A genetic screen identifies *dreammist* as a regulator of sleep

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Significance statement: Sleep is an essential component of behaviour, but the genes that regulate sleep and wake states are still being uncovered. A viral insertion screen in zebrafish identified a novel sleep mutant called *dreammist*, in which a small, highly-conserved transmembrane protein is disrupted. The discovery of *dreammist* highlights the importance of a class of small transmembrane-protein modulators of the sodium pump in setting appropriate sleep duration.
ABSTRACT

Sleep is a nearly universal feature of animal behaviour, yet many of the molecular, genetic, and neuronal substrates that orchestrate sleep/wake transitions lie undiscovered. Employing a viral insertion sleep screen in larval zebrafish, we identified a novel mutant, dreammist (dmist), with altered sleep-wake dynamics. CRISPR/Cas9-mediated disruption of dmist also led to behavioural hyperactivity and reduced sleep at night. The neuronally expressed dmist gene is conserved across vertebrates and encodes a small single-pass transmembrane protein that is structurally similar to the Na⁺,K⁺-ATPase regulator, FXYD1/Phospholemman. Disruption of either fxyd1 or atp1a3a, a Na⁺,K⁺-ATPase alpha-3 subunit associated with several heritable movement disorders in humans, led to decreased night-time sleep. As intracellular Na⁺ concentration is disrupted in dmist mutant brains after high neuronal activity similarly to atp1a3a mutants, but is also elevated specifically at night, we propose that sleep-wake stability is modulated by Dmist-dependent changes to Na⁺ pump function during sleep homeostatic challenge and at specific times of the day-night cycle.

INTRODUCTION

The ability for animals to switch between behaviourally alert and quiescent states is conserved across the animal kingdom (Cirelli, 2009; Joiner, 2016). Fundamental rules that govern the regulation of sleep-like states are shared across species, such as the role of circadian and homeostatic cues in regulating the time and amount of sleep, stereotyped postures, heightened arousal thresholds, and the ready reversibility to a more alert state (Joiner, 2016). The near ubiquity of behavioural sleep implies that it serves ancient functions and is subject to conserved regulatory processes. However, many key molecular components that modulate sleep and wake states remain undiscovered.

Over the past two decades, investigations into sleep and arousal states of genetically tractable model organisms, such as Drosophila melanogaster, C. elegans, and zebrafish have
uncovered novel molecular and neuronal components of sleep regulation through gain- and loss-of-function genetic screens (reviewed in Barlow and Rihel, 2017; Sehgal and Mignot, 2011). The power of screening approaches is perhaps best exemplified by the first forward genetic sleep screen, which identified the potassium channel shaker as a critical sleep regulator in Drosophila (Cirelli et al., 2005). This result continues to have a lasting impact on the field, as not only did subsequent sleep screening efforts uncover the novel SHAKER-regulator sleepless, (Koh et al., 2009), but investigations into SHAKER’s beta subunit HYPERKINETIC ultimately revealed a critical role for this redox sensor linking metabolic function to sleep (Bushey et al., 2007; Kempf et al., 2019). Other Drosophila loss of function screens have highlighted the sleep-regulatory roles of the dopamine transporter, fumin, (Wu et al., 2008) and the Cullin-3 ubiquitin ligase adaptor, insomniac (Pfeiffenberger and Allada, 2012; Stavropoulos and Young, 2011), among others.

Disparate screening strategies across model organisms continue to unveil novel sleep modulators in both invertebrate and vertebrate model systems. For example, the roles of RFamide receptor DMSR-1 in stress-induced sleep in C. elegans (Iannacone et al., 2017) and SIK3 kinase in modulating sleep homeostasis in mice (Funato et al., 2016) were identified in genetic screens. Moreover, a gain of function screening strategy in Drosophila revealed the novel sleep and immune regulator, nemuri (Toda et al., 2019), and a zebrafish overexpression screen uncovered the secreted neuropeptides Neuromedin U and Neuropeptide Y, which decrease and increase sleep, respectively (Chiu et al., 2016; Singh et al., 2017). The continued success of screening strategies in revealing novel sleep-wake regulatory genes suggests that more sleep signals likely remain to be discovered.

One of the lessons from these genetic screens is that many of the uncovered genes play conserved roles across species—for example, Shaker also regulates mammalian sleep (Douglas et al., 2007) and RFamides induce sleep in worms, flies, and vertebrates (Lee et al.,
Nevertheless, not every invertebrate sleep-regulatory gene has a clear homolog in vertebrate species, while known human sleep/wake regulators, such as the narcolepsy-associated neuropeptide hypocretin/orexin and its receptors, lack invertebrate orthologs (Chemelli et al., 1999; Lin et al., 1999; Prober et al., 2006; Sakurai, 2013; Yokogawa et al., 2007). Therefore, genetic sleep screens in vertebrates are likely to provide added value in uncovering additional regulatory components required to control the initiation and amount of sleep.

