et al., 2010). By RNA-seq, we identified minor expression (Lin et al., 2010; Kleine-Kohlbrecher et al., 2010; Fortscheberger et al., 2010). Among the upregulated genes, we identified those encoding the Hh-related proteins WRT-8 and GRL-16, and showed that their transient overexpression in wild-type embryos induces axon guidance defects similar to those identified in *jmjd-1.2* mutant animals. This strongly suggests that dysregulation of wrt-8 and grl-16 is causal for the *jmjd-1.2* axonal phenotype. Further, we have shown that loss or reduction of genes encoding homologs of components of the Hh pathway, including wrt-8 and grl-16, is sufficient to rescue the *jmjd-1.2* defects. These results support a previously unappreciated role of Hh-like signaling in axon guidance in *C. elegans*. Two other genes were found to be upregulated in *jmjd-1.2* mutant animals, and their loss was sufficient to restore normal axon guidance in *jmjd-1.2* mutants: clec-230, which encodes a protein of 179 amino acids that contains a lectin domain, a carbohydrate-binding module often found in proteins with functions in adhesion (Drickamer and Dodd, 1999); and cut-3, which encodes a component of the worm cuticle and is specifically expressed in the embryo (Sapió et al., 2005). Neither cut-3 nor clec-230 has clear homologs in mammals and they were not investigated further. Although it is possible that these genes are Hh transcriptional target genes, it is also conceivable that, being molecules implicated in adhesion and extracellular matrix composition, they could play a role in Hh diffusion.

The role of the Hh pathway in axon guidance remains poorly understood, but some studies indicate that in this context Hh triggers an alternative, transcription-independent signaling cascade that acts rapidly and locally at the growth cone to induce actin cytoskeleton remodeling (Charron and Tessier-Lavigne, 2005; Bijlsma et al., 2007; Yam et al., 2009; Sasaki et al., 2010). Interestingly, our genetic interaction analyses suggest that molecules regulating actin dynamics play a role in the establishment of aberrant axonal migration in *jmjd-1.2* mutants. In support of this, it has previously been reported that downregulation of PHF8 results in cytoskeleton disorganization and in cell adhesion defects (Asensio-Juan et al., 2012). It is possible that altered actin dynamics counteract the effects of increased Hh signaling by regulating Hh trafficking and secretion. However, based on our previous findings that misexpression of actin regulators in PVQ neurons causes aberrant axon guidance (Mariani et al., 2016) and on our overexpression experiments in the ***wsp-1*** mutant background, we favor a hypothesis (Fig. 5) in which JMJD-1.2 modulates the expression of Hh-related proteins in neuronal and hypodermal cells, which, after secretion, activate a signaling pathway controlling actin remodeling and axon guidance in target neurons. How dysregulation of the Hh pathway impacts actin dynamics, thereby leading to incorrect axon guidance, is a key aspect that remains to be investigated. As we did not identify any transcriptional dysregulation of actin regulators in *jmjd-1.2* mutants by RNA-seq, it is tempting to speculate that JMJD-1.2 might modulate the activity and/or cellular localization of actin regulators. Similarly, the tissue specificity of Hh signaling components remains unknown and further analyses will be required to identify not only the specific cells secreting the ligands (signaling cells) but also those expressing Hh receptors (receiving cells). Theoretically, despite being obtained in
**C. elegans**, our results suggest that an aberrant Hh pathway could be related to XLMR and other cognitive diseases, such as intellectual disability (ID) or autism spectrum disorder (ASD). Supporting this possibility, the Hh acyltransferase HHAT has been identified as mutated in whole-exon sequencing and proposed as a candidate gene for ID (Agha et al., 2014). Similarly, PTC1D1, which shares high homology with the Patched receptors, has been suggested as a candidate gene for ASD and ID (Noor et al., 2010; Filges et al., 2011; Chaudhry et al., 2015).

We recently identified RBR-2, an H3K4me3/2 histone demethylase, as being implicated in the regulation of axon guidance. rbr-2 is the sole nematode homolog of the human XLMR gene JARIDIC (KDM5C), and controls axon guidance in a similar subset of neurons by reducing the expression of actin-related genes, including wsp-1 (Mariani et al., 2016). The fact that rbr-2 and jmjd-1.2 share similar phenotypes when ablated, are both required during embryogenesis and bind H3K4me3 through their PHD domains, suggests that these histone demethylases might regulate axon guidance in a concerted manner. Indeed, we previously reported that rbr-2/jmjd-1.2 double mutants show similar levels of PVQ defects as the single mutants (Mariani et al., 2016), confirming that rbr-2 and jmjd-1.2 act in a common pathway regulating axon guidance. Altogether, these findings confirm that epigenetic factors are essential for the correct execution of developmental processes, providing novel insights into how neuronal development is achieved.

