Regulators of H3K4 methylation mutated in neurodevelopmental disorders control axon guidance in *C. elegans*.

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ABSTRACT

Post-translational histone modifications regulate chromatin compaction and gene expression to control many aspects of development. Mutations in genes encoding regulators of H3K4 methylation are causally associated with neurodevelopmental disorders characterized by intellectual disability and deficits in motor functions. However, it remains unclear how H3K4 methylation influences nervous system development and contributes to the aetiology of disease. Here, we show that the catalytic activity of set-2, the C. elegans homolog of the H3K4 methyltransferase KMT2F/G (SETD1A/B) genes, controls embryonic transcription of neuronal genes and is required for establishing proper axon guidance and for neuronal functions related to locomotion and learning. Moreover, we uncover a striking correlation between components of the H3K4 regulatory machinery mutated in neurodevelopmental disorders and the process of axon guidance in C. elegans. Thus, our study supports an epigenetic-based model for the aetiology of neurodevelopmental disorders, based on aberrant axon guidance process originating from deregulated H3K4 methylation.

INTRODUCTION

Development of the nervous system requires the coordination of several events, including neuronal progenitor self-renewal, cell migration and differentiation along different lineages, directional neurite outgrowth, and synapse formation. During each step, tight transcriptional control of neurodevelopmental genes is crucial, with chromatin factors playing a major regulatory function by controlling chromatin accessibility. The contribution of chromatin factors to synaptic plasticity, learning, and memory testifies the broad role of epigenetic mechanisms in the formation and functionality of the nervous system.
The relevance of chromatin factors in nervous system development is highlighted when considering neurodevelopmental disorders, conditions characterized by intellectual disability where social/motor and learning skills are variably affected. Advances in next generation sequencing have allowed a thorough analysis of individuals affected by neurodevelopmental disorders, generating valuable insights for inferring the molecular basis of these diseases. Strikingly, chromatin regulators have emerged as the second-most associated category, outside genes directly involved in synaptic function. In particular, regulators of histone 3 lysine 4 (H3K4) methylation are well-represented among mutated chromatin factors. The levels of H3K4 methylation are dynamically regulated by the action of lysine methyltransferases (KMTs), the majority belonging to the KMT2 family (KMT2A-D, or MLL1-4, and KMT2F/G, or SETD1A/B) and lysine demethylases (KDMs) of the KDM1 and KDM5 families. KMT2 members are the catalytic subunits of COMPASS-like complexes (COMplex of Proteins ASsociated with Set-1) that include WDR5, RBBP5, ASH2L, DPY30 as core components, and are required for optimal catalytic activity of each complex.

Mutations in KMT2 members have been identified in cases of Wiedemann-Steine syndrome, Kleefstra syndrome, Kabuki syndrome, and are associated with schizophrenia, autism, and neurodevelopmental disorders. KDM1 and KDM5 members are found mutated in autism spectrum disorders, X-linked mental retardation, non-syndromic intellectual disability and Kabuki syndrome. Finally, PHF8, a H3K4me3 binder is altered in cases of X-linked retardation. Taken together, these results strongly suggest that tight control of H3K4 methylation is crucial for brain development and functionality and that its deregulation is implicated in the pathogenesis of neurodevelopmental disorders. However, the roles of the H3K4 regulatory machinery in key aspects of neuronal development remain poorly characterized. In particular, how H3K4 methylation impacts axon guidance, a process required to direct the
axons to their targets and establishing functional neuronal circuits is unknown. Investigation of this process is limited by the complexity of the mammalian nervous system and by the inadequacy of in vitro systems to reproduce physiological conditions. Thus, in vivo studies in tractable model organisms could help to dissect the role of histone methylation in this highly-conserved biological process.

_Caenorhabditis elegans_, in which the H3K4 methylation machinery is well-conserved, is an amenable model system for studying neurodevelopmental mechanisms. Factors such as a well-defined neuronal connectome and a simple body plan make this organism ideal for unveiling the roles of chromatin factors and to assess functional relevance of genetic variations observed in neurodevelopment diseases. In _C. elegans_, the process of axon guidance can be studied by following the trajectory of PVQ axons which run along the entire animal body in a stereotyped manner. Due to this invariant pattern of development, the PVQs have been used to identify genes and pathways implicated in axon guidance. In this study, we have directly tested the role of H3K4 methylation in regulating axon guidance by analysing mutant animals lacking the majority of known H3K4 regulators. The results show that H3K4 methylation regulation is strictly required for the establishment of axon trajectories and that the deposition of methylation on H3K4 is crucial for neuronal functions related to locomotion and learning.

RESULTS

Multiple regulators of H3K4 methylation are required for axon guidance

The PVQs are a pair of interneurons located at the posterior region of the animal with axons projecting anteriorly during mid-embryogenesis along the ventral nerve cord in two distinct and parallel bundles, separated by the ventral midline (Fig. 1A). To test the hypothesis that regulation of H3K4 methylation is relevant in the establishment of proper axon guidance, transgenic animals expressing a GFP reporter in PVQ neuronal cell bodies
and axons were crossed with deletion mutants of components of the H3K4 regulatory machinery. Based on H3K4-related functions (Table 1), we included in our analysis alleles for set-2, set-16, set-17, set-30, previously reported to act as H3K4 methyltransferases 54-56. We also tested mutant alleles for a H3K4 demethylase, spr-5 57, and for genes encoding for components of the COMPASS-like complexes 58-60, wdr-5.1, rbbp-5 and ash-2. Mutants for the H3K4 demethylase rbr-2 and for the H3K4 binder jmjd-1.2 were used as positive controls for phenotypic changes 52,53. Deletion mutants for set-2, set-16, spr-5, wdr-5.1, ash-2 and rbbp-5 display defects in PVQ axon guidance (Fig. 1B), resulting in aberrant midline crossover of the axons often occurring in the posterior part of the body (Fig. 1A). The axonal defects observed in all mutants are noticeably similar in terms of pattern and penetrance. In contrast, we found that deletions of set-17 and set-30 did not compromise the PVQ patterning (Fig. 1B). Interestingly, while human homologues of set-2 (KMT2F/G), set-16 (KMT2A-D), spr-5 (KDM1A), rbr-2 (KDM5A-D), jmjd-1.2 (PHF8), ash-2 (ASH2L), wdr-5.1 (WDR5) and rbbp-5 (RBBP5) are mutated in neurodevelopmental diseases (Table 1), no alterations have been reported for the homologues of set-17 and set-30, corresponding to PRDM7/9 and KMT3C, respectively, previously reported to methylate H3K4 and H3K36 61-65. Thus, our analysis reveals that the majority of H3K4 methylation regulators in C. elegans contribute to the establishment of correct axon guidance, indicating that the regulation of H3K4 methylation is crucial in this process. More importantly, these results highlight a striking and previously unknown correlation between genes regulating H3K4 methylation mutated in neurodevelopmental diseases and genes involved in axon guidance in C. elegans.
SET-2 controls axon guidance of a subset of neurons