While sleep screening in mammals is feasible (Funato et al., 2016), it remains an expensive and technically challenging endeavour. With its genetic tractability, availability of high-throughput sleep assays (Rihel and Schier, 2013), and conserved sleep genetics, such as the hypocretin, melatonin, and raphe systems (Gandhi et al., 2015; Oikonomou et al., 2019; Prober et al., 2006), the larval zebrafish offers an attractive alternative vertebrate system for sleep screens. We took advantage of a collection of zebrafish lines that harbour viral-insertions in >3500 genes (Varshney et al., 2013) to perform a targeted genetic screen. We found one short-sleeping mutant, dreammist, with a disrupted novel, highly conserved, vertebrate gene that encodes a small single pass transmembrane protein. Sequence and structural homology to the Na⁺/K⁺ pump regulator FXYD1/Phospholemman suggests that Dreammist is a neuronal-expressed member of a class of sodium pump modulators that is important for regulating sleep-wake behaviour.
**RESULTS**

**Reverse genetic screen identifies *dreammist*, a mutant with decreased sleep**

We used the availability of the ‘Zenemark’ viral-insertion based zebrafish gene knock-out resource (Varshney et al., 2013) to perform a reverse genetic screen to identify novel vertebrate sleep genes. This screening strategy offers several advantages compared to traditional chemical mutagenesis-based forward genetic screening approaches. First, unlike chemical mutagenesis, which introduces mutations randomly, viral insertions tend to target the 5' end of genes, typically causing genetic loss of function (Sivasubbu et al., 2007). Second, because the virus sequence is known, it is straightforward to map and identify the causative gene in mutant animals. Finally, since viral insertions in the Zenemark collection are already mapped and sequenced, animals harbouring insertions within specific gene classes can be selected for testing (Figure S1A). This allowed us to prioritise screening of genes encoding protein classes most often linked to behaviour, such as G-protein coupled receptors, neuropeptide ligands, and ion channels and transporters (Supplemental Data 1).

For screening, we identified zebrafish sperm samples from the Zenemark collection (Varshney et al., 2013) that harboured viral insertions in genes of interest and used these samples for *in vitro* fertilization and the establishment of F2 families. For each viral insertion line, clutches from heterozygous F2 in-crosses were raised to 5 days post-fertilisation (dpf) and tracked using videography (Figure S1A) to quantify the number and duration of sleep bouts and the average waking activity (time spent moving per one-minute active bouts) over 48 hours. The genotypes of individual larvae were determined by PCR after behavioural tracking, with each larva assigned as wild type, heterozygous, or homozygous for a given viral insertion to assess the effect of genotype on sleep/wake behaviour. While most lines screened had minimal effects on sleep-wake behavioural parameters (Figure S1B-C), one homozygous viral insertion line, 10543/10543, had a reduction in daytime sleep (Figure S1B) and an increase in daytime
waking activity (Figure S1C) relative to their wild type sibling controls. We re-named this 10543
viral insertion line *dreammist (dmist).*

In follow-up studies, we observed that animals homozygous for the viral insertion at this
locus (*dmist*_{vir/vir}) showed a prominent decrease in sleep during the day and a trend to sleep
less at night compared to their wild-type siblings (*dmist*_{+/+}) (Figure 1A). There was an almost
50% reduction in average daytime sleep (Figure 1C) due to a decrease in the number of sleep
bouts (Figure 1D), and sleep bout length at night was significantly reduced (Figure 1E). *dmist*_{vir/vir} larvae also exhibited significantly increased daytime waking activity (Figure 1B, F).

Because Zenemark lines can contain more than one viral insertion (17.6% of lines have >=2
insertions; Varshney et al 2013), we outcrossed *dmist*_{vir/+} fish to wild-type fish of the AB-TL
background and re-tested *dmist* mutant fish over several generations. Normalising all the
behavioural parameters to *dmist*_{+/+} controls with a linear mixed effects (LME) model showed
consistent sleep changes in *dmist*_{vir/vir} fish over N=5 experiments. The *dmist*_{vir/vir} larvae
consistently show a more than 50% decrease in sleep during the day due to a significant
reduction in the number and duration of sleep bouts, as well as a strong increase in waking
activity (Figure 1G). The *dmist*_{vir/vir} mutants also had a significant reduction in sleep at night
compared to wild type siblings (Figure 1G). These effects on sleep and wakefulness are not
due to alterations in circadian rhythms, as behavioural period length in fish that were entrained
and then shifted to free-running constant dark conditions was unaffected in *dmist*_{vir/vir} compared
to wild-type sibling larvae (Figure S2A-C).