In conclusion, our study reveals a novel function of jmjd-1.2 in axon guidance and provides the first evidence that the Hh-like pathway, as conserved in **C. elegans**, contributes to the correct establishment of axon migration.

**MATERIALS AND METHODS**

**Genetics and strains**

**C. elegans** strains were cultured using standard growth conditions at 20°C on Escherichia coli OP50 (Brenner, 1974). Strains were as follows: wild-type Bristol: N2; jmjd-1.2(tm3713) I; CV3334: oys141[prsa-6::GFP+ lin-15+] V; MU1085: bvdx2[Ppfp-1::GFP+](pRF4)rol-6(sa1006); OH4887: ods182[Pinx-18::GFP+]; VH468: hds62(Podr-2::CFP); Psa-6::DsRed2) III; SK4013: zdls13[Ppfr-1::GFP+]; EV1285: oxs121[Punc-47:: GFP+]; lin-15+] X; NW1100: evl-20&cut-3(ok1819)/mIn1[mIs14 dpy-20(e128)].

**Generation of transgenic constructs**

The jmjd-1.2::GFP construct, which includes a 4715 bp fragment containing 786 bp of promoter and the entire genetic locus of jmjd-1.2, has been described previously (Kleine-Kohlbrecher et al., 2010). Plasmids carrying the jmjd-1.2 gene fused to GFP and under the control of specific promoters were constructed using the MultiSite Gateway Three-Fragment Vector Construction Kit (Life Technologies). The constructs used in Fig. 3B and Fig. 4B,D,F were generated as described in the supplementary Materials and Methods. The DNA sequences of all constructs were verified by sequencing.

**Microinjection and production of transgenic lines**

Transgenic lines were obtained through microinjection (Mello et al., 1991). Constructs were injected into young adult hermaphrodites as extrachromosomal arrays at 10-50 ng/µl with Ptx5-3::RFP (50-90 ng/µl) or Pmyo-2::mCherry (0.5-5 ng/µl) as injection markers.

**Fluorescence microscopy**

For all transgenic markers, neuronal phenotypes were scored in L4 and young adult hermaphrodites grown at 25°C. Images were taken using an automated fluorescence microscope (Zeiss AXIO Imager M2) and Micro-Manager open source software (version 1.4.11). All pictures were exported using Photoshop (Adobe).

**DII staining of amphid and phasmid neurons**

Staining was performed as described previously (Mariani et al., 2016).

**Heat shock experiments**

For overexpression experiments, embryos or L1 larvae were heat shocked at 37°C for 30 min each. After heat shock, worms were kept at 25°C overnight and L2/L3 larvae were analyzed by fluorescence microscopy the following day.

**RNAi analysis**

RNAi by feeding was carried out as described previously (Timmons et al., 2001). Constructs were obtained from the **C. elegans** RNAi Feeding Library (J. Ahringer’s laboratory, Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK) except for ptc-1, ptc-3, tra-1 and phg-1, which were cloned into pCR2.1 TOPO vector using the TOPO TA Cloning Kit (Life Technologies). Empty L4440 vector was used as negative control. F2 progeny was analyzed for axon guidance defects at L4 stage. Three individual plates were scored for each variable, in three independent experiments. We observed a decreased percentage of defects in jmjd-1.2(tm3713) and wild-type animals treated with L4440 as compared with jmjd-1.2(tm3713) mutants (Table 3), suggesting that the phenotype measured is, for unclear reasons, sensitive to the bacterial strain used as a source of food. However, jmjd-1.2(tm3713) mutants treated with L4440 still have a significantly increased percentage of defects compared with wild-type animals grown under similar conditions (P<0.0002).

**RNA-seq**

Gravid hermaphrodites cultured at 25°C were treated with hypochlorite solution and embryos were flash-frozen in liquid nitrogen and stored at −80°C before RNA extraction. RNA was isolated from two independent cultures using TRIzol reagent (Life Technologies) and the NEnasy Mini Kit (Qiagen). RNA amplification and sequencing were performed by the Beijing Genomics Institute (BGI).