To gain insight into the molecular mechanisms underlying the axonal defects observed in H3K4 regulator mutants, we characterized the role of set-2 in detail. set-2 is homologous to KMT2F/G (also called SETD1a/b) which have essential roles during early mouse embryogenesis. In human, a role for KMT2F/G mutations in neurodevelopmental disorders has been recently suggested by the identification of variants in KMT2F and KMT2G in individuals with intellectual disability, autism, epilepsy, and schizophrenia. SET-2 is considered the major methyltransferase for H3K4 in C. elegans, but its role in neuronal development has not been investigated. The set-2(tm1630) and set-2(n4589) alleles carry large deletions at the 5' of the gene, including the start codon (Fig. 2A), and show similar defective axon guidance phenotypes (Fig. 2B). Furthermore, transgenic expression of a fosmid containing the set-2 gene in set-2(tm1630) allele rescues the axon guidance phenotype (Fig. 2B). These results strongly suggest that the axonal defect observed is linked to aberrations of set-2.

We investigated the focus of action of set-2 by testing the ability of set-2 expression in different tissues to rescue the defects observed in set-2(tm1630) mutants. Our results show that SET-2 acts specifically in the nervous system to control PVQ development (Fig. 2C and Fig. S1). However, re-expression of set-2 in PVQ neurons is not sufficient to rescue the phenotype (Fig. 2B). This result is consistent with a non cell-autonomous function of set-2, however, it should be noted that several technical issues (inappropriate time and/or level of expression) may also account for this negative outcome. To determine whether SET-2 is required in embryos to establish correct axon guidance, or during larval development to maintain PVQ axonal position, we analyzed the defect of PVQ axons in freshly hatched larvae. The percentage of axon defects identified in L1 is similar to the one observed in mutant adult animals (16% ± 2) (Fig.S2), suggesting that SET-2 is required during embryogenesis to ensure proper PVQ axon guidance. In agreement with this,
transgenic animals carrying a mCherry-tagged transcriptional reporter show set-2 expression in early embryo (Fig. S3). Loss of set-2 also impacts the projection of HSN neurons, which extend during larval development, and the axon trajectory of VD and DD neurons in the dorsal nerve cord (Table 2). In contrast, other neurons such as the mechanosensory neurons (AVM, ALM, PVM and PLM), and the AVK interneuron display normal axon guidance pattern in set-2 mutant animals (Table 2). Of note, abrogation of set-2 does not impact the migration of AVM, PVM and HSN neurons (Table 2). These results indicate that SET-2 regulates the projection of several neurons but is not required to organize the overall architecture of the C. elegans nervous system.

The catalytic activity of SET-2 is required to control axon guidance

SET-2 mainly catalyzes the tri-methylation of H3K4\(^{59,68}\). In agreement with this, we observed strongly reduced levels of H3K4me3 in the set-2(tm1630) mutant embryos, indicating that SET-2 is the main enzyme catalyzing H3K4me3 in embryos (Fig 3A and Fig. S4). Several point mutations in the SET domain have been shown to perturb the activity without compromising the stability of the protein\(^{69,70}\). To directly assess the relevance of the enzymatic activity of SET-2, and therefore of H3K4me3, in the context of axonal guidance, we introduced mutations in the set-2 gene giving rise to two mutated alleles set-2(zr1504) and set-2(zr2012), in which conserved amino acids located in the SET domain were mutated (H1447K and R1426W, respectively, Fig. 3A). In the set-2(zr2012) allele, we introduced a mutation leading to the same amino acid substitution found in SETD1B/KMT2G in a case of intellectual disability linked to epilepsy and autism\(^{67}\). Therefore, the set-2(zr2012) allele provided a simple model to test the effects of a disease-associated mutation of set-2 in an in vivo context. In both mutants animals we observed a strong reduction of H3K4me3 levels, similar to the one detected in the set-2(tm1630) deletion allele both by Western blot and immunofluorescence in embryos (Fig. 3A and Fig. S4). Importantly, these mutant alleles showed axonal defects with similar
penetrance to the \textit{set-2(tm1630)} deletion mutant (Fig. 3B). Therefore, our result, together with the evidence (Fig. S5) that no axon abnormalities are observed in \textit{set-2(ok952)} allele, an in-frame deletion in which the levels of H3K4me3 in embryos were not affected \textsuperscript{68}, suggests the catalytic activity of SET-2 is crucial for proper axon guidance.

\textbf{SET-2 genetic interactions with pathways regulating axon guidance}

Multiple conserved redundant pathways control axon guidance. The role of several signalling pathways like Netrin, Slit, Ephrins and Semaphorin in this context is well characterised in \textit{C. elegans}. Similarly, the relevance of transmembrane proteins like Syndecan and other proteoglycans is well-established \textsuperscript{51}. Genetic interaction assays have been used to determine the components of these pathways and to establish functional relationships amongst the involved genes \textsuperscript{46,71}. In order to assess if \textit{set-2} acts within known pathways, we generated animals carrying the \textit{set-2(tm1630)} allele together with mutations of genes belonging to the major axon guidance pathways and analysed the trajectories of the PVQ neurons. Concomitant abrogation of \textit{set-2} and components of Ephrin (\textit{vab-1}) or Semaphorin (\textit{plx-2}) pathways resulted in a phenotype whose penetrance was similar to the one observed in the single mutants (Table 3). Analogous results were obtained in \textit{sdn-1;set-2}. To the contrary, when Netrin (\textit{unc-5}) and sax-3/ROBO pathways (\textit{sax-3}) were ablated in \textit{set-2} genetic background, we observed an exacerbation of the phenotype (Table 3). Therefore, \textit{set-2} appears to act in parallel to the Netrin and sax-3/ROBO pathways and in concert with Ephrins and Semaphorin, the main antero-posterior signalling pathways involved in axon guidance.