The *dmist* gene encodes a novel, small transmembrane protein

Having identified a sleep mutant, we next sought to investigate the target gene disrupted by
the viral insertion. Line 10543 (*dmist*_{vir}) was initially selected for screening due to a predicted
disruption of a gene encoding a serotonin transporter (*slc6a4b*) on chromosome 5. However,
mapping of the dmist viral insertion site by inverse-PCR and sequencing revealed that the virus was instead inserted into the intron of a small two-exon gene originally annotated in the Zv6 genome assembly as a long intergenic non-coding RNA (lincRNA; gene transcript ENSDART00000148146, gene name si:dkey234h16.7), which lies approximately 6 kilobases (kb) downstream of the slc6a4b gene in zebrafish. At least part of this region is syntenic across vertebrates, with a small two-exon gene consistently identified adjacent to the genes ankrd13a and GIT in several vertebrates, including human and mouse (Figure 2A). Amplifying both 5’ and 3’ ends of zebrafish si:dkey234h16.7 and mouse E13.5 1500011B03-001 transcripts with Rapid Amplification of cDNA ends (RACE) confirmed the annotated zebrafish and mouse transcripts and identified two variants with 3’ untranslated regions (3’UTR) of different lengths in zebrafish (Figure S3B). To test whether the viral insertion in dmist^ir^/ir^ disrupts expression of si:dkey234h16.7 or neighbouring genes, we performed quantitative analysis of gene transcript levels in wild type and mutant dmist larvae by RT-qPCR. This revealed that the dmist viral insertion caused a more than 70% knockdown in the expression of si:dkey234h16.7 while the expression of the most proximal 5’ or 3’ flanking genes, slc6a4b_Dr and ankrd13a_Dr, were unaffected (Figure 2B, Figure S3A). Since this disruption of expression is most consistent with si:dkey234h16.7 being the causal lesion of the dmist mutant sleep phenotype, we renamed this gene dreammist (dmist).

Although originally annotated as a lincRNA, computational predictions indicated that the dmist transcripts contain a small open reading frame (ORF) encoding a protein of 70 amino acids (aa) (Figure 2C, Figure S3B). Furthermore, mining of ribosome profiling datasets in zebrafish has shown that dmist is found in polyribosomes (Chew et al., 2013), and chromatin immunoprecipitation sequencing data (Pauli et al., 2015; Ulitsky et al., 2011) had identified that the locus contains histone methylation states associated with protein-coding genes (Aday et al., 2011). Incorporating this evidence, updated zebrafish genome assemblies (Zv9-11) have
annotated dmist as a small protein-coding gene. Querying the human and vertebrate protein databases by BLASTp using the C-terminal protein sequence of Dmist identified orthologs in most other vertebrate clades, including other species of teleost fish, birds, amphibians, and mammals (Figure 2A, C). All identified orthologs encoded predicted proteins with an N-terminal signal peptide sequence and a C-terminal transmembrane domain (Figure 2C). The peptide sequence identity across orthologs ranged from 38 to 84%, with three peptide motifs (QNLV, CVYKP, RRR) showing high conservation across all vertebrates (Figure 2C, Figure S3D). In summary, we found that the dreammist gene, the expression of which is disrupted in dmistvir/vir fish with sleep phenotypes, encodes a protein that is highly conserved across vertebrates at both the genomic and molecular levels.

Genetic molecular analysis of dmist expression in zebrafish and mouse

Because the viral insertion disrupts dmist throughout the animal’s lifetime, we examined both the developmental and spatial expression of dmist to assess when and where its function may be required for proper larval sleep. Using the full-length transcript as a probe (Figure S3B), we performed in situ hybridization across embryonic and larval development. Maternally deposited dmist was detected in early embryos (2-cell stage) prior to the maternal to zygotic transition (Giraldez et al., 2006) (Figure 2D). Consistent with maternal deposition of dmist transcripts, inspection of the 3’ end of the dmist gene revealed a cytoplasmic polyadenylation element (‘TTTTTTAT’; Supplemental Information 2) that is required for zygotic translation of maternal transcripts (Villalba et al., 2011). At 24hpf, transcripts were detected in regions that form the embryonic brain, such as ventral telencephalon, diencephalon and cerebellum, and in the developing eye (Figure 2D, S3C). By 5dpf, dmist transcripts were detected throughout the brain (Figure 2D). To test whether dmist transcripts are under circadian regulation, we performed RT-qPCR in fish that were entrained and then shifted to free-running constant dark
conditions. In contrast with the robust 24-hr rhythmic transcription of the circadian clock gene per1, we did not detect any changes in dmist expression throughout the 24 hour circadian cycle (Figure S2D).