**RNA-seq data analysis**

Barcode and adaptor-cleaned sequences were checked for quality using FastQC (Babraham Bioinformatics) and mapped to the C. elegans genome (WS220) with TopHat 2.0.9 (Trapnell et al., 2012) using parameters described previously (Petlona et al., 2013). Reads successfully mapped were >95% using a criterion of two mismatches. The number of reads processed and percentage aligned were: wild-type replicate 1, 43.2 M, 95.8%; wild-type replicate 2, 37.8 M, 96.0%; jmjd-1.2 replicate 1, 39.9 M, 96.0%; jmjd-1.2 replicate 2, 36.3 M, 95.8%; wild-type replicate 3, 34.2 M, 95.8%; wild-type replicate 4, 33.8 M, 95.8%; wild-type replicate 5, 33.2 M, 95.8%; wild-type replicate 6, 32.8 M, 95.8%; and wild-type replicate 7, 32.4 M, 95.8%.
95.8%; jmjd-1.2 replicate 2, 34.7 M, 95.9%. Mapped reads were analyzed for transcript assembly and differential expression using Cufflinks 2.1.1 (Trapnell et al., 2012) with a filter of 1.5-fold difference and FDR correction (P<0.05). Differentially expressed genes were used for gene set enrichment analysis (GSEA) performed using DAVID 6.7 (Huang da et al., 2009).

Real-time quantitative PCR (qPCR)
RNA isolation, cDNA synthesis and qPCR were performed as described previously (Mariani et al., 2016). The measures were normalized to Y45F11D.4 (Zhang et al., 2012). All reactions were performed in duplicate, in two independent experiments.

Generation of antibody against JMJD-1.2
A polyclonal antibody against JMJD-1.2 was generated by Innovagen AB (IDON Gamma Rec., Lund, Sweden), by immunizing rabbits with a purified bacterial GST-tagged fragment of the JMJD-1.2 protein encoded by the first four exons of the gene. The antibody was affinity purified and validated by western blot on lysate from mutant animals.

Western blot analysis
Whole-worm lysates for SDS-PAGE were prepared by boiling mixed-stage worms in SDS-PAGE loading buffer for 5 min. Protein concentration was estimated using the modified micro-Lowry assay. The following antibodies were used: rabbit polyclonal anti-JMJD-1.2 (Innovagen; 1:1000); mouse monoclonal anti-actin (Millipore, MAB1501; 1:15,000); peroxidase-labeled anti-rabbit and anti-mouse secondary antibodies (Vector Labs, PI-2000; 1:10,000).

Immunofluorescence
Whole worms were stained according to Finney and Ruvkun (1990). Primary antibody [polyclonal anti-JMJD-1.2 (Innovagen)] was incubated overnight at 4°C in a humid chamber and secondary antibody [donkey anti-rabbit IgG (Alexa Fluor 594, Invitrogen, A21207)] was incubated for 1 h at room temperature. Washes were in PBS/0.2% Tween 20. Vectashield H1200 mounting medium for fluorescence with DAPI was used to counterstain DNA.

Statistical analyses
All phenotypes were scored as percentages of defective animals among total observed. Statistical analyses were performed in GraphPad Prism 6 using Fisher’s exact test for pairwise comparisons, or one-way ANOVA followed by Tukey’s multiple comparison test for multiple comparisons. In rescue and overexpression experiments, significance was calculated in relation to non-transgenic controls for each transgenic line. As the penetrance of defects in non-transgenic siblings was always consistent with the phenotype of jmjd-1.2(tm3713), these data are not shown in Fig. 2B,C, Fig. 3B and Fig. S3C. qPCR data were compared using Student’s t-test. P<0.05 was considered significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.R.R., L.M., E.M. and P.G.A. carried out the experimental work. J.P. and G.W. analyzed the RNA-seq data. A.R.S., L.M., E.M., P.G.A. and A.E.S. designed the experiments and analyzed the data. L.M. and A.E.S. wrote the manuscript.

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Data availability
RNA-seq data are available at the NCBI Sequence Read Archive (SRA) public database with the following accession numbers: BioProject, PRJNA354814; BioSample, SAMN06052359; Data, SRR5051663, SRR5051664, SRR5051665, SRR5051665.

Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.142695.supplemental

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