A similar experimental approach was performed to analyse the cross-talk among the H3K4 methylation regulators we found involved in axon guidance. To investigate the relationship of \textit{set-2} with \textit{rbr-2}, \textit{spr-5} and \textit{jmjd-1.2}, we analysed the PVQ defects of animals lacking a combination of these genes. None of the double mutants showed an
additive effects (Fig. 4A), suggesting that the regulators act jointly to ensure the correct levels of H3K4 methylation and normal axon guidance. However, the abrogation of \textit{rbr-2} in the \textit{set-2(tm1630)} background led to an amelioration of the axon phenotype, suggesting that \textit{rbr-2}, likely through its H3K4 demethylase activity, can counteract the effect of \textit{set-2} in axon guidance. A similar neutralizing effect of \textit{rbr-2} mutations has been observed previously for the lifespan phenotype of \textit{set-2} \textsuperscript{56}. Finally, we analysed the penetrance of the defects in compound mutants of \textit{set-2} with \textit{rbbp-5} or \textit{ash-2}, components of the COMPASS complexes. Double mutants showed levels of defects similar to those observed in single mutants (Fig. 4B), suggesting that \textit{set-2} controls the axon guidance process in the context of the COMPASS complex.

**\textit{set-2} controls axon guidance by regulating actin remodelling through \textit{wsp-1}**

Actin remodelling at the growth cone is ultimately the key process directing axon guidance. We therefore tested if the defect observed in \textit{set-2} mutant animals could be related to aberrant regulation of actin dynamics. We generated double mutants eliminating \textit{set-2} in concomitance with \textit{wsp-1/WASP}, \textit{wve-1/WAVE}, \textit{unc-43/Ena/VASP}, known actin regulator genes\textsuperscript{72}. While we observed no effect with \textit{unc-34} or \textit{wve-1}, ablation of \textit{wsp-1} fully rescued the \textit{set-2} axon guidance phenotype (Fig. 4C). This result suggests that \textit{set-2} controls axon guidance by regulating actin remodelling specifically through \textit{wsp-1}. We also tested the effect of \textit{cdc-42} and \textit{nck-1} ablation, whose mammalian homologs are known activators of N-WASP \textsuperscript{73}. As only loss of \textit{cdc-42} rescued the \textit{set-2} phenotype (Fig 4C), we conclude that an aberrant CDC-42-dependent activation of WSP-1 is likely at the base of the axonal defect observed in \textit{set-2} mutant animals.
Transcriptional regulation mediated by SET-2

H3K4me3 is a post-translational modification identified at promoter regions of transcriptionally active genes and, in agreement with this, loss of set-2 has been reported to deregulate transcriptional activity\textsuperscript{58,74}. To gain insight into the mechanisms of action of SET-2 in axon guidance, we analysed the transcriptome of set-2(tm1630) mutants at mid-embryonic stage when PVQ axon development occurs. Principal component analysis (PCA) of RNA sequencing datasets from wild-type and set-2(tm1630) animals indicated that the gene expression patterns in set-2 mutant embryos were significantly different from wild-type embryos (Fig. 5A), with 6444 genes (FDR<0.05) differentially expressed (DE) (Fig. 5B and Table S1). A similar number of genes were downregulated and upregulated in comparison to wild-type animals (Table S1). The median log2 fold changes of gene expression are 2.27±0.018 (Mean±SEM) for upregulated genes and 1.78±0.01 (Mean±SEM) for downregulated genes. Strikingly, among the downregulated genes, Gene Ontology (GO) analysis identified genes associated with neuronal function categories, including neuronal development, locomotion, chemotaxis, neuronal cell projection, axon guidance and synaptic transmission (Fig. 5C and Table S1). And exception of sax-3, all the other genes involved in axon guidance pathways tested for genetic interactions are listed as downregulated genes in the RNA sequencing dataset. Among the upregulated genes, categories related to germ cell biology and DNA replication/repair were significantly enriched (Fig. 5D), corroborating previous studies that implicated set-2 in fertility and genome stability\textsuperscript{68,75}. We also analyzed the transcriptome of the set-2(zr2012) allele that expresses a mutant SET2 protein with an amino acid substitution found in SETD1B in a case of intellectual disability\textsuperscript{67}. Despite a smaller number of DE genes identified in animals carrying this allele (3053 DE genes, FDR<0.05) (Table S1 and Fig. S6), the intersection of DE genes and consistently downregulated genes in the two set-2 alleles was significant (p<0.001 and p<0.0001, respectively). Importantly, GO analysis of
downregulated genes in set-2(zr2012) and consistently downregulated genes in set-2(tm1630) and set-1(zr2012) identified neuronal categories as enriched, confirming the relevance of set-2 in positively regulating the transcription of neuronal genes (Fig. S6 and Table S1). These results suggest that, in agreement with its catalytic activity, SET-2 contributes substantially to the regulation of gene expression in embryos. Furthermore, the identification of several downregulated genes belonging to the cell projection/axon guidance class corroborates our finding that regulation of H3K4 methylation is required for the establishment of proper axon trajectory.