Consistent with brain expression in larval zebrafish, we identified the expression of Dmist_Mm in a published RNAseq dataset of six isolated cell types from mouse cortex (Zhang et al., 2014). We confirmed that Dmist_Mm is specifically enriched in neurons by hierarchical clustering of all 16,991 expressed transcripts across all six cells types, which demonstrated that Dmist_Mm co-clusters with neuronal genes (Figure S3E). Pearson correlation of Dmist_Mm with canonical markers for the six cell types showed that Dmist_Mm expression is highly correlated with other neuronal genes but not genes associated with microglia, oligodendrocytes, or endothelia. This indicates that dmist is specifically expressed in neurons in both zebrafish and mouse (Figure S3F).

Dmist localises to the plasma membrane

Although the dreammist gene encodes a conserved ORF with a predicted signal peptide sequence and transmembrane domain (Figure 2C; Figure S3G-I), we wanted to confirm this small peptide can localise to the membrane and if so, on which cellular compartments. To test these computational predictions, we transiently co-expressed GFP-tagged Dmist (C-terminal fusion) with a marker for the plasma membrane (myr-Cherry) in zebrafish embryos. Imaging at 90% epiboly revealed Dmist-GFP localised to the plasma membrane (Figure 2E). Conversely, introducing a point mutation into Dmist’s signal peptide cleavage site (DmistA22W-GFP) prevented Dmist from trafficking to the plasma membrane, with likely retention in the endoplasmic reticulum (Figure 2F). Together, these data indicate that Dmist localises to the plasma membrane despite its small size, as computationally predicted.
CRISPR/Cas9 generated dmist<i>/i</i>8 mutant exhibits decreased night-time sleep

The original viral insertion line had reduced dmist expression by 70%, suggesting that dmist<sub>vir</sub> is a hypomorphic allele. To confirm that the sleep phenotypes observed in dmist<sup>vir/vir</sup> animals are due to the disruption of Dmist function, we used CRISPR/Cas9 to create an independent dmist loss of function allele. We generated a zebrafish line in which the dmist gene contains an 8bp insertion that causes a frameshift and early stop (dmist<i>/i</i>8, Figure 3A). The dmist<i>/i</i>8 allele is predicted to encode a truncated protein lacking the complete signal peptide sequence and transmembrane domain (Figure 3B), indicating this is likely a null allele. RT-qPCR showed that dmist transcript levels were 60% lower in dmist<i>/i</i>8 fish compared to wild type siblings, consistent with nonsense-mediated decay (Figure S4A, B).

We next assessed the sleep and activity patterns of dmist<i>/i</i>8 fish. As seen in exemplar individual tracking experiments, dmist<i>/i</i>8 larvae sleep less at night due to fewer sleep bouts as well as an increase in waking activity relative to wild type and heterozygous mutant siblings (Figure 3C-H). This significant night-time reduction in sleep and increase in wakefulness is also apparent when combining N=5 independent experiments with an LME model to normalize behaviour across datasets (Figure 3I). Although dmist<sup>vir/vir</sup> larvae also slept less at night (Figure 1G), the large day-time reduction in sleep observed in dmist<sup>vir/vir</sup> larvae is absent in dmist<i>/i</i>8 animals, perhaps because of differences in the dmist<sup>vir</sup> genetic background affecting behaviour. In addition, because the dmist<sup>vir</sup> is likely a hypomorphic allele, we focused subsequent experiments on the CRISPR-generated dmist<i>/i</i>8 larvae.

To test whether the increased night-time activity of dmist<i>/i</i>8 mutants persists in older animals, we raised dmist<i>/i</i>8 mutants with their heterozygous and wild type siblings to adulthood in the same tank and tracked individual behaviour for several days on a 14:10 light:dark cycle. As in larval stages, dmist<i>/i</i>8 adults were hyperactive relative to both dmist<i>/i</i>8/ and dmist<i>/+</i>
siblings, maintaining a higher mean speed at night (Figure 3L-M, S4D). This suggests that
either Dmist affects a sleep/wake regulatory circuit during development that is permanently
altered in dmist mutants or that Dmist is continuously required to maintain normal levels of
night-time locomotor activity.

dmist mutants have altered sleep homeostasis

The timing and duration of sleep is regulated by both circadian and homeostatic processes
(Borbély, 1982). Since the disruption of dmist does not affect circadian regulation of sleep
(Figure S2A-C), we tested whether dmist mutants have deficits in sleep homeostasis.

We have previously shown in zebrafish that a brief exposure to hyperactivity-inducing drugs
such as the epileptogenic pentylentetrazol (PTZ) or wake-promoting caffeine induces a dose-
dependent increase in homeostatic rebound sleep following drug washout that is
phenotypically and mechanistically similar to rebound sleep following physical sleep
deprivation (Reichert et al., 2019). Upon wash-on/wash-off of 5mM PTZ, sleep rebound occurs
in approximately 50% of wild type larvae (Reichert et al., 2019; Figure 