**Loss of set-2 impairs nervous system functionality**

Besides the genes required for the establishment of proper axon guidance, the transcriptome analysis of set-2 mutant alleles reveal that numerous neuronal genes are differentially expressed, suggesting a broad role of set-2 in the nervous system. Therefore, we tested whether the loss of set-2 would result in abnormal neuronal functionality. Despite set-2(tm1630) and set-2(zr2012) mutant animals appear superficially wild-type, we detected differences when compared to control animals in specific functional tests. Locomotion in *C. elegans* is controlled by excitatory cholinergic and inhibitory GABAergic motor neurons, which functionality can be determined by observing animals swimming in liquid. set-2(tm1630) and set-2(zr2012) mutant animals display a reduced rate of body bends in liquid compared to wild-type animals (Fig. 6A). Furthermore, when left moving on plate for 1 hour at 20°C, set-2(tm1630) and set-2(zr2012) mutants appear to explore fewer regions of the plate (Fig. 6B) and to move in a tighter circular pattern compared to control animals (Fig. S7). A closer analysis into the crawling tracks reveals differences in waves amplitude between control animals and mutants, but no significant differences were observed when comparing wave lengths (Fig. 6C). Overall, these results suggest abnormal locomotion behaviour in set-2(tm1630) and set-2(zr2012) mutant animals. Defecation in *C. elegans* is the result of a stereotyped and tightly regulated motor program
involving the subsequent contraction of three distinct sets of enteric muscles. Monitoring this relatively simple process is a powerful method in determining neuronal system functionality and synaptic transmission. We found that the rate of defecation in both set-2 mutants is significantly reduced compared to wild type animals (Fig. 6D). Lastly, we assessed chemotaxis responses towards attractive stimuli by testing the response of set-2 mutant animals to sodium chloride. No differences were observed in the set-2(tm1630) and set-2(zr2012) mutant animals compared to control animals, suggesting that set-2 mutants have intact chemotactic response (Fig. 6E). In C. elegans the chemotactic response changes according to previous experiences. When animals are grown in presence of food and sodium chloride they are attracted to the salt. In contrast, when worms are starved in the presence of salt, they learn to avoid it as they associate the salt with an unfavourable condition. We tested if this associative learning process is affected in set-2(tm1630) and set-2(zr2012) mutant animals by conditioning animals in unseeded plates containing sodium chloride and subsequently testing their reaction to the salt. In contrast to wild type animals, conditioned set-2 mutants are still attracted to sodium chloride, suggesting a defect in the associative learning process (Fig. 6E). Overall, these results indicate that mutations in set-2 gene, in correlation with aberrant expression of neuronal genes, results in compromised neuronal functions.

**DISCUSSION**

Despite the recognition of H3K4 methylation as a critical epigenetic modification in neuronal development, its biological role in this tissue is only marginally understood. In this study we specifically addressed the role of the main H3K4 methyltransferase, SET-2 in neuronal development using C. elegans as a model system. Our results demonstrate the requirement of the catalytic activity of SET2, and therefore for H3K4 methylation, in the process of axon guidance. Moreover, we show that several proteins involved in the regulation of H3K4 methylation are also required for the establishment of axon trajectories,
including set-16, another H3K4 methyltrasferase. We don’t know if set-2 and set-16 have a redundant role in axon guidance and share common targets. Nevertheless, the requirement of multiple H3K4 regulators likely reflects the notion that axon guidance is a complex process regulated by a multitude of extracellular cues and signalling pathways that need to be integrated and temporally and spatially coordinated. We propose that such orchestration is, at least in part, epigenetically controlled and occurs by fine-tuning the transcription of the implicated genes through H3K4 methylation regulation.

Our analysis showed that catalytic activity of SET-2 is required for the correct axon guidance through a mechanism that involves the regulation of cytoskeleton dynamics. Remarkably, the axon guidance defects observed in the H3K4 methylation regulators investigated in detail so far (rbr-2, jmjd-1.2, set-2) are all suppressed by wsp-1 ablation (this study and 52,53), suggesting that, in line with the genetic interaction observed among set-2, rbr-2 and jmjd-1.2, wsp-1 is a shared target (Fig. 7). Our results, showing that only wsp-1 but not other known actin regulators like unc-34 or wve-1 suppresses the axonal defects, suggests that the H3K4 regulatory machinery controls a specific branch of actin remodelling. It should also be noted that the modalities by which H3K4 regulators control WSP-1 functionality are likely different, as rbr-2 controls wsp-1 expression 52, while jmjd-1.2 appears to regulate its activation through nck-1 and cdc-42 53. set-2 action on wsp-1 activation seems to strictly depend on cdc-42, as ablation of cdc-42, but not nck-1, ameliorates the axonal phenotype. Puzzlingly, in set-2(tm1630), the levels of wsp-1 expression appears reduced (Table S1). Since loss of wsp-1 is not resulting in axon defects, the wsp-1 dowregulation observed is likely due to a compensatory mechanism to reduce the effects of its aberrant activation. The identification of direct targets of SET-2 will help to clarify the specific mechanism underlying the genetic interactions observed.

It is intriguing that only a subset of neurons is invariantly disturbed by the loss of H3K4 regulator genes 52,53 (and this study), suggesting that in these neurons the process
of axon guidance is particularly vulnerable and under epigenetic control. This possibility is also suggested by a study showing that defects in axon guidance of the same subset of neurons are observed in animals experiencing oxygen deprivation during embryonic development \(^\text{49}\). Further analyses testing other adverse environmental conditions and cell-specific studies related to expression patterns and lineage will help to understand the origin of this susceptibility.

*set-2* mutant alleles show phenotypes related to locomotion and learning. It is likely that these phenotypes might only in part depend on axon guidance defects. Indeed, transcriptional profiles and gene ontology analyses of *set-2* alleles identified several neuronal categories among differentially expressed genes, suggestive of a novel and broad role of *set-2* in the *C. elegans* nervous system. The role of *set-2* mammalian homologues in neurons has not been investigated due to the essential nature of these genes during mouse embryogenesis. However, considering our results and the frequency of mutations in these genes in cases of intellectual disability, autism, epilepsy, and schizophrenia \(^\text{22,24,67}\), it is tempting to hypothesise an evolutionary-conserved role for KMT2F and KMT2G in nervous system development, which deserves further investigation.

In the context of disease, the recent identification by genome-wide association studies of a H3K4 regulation domain affected in neuronal disorders has emphasised the potential impact of epigenetic regulation in the developing nervous system and in illness.

By demonstrating that *C. elegans* homologs of H3K4 regulators mutated in disease are all required for proper axon guidance (Fig. 7) and that a disease-associate mutation of SETD1B reproduces the axonal phenotype in the nematode, our studies provide evidence for a common denominator among these genes. All together, our results suggest that aberrant axon guidance is a shared trait in neurodevelopmental diseases and offer experimental support to a recently suggested hypothesis proposing that dysregulated axonal guidance underlines neurological disorders \(^\text{80-82}\).
MATERIAL AND METHODS

Genetics and strains

C. elegans strains were cultured using standard growth conditions at 20°C on Escherichia coli OP50. Double mutant animals were generated by standard crossing procedure. set-2(tm1630) was backcrossed six times, set-2(zr2012) was backcrossed two times. Neuronal marker strains were backcrossed three times. Strains used are listed in Table S2. set-16(n4526) is a balanced strain. Heterozygotes segregate Dpy sterile animals (+/+, larval lethal animals (-/-) and wild type animals (+/-).

Generation of constructs

The set-2 transcriptional reporter includes a ≈400bp region located at the 5' end of set-2 gene amplified using the following primers: 5'ccgatatcagtagaaatctg and 5'gcaacttatcatccagaccata. The PCR product was cloned into pD95.75mCherry. Tissue-specific rescue constructs were constructed using MultiSite Gateway Three-Fragment Vector Construction Kit (Life Technologies) as previously described. set-2 cDNA was a kind gift from Francesca Palladino (ENS, Lyon, France).

Generation of transgenic lines

Transgenic lines were created by microinjection. The set-2 transcriptional reporter line was obtained by injecting 50ng/µl of reporter construct into N2. Tissue-specific rescue constructs were injected at 10ng/µl with with myo-2::mCherry as co-injection marker at 5ng/µl into set-2(tm1630). Fosmid was injected at 5 ng/µl with a co-injection marker myo-2::mCherry at 5ng/µl. Fosmid (WRM0638aG05) was a kind gift from Roger Pocock (Dep. Of Anatomy and Development, Monash University, Melbourne, Australia). Transgenic lines used in the study are listed in Table S2.
CRISPR lines

CRISPR lines was created by injecting ssDNA repair templates for set-2 with desired substitutions cloned into pJJR50 (zr1504 sgRNA sequence CCTTCGCGTAGCAATTAGGT and zr2012 sgRNA sequence TCACATGATGCAGATCAATT). The mix contained a pha-1 repair template and pJW1285 (driving the expression of Cas9) was injected into pha-1(e2123) mutants. All constructs were injected at 50ng/µl. Selection for pha-1 wild type clones was performed at 25°C. Mutations were confirmed by sequencing. The mutation in zr1504 allele was selected based on the following criteria: 1) residue conserved from yeast to human, 2) outside of the interaction surface with other components of the MLL-complex based on 88, 3) conservative substitution (H to K). The mutation in the zr2012 allele reproduces an alteration identified in SETD1B/KMT2G in a case of intellectual disability linked to epilepsy and autism 67.

Western blot and Immunostaining

Protein extracts were prepared from embryos obtained after hypochlorite treatment of animals grown at 20°C. Samples were boiled in SDS-PAGE buffer for 5 minutes and sonicated for 10 minutes using Diagenode Bioruptor (UCD-300). The following antibodies were used: anti H3K4me3 (Cell Signaling, C42D8, lot: 10; 1:750); polyclonal anti H3 (Abcam,ab1791, lot: GR234589-1; 1:10.000), peroxidase-labeled anti-rabbit secondary antibody (Vector, lot: ZE1026; 1:10.000). Western blots were quantified using ImageJ (National Institutes of Health).

Embryo staining was performed as described in 89. Primary antibody H3K4me3 (Cell Signaling, C42D8, lot: 10; 1:500), was incubated over night at 4°C and secondary antibody [goat anti-rabbit IgG (Alexa Fluor 488, Invitrogen, A11008; 1:500)] was incubated for 2 h at room temperature. Embryos were stained with DAPI and slides were mounted using mounting media.
**Axon guidance analyses**

Axon guidance phenotype was scored at 20°C at L4 stage, unless otherwise stated. Worms were immobilized in NaN₃ and placed on microscope slides with a 5% agarose pad. Results from at least three biological independent experiments were used for statistical analyses. Images were obtained using a Zeiss, AXIO imager M2 fluorescence microscope. The scoring of the *set-16* mutant was done at early larval stage, due to the larval lethality of the strain. Arrested larvae were considered *set-16* null. Investigators were not blinded during the experiments.

**Statistical analyses**

Statistical analysis for all neuronal scoring were performed in Graphpad Prism 8 using Student’s t-test or one-way ANOVA (Tukey’s multiple comparison). All values are presented as mean percentages.

**RNA sequencing**

RNA was isolated from three independent experiments per genotype. Hermaphrodites were bleached two times to achieve better synchronization. Eggs recovered from the second hypochlorite treatment of highly synchronized young adult animals were kept at 20°C for 4 hours in M9 to reach mid-embryogenesis (the majority of the eggs were at comma-stage) and freeze-cracked in liquid nitrogen. Wild type and *set-2* samples were prepared and analysed in parallel, to minimise, as much as possible, synchronization and batch issues. RNA was extracted using Arcturus™ Picopure™ RNA isolation kit (Thermo fisher, cat KIT0204). Sequencing libraries was constructed using Truseq RNA library Prep Kit v2 (Illumina, cat RS-122-2001). Libraries were sequenced using NextSeq 500 and NextSeq® 500/550 High Output Kit v2 (Illumina, cat FC-404-2005). Dataset produced in this study are available at Gene Expression Omnibus (GEO): GSE143811 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143811).
RNA sequencing analysis

RNA sequencing results were analysed by Galaxy (v19.05). Reads were mapped to the *C. elegans* genome (WS220) using a criterion of two mismatches. Number of reads aligned for each replica was between 14.6 to 53.1 millions. Deseq2 (v2.1.8.3) was used to determine differentially expressed genes and to generate PC plot and scatter plot. Differentially expressed genes with FDR<0.05 were analyzed using g-Profiler (http://biit.cs.ut.ee/gprofiler/gost) with Bonferroni correction. P value for overlapping gene lists was calculated at http://www.nemates.org/MA/progs/overlap_stats.html

Neuronal function analyses

**Thrashing assay in liquid.** Wells of a 96-well microtiter plate with 400 µL of M9 at a temperature of 20°C were used. Three young adult stage worms of the same strain were placed in each well and left for 10 minutes at 20°C to adapt. Body bends were counted for 30 seconds. One bend was counted every time the mid body of the animal returned to the same position. The experiment was carried out for at least 60 worms per strain.

**Tracking and exploration assay.** NGM plates (6cm) were seeded with 600 µL of OP50 grown over night in LB media at 37°C and stored at 25°C for one night. One young adult stage worm was placed on the centre of the bacteria lawn and left to crawl for 1 hour at 20°C. For the tracking assay, animals were removed after 1h of crawling and the body length was measured for normalization. The tracks that the animals left onto the plates were visualized using a dissecting microscope and a digital camera using the same magnification settings. Amplitude and wavelength of the tracks were measured using ImageJ. For the exploration assay, animals were removed after 1 hour of crawling and plates were superimposed on a grid containing 3x3 mm wide squares and the number of squares entered by the worm were manually counted as previously described. Both assays were performed using at least 30 worms per strain.
**Defecation assay.** Defecation was assessed as previously described\(^9\). Each DMP cycle was counted as the interval between two posterior body wall muscle contraction. Five full cycles for each animal were counted. This assay was carried out for at least 30 worms per strain.

**Chemotaxis and chemotaxis plasticity assay.** The chemotactic response to NaCl was conducted as previously described\(^7\). Briefly, 20 mL of buffered agar was poured into 10 cm diameter Petri dishes. To set up a salt gradient, 10 µL of 2.5 M NaCl solution were applied to the attractant spot, and 10 µL of ddH2O were applied to the control spot 16 h before the assay. Another 10 µL of 2.5 M NaCl solution or water were applied 4h before the assay onto the same spots. 1µL of NaN\(_3\) was applied to both spots 1 minute before the assay. Synchronized young adult animals were washed 3 times with CTX solution (5mM KH2PO4/K2HPO4 pH 6, 1mM CaCl and 1mM MgSO4), and 40-50 worms were put in the center of the assay plate in a minimal volume buffer. Animals were left to crawl for 45 minutes at 20°C, then the plates were placed at 4°C overnight and the chemotactic index was calculated. The chemotaxis index was defined as the number of animals in the NaCl area (within 1.5 cm from the solution spot) minus the number of animals in the control area, divided by the total number of animals in the plate. Worms unable to leave the centre of the assay plate were censored. The chemotaxis was assessed with assay plates prepared in the same way described above. Synchronized young adult animals were washed 3 times with CTX solution, and 40-50 worms were placed onto conditioning plates, prepared with NGM media (containing NaCl) and without OP50. Animals were conditioned for 1h, washed again 1 time with CTX solution and placed in the centre of the assay plates. Worms were left to crawl for 45 minutes at 20°C, then the plates were placed at 4°C overnight. The chemotactic index was calculated as before. Several independent biological replica were analysed. Investigators were not blinded during the experiments.
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DATA AVAILABILITY

All the data that support the findings presented in the study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

S.A-N, B.A and L.B carried out the experimental work and analyzed the data. S.A-N and A.E.S. designed the experiments. A.E.S analysed the data, and wrote the manuscript.

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Figures

A. Top: Schematic of PVQ neurons in wild type at L4 stage. Bottom: representative image of the posterior section of wild type and set-2(tm1630) L4 worms expressing GFP in PVQ neurons (transgene oyls14). White arrows indicate the most common defect observed in mutant animals, where the left PVQ neuron defasciculates and erroneously migrates to the contralateral side of the ventral nerve cord and back again. Scale bar 50 μm.

B. Quantification of PVQ defects at L4 stage in wild type and in mutants of genes involved in H3K4me regulation. Mutants for rbr-2, a H3K4 demethylase, and jmjd-1.2, a H3K4me3 binder, previously reported to exhibit axon guidance defects, were used as positive controls. All the alleles used carry large deletions and are most likely null mutants.

Figure 1. Loss of H3K4me regulators causes axon guidance defects. A. Top: Schematic of PVQ neurons in wild type at L4 stage. Bottom: representative image of the posterior section of wild type and set-2(tm1630) L4 worms expressing GFP in PVQ neurons (transgene oyls14). White arrows indicate the most common defect observed in mutant animals, where the left PVQ neuron defasciculates and erroneously migrates to the contralateral side of the ventral nerve cord and back again. Scale bar 50 μm. B. Quantification of PVQ defects at L4 stage in wild type and in mutants of genes involved in H3K4me regulation. Mutants for rbr-2, a H3K4 demethylase, and jmjd-1.2, a H3K4me3 binder, previously reported to exhibit axon guidance defects, were used as positive controls. All the alleles used carry large deletions and are most likely null mutants.
set-16(n4526) mutant was scored at L1 stage, due to larval lethality. n>150 for all strains, except for set-16(n4526), n=56. Statistical analysis (one-way ANOVA – Tukey’s multiple comparison test). **p<0.005; ***p<0.0005; ****p<0.0001; n.s, not significant compared to wild type. Black dots represent independent scorings. Error bars represent standard error of mean (SEM).
Figure 2. SET-2 is required in the nervous system to ensure proper PVQ axon guidance.  

A. Schematic of the set-2. RRM, RNA recognition motif. SET, methyltransferase domain. Deletions and point mutation alleles used in this study are shown.  

B. Quantification of PVQ defects at L4 stage in wild type, set-2 deletion alleles and in set-2(tm1630) animals ectopically expressing a genomic region that includes the set-2 gene (Fosmid WRM0638aG05).  

C. Quantification of PVQ defects at L4 stage in wild type and set-2(tm1630) mutants expressing set-2 cDNA in different tissues. Promoters used to drive set-2 expression: Neuronal, rgef-1. Hypodermal, dpy-7. PVQ, sra-6. n>150. Statistical analysis (one-way ANOVA – Tukey’s multiple comparison test). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001; n.s, not significant. In C, comparisons are with set-2. Black dots represent independent scorings. Error bars represent standard error of mean (SEM).
Figure 3. The catalytic activity of SET-2 is paramount for correct axon guidance. A. Top: Alignment of a portion of the SET domain of SET-2 with homologues in different species. Grey shade denotes conserved amino acids. In red the conserved amino acids substituted in set-2 alleles zr2012 and zr1504. Corresponding amino acid substitutions in human are shown. In zr2012 allele, Arg1426 is changed to Trp, reproducing a disease-associated mutation (R1842W). Bottom: representative western blot showing embryonic H3K4me3 level in the indicated strains. H3 is used as loading control. Numbers indicate the average of H3K4me3 relative to wild type, from three independent experiment ±SEM. 

B. Quantification of PVQ defects at L4 stage in wild type and indicated set-2 mutant alleles. Statistical analysis (one-way ANOVA – Tukey’s multiple comparison test). n>150, ****p<0.0001 compared to wild type. Black dots represent independent scorings. Error bars represent standard error of mean (SEM).
Figure 4. set-2 genetic interactions with other H3K4me regulators and actin modulators. A. Quantification of PVQ defects at L4 stage in wild type, single and compound mutants of H3K4 regulators. B. Quantification of PVQ defects at L4 stage in wild type, single and compound mutants of COMPASS complex members. C. Quantification of PVQ defects at L4 stage in wild type, single and compound mutants of actin modulators. n>150 for all strains (except for cdc-42(gk388); set-2(tm1630) n=63). Statistical analysis (one-way ANOVA – Tukey’s multiple comparison test). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001; n.s, not significant, compared with single mutants with the highest penetrance. Black dots represent independent scorings. Error bars represent standard error of mean (SEM).
**Figure 5.** Transcriptional regulation mediated by SET-2.  

**A.** Principal component analysis (PCA) plot of wild type and set-2(tm1630) mid-embryos. Each dot represents one sample and each colour a genotype.  

**B.** MA-plot showing gene expression changes in set-2(tm1630). The X axis represents the mean of counts, the Y axis represents log2 fold change. DE genes with FDR<0.05 are shown in red.  

**C.** Gene ontology analysis of biological processes of down- (left) and up- (right) regulated genes in set-2(tm1630) allele by g-Profiler, using adjusted P-values (Bonferroni correction). Selected top scoring categories are presented together with the number of genes identified in each category.
Figure 6. Loss of set-2 impairs nervous system functionality. A. Quantification of body bends in liquid in wild type, set-2(tm1630) and set-2(zr2012). n > 60, ****p<0.0001 (Student’s t-test). B. Quantification of exploratory behaviour in wild type, set-2(tm1630) and set-2(zr2012). n> 30, ****P<0.0001 (Student’s t-test). C. Left and middle: Quantification of wavelength and amplitude of tracks left on a bacterial lawn by wild type, set-2(tm1630) and set-2(zr2012). n> 30, *p<0.05, ****p<0.0001, n.s., not significant compared to wild type (Student’s t-test). Right: representative images of crawling tracks in wild type, set-2(tm1630) and set-2(zr2012) mutant animals. D. Quantification of defecation
motor program (DMP) length in wild type, set-2(tm1630) and set-2(zr2012). n > 30, ***p<0.001, ****p<0.0001 (Student’s t-test). 

**E.** Quantification of chemotactic response to NaCl and plasticity in set-2(tm1630) and set-2(zr2012). Worms were washed and put directly onto assay plates (naive) or onto conditioning plates for 1h before being tested (conditioned). Chemotactic indexes of mutant strains in conditioned state were compared to wild type in the same conditions. Comparison of chemotactic indexes of wild type in naive and conditioned state is marked with a line. n > 200, Statistical analysis (one-way ANOVA – Tukey’s multiple comparison test). ****p<0.0001, **p<0.01, *p<0.05. In A-D, black dots represent single animals. In E, black dots represent independent replica.
Figure 7. *C. elegans* homologs of H3K4 regulators mutated in disease all contribute to axon guidance. Schematic model depicting the role of *rbr-2*, *set-2* and *jmjd-1.2* in regulating *wsp-1*-mediated actin remodelling and axon guidance. *rbr-2* directly influences *wsp-1* transcription\(^5^2\), while *set-2* and *jmjd-1.2* control *wsp-1* activity through *cdc-42* and *nck-1* (this study and \(^5^3\)). The action of *set-16*, COMPASS complex components and *spr-5* on *wsp-1* remain to be elucidated. In red, mammal homologs found mutated in neurodevelopmental diseases.
# Tables

## Table 1. *C. elegans* H3K4me regulators analysed

| *C. elegans* genes | PVQs axon guidance defects | H3K4 activity | Human homologues | Mutations in neuronal disorders |
|-------------------|-----------------------------|---------------|------------------|-------------------------------|
| set-2             | Yes                         | Methyltransferase | KMT2F/G       | Yes*                          |
| set-16            | Yes                         | Methyltransferase | KMT2A-D        | Yes*                          |
| set-17            | No                          | Methyltransferase | PRDM7/9        | No*                           |
| set-30            | No                          | Methyltransferase | KMT3C          | No*                           |
| rbr-2             | Yes                         | Demethylase     | KDM5A-D        | Yes*                          |
| spr-5             | Yes                         | Demethylase     | KDM1A          | Yes*                          |
| jmjd-1.2          | Yes                         | Binder         | KDM7A          | Yes*                          |
| wdr-5.1           | Yes                         | COMPASS Complex | WDR5           | Yes*                          |
| rbbp-5            | Yes                         | COMPASS Complex | RBBP5          | Yes*                          |
| ash-2             | Yes                         | COMPASS Complex | ASH2L          | Yes*                          |
| dpy-30            | ND                          | COMPASS Complex | DPY30          | Yes*                          |

Table 1. *C. elegans* H3K4me regulators analysed. *C. elegans* genes, axonal defects in mutant alleles, H3K4-related activities, human homologs. Association of human homologs to neuronal disorders is based on the human disease database MalaCards and literature, as indicated. *According to MalaCards (www.malacards.org), a83, b84, c85*
### Table 2. Neuroanatomical analysis of set-2(tm1630) mutants

| Neurons (marker used) | wild type | set-2(tm1630) |
|-----------------------|-----------|---------------|
| **Internaerons**      |           |               |
| PVQ (oyls14)<sup>a</sup> | 5%        | 21%***        |
| AVK (bwls2)<sup>a</sup>  | 1%        | 1% n.s        |
| **Moteoners**         |           |               |
| HSN (zdls13)          |           |               |
| Axon guidance<sup>a</sup> | 4%        | 21%**        |
| Cell migration<sup>b</sup> | 7%        | 9% n.s        |
| VD/DD (oxls12)        |           |               |
| Midline L/R choice<sup>c</sup> | 27%      | 61%**        |
| Guidance<sup>d</sup>  | 1%        | 17%**        |
| **Mechanosensory neurons** |              |               |
| AVM (zdls5)           |           |               |
| Axon guidance<sup>e</sup> | 0%        | 1% n.s        |
| Cell migration<sup>f</sup> | 0%        | 0% n.s        |
| ALM (zdls5)<sup>f</sup> | 0%        | 0% n.s        |
| PVM (zdls5)           |           |               |
| Axon guidance<sup>e</sup> | 1%        | 1% n.s        |
| Cell migration<sup>h</sup> | 0%        | 0% n.s        |
| PLM (zdls5)<sup>f</sup> | 3%        | 5% n.s        |

Table 2. Neuroanatomical analysis of *set-2(tm1630)* mutants. Different morphological classes of neurons examined in wild type and *set-2(tm1630)* mutant animals, carrying specific transgenic markers, as indicated. n>150. Student t-test was used to assess for statistical significance **p<0.005; p<0.005; n.s, compared to wild type. <sup>a</sup> Axons crossing the midline of the animal. <sup>b</sup> Cell body not reaching the correct position at the midbody. <sup>c</sup> Axons extending to the left side instead of the right side. <sup>d</sup> Axons extending anterior or posterior during circumferential growth to the dorsal nerve cord. <sup>e</sup> Axons extending anterior before extending into the ventral nerve cord. <sup>f</sup> Axons extending towards ventral or dorsal nerve cord. <sup>g</sup> Cell body positioned lateral right side of the anterior part of the animal between vulva and head. <sup>h</sup> Cell body positioned lateral left side of posterior part of the animal between vulva and tail.
Table 3. Genetic interaction with classical guidance genes

| Genotype                                      | PVQ defects |
|-----------------------------------------------|-------------|
| wild type                                     | 6%          |
| set-2(tm1630)                                 | 22%         |
| unc-5(e53)                                    | 22%         |
| set-2(tm1630); unc-5(e53)                     | 46%****     |
| vab-1(dx31)                                   | 22%         |
| vab-1(dx31); set-2(tm1630)                     | 28% n.s     |
| plx-2(ev773)                                  | 20%         |
| plx-2(ev773); set-2(tm1630)                    | 27% n.s     |
| sax-3(ky123)                                  | 53%         |
| sax-3(ky123); set-2(tm1630)                    | 78%****     |
| sdn-1(zh20)                                   | 48%         |
| sdn-1(zh20); set-2(tm1630)                     | 47% n.s     |

Table 3. Genetic interaction of set-2 with classical axon guidance pathways.

PVQ defects at L4 stage in wild type, single and compound mutants of classical axon guidance genes. n>150. Student t-test was used to assess for statistical significance ****p<0.0001; n.s, not significant compared to the single mutant with the highest penetrance.
Figure S1. Expression of transgenic SET-2::GFP in specific tissues. Top: Expression pattern of SET-2::GFP under the control of the neuronal specific rgef-1 promoter. Pharynx is visible as myo-2::mCherry was used as co-injection marker. Middle: Expression pattern of SET-2::GFP under the control of the hypodermal specific dpy-7 promoter. Depicted is the posterior of the animal. Bottom: Expression pattern of SET-2::GFP under the control of the PVQ promoter sra-6. The posterior extremity of the animal is outlined with white-dotted line. Strong fluorescence in the left of the picture is autofluorescence from the gut. Scale bars: 50 μm.
Figure S2: Axon guidance defect in set-2(tm1630) at L1 stage. Representative image of a set-2(tm1630) L1 carrying the oyls14 transgene. Arrow indicates an aberrant pattern of PVQ axon guidance in the posterior part of the animal. Scale bar: 50μm.
**Figure S3. set-2 transgene expression.** 

**A.** Representative DIC (top) and fluorescence (bottom) images of a transgenic embryo expressing a *set-2* transcriptional construct (ZR1053). Ubiquitous expression of *set-2* during embryonic development is evident around the presumptive time of PVQ axon development. Scale bar 20 µm  

**B.** Representative fluorescence images of one-day adult transgenic animals. Top: Lateral anterior view. *set-2* expression in the pharynx and in the nerve ring (arrowhead). Bottom: Ventral midbody view. *set-2* expression in neurons of the ventral nerve cord (arrowheads). HSNs are indicated by asterisks. Scale bar 30 µm.
**Figure S4. H3K4 levels in set-2 alleles.** Representative images of wild type, set-2(tm1630), set-2(zr2012), set-2(zr1504) embryos at comma stage, stained with H3K4me3 antibody. DNA was stained with DAPI.
Figure S5. PVQ defects in a weak allele of set-2. Quantification of PVQ defects at L4 stage in wild type and set-2(ok952) mutants (n>150). Statistical analysis (Student’s t-test), n.s, not significant compared to wild type. Black dots represent independent scorings. Error bars represent standard error of mean (SEM).
GO downregulated genes set-2(zr2012)

- response to external stimulus: 79 genes
- cilium organization: 18 genes
- neurogenesis: 45 genes
- neuron differentiation: 42 genes
- nervous system development: 54 genes
- cell fate specification: 33 genes
- transcription, DNA-templated: 135 genes
- RNA metabolic process: 145 genes

PC1: 54% variance  
PC2: 44% variance

GO upregulated genes set-2(zr2012)

- chaperone-med. protein folding: 9 genes
- peptidyl-amino acid modification: 54 genes
- protein folding: 24 genes

PC1: 54% variance  
PC2: 44% variance

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**Figure S6. set-2(zr2012) analyses.**

**A.** Principal component analysis (PCA) plot of wild-type and set-2(zr2012) mid-embryos. Each dot represents one sample and each colour a genotype. **B.** Gene ontology analysis of biological processes of down- (left) and up- (right) regulated genes in set-2(zr2012) allele by g-Profiler, using adjusted P-values (Bonferroni correction). Selected top scoring categories are presented together with the number of genes identified in each category.
Figure S7. Crawling pattern of set-2 mutant animals. Representative images of crawling tracks onto an uniform bacterial lawn left by wild type and indicated set-2 mutant animals.
Table S1.

Click here to Download Table S1

Table S2. List of strains used in this study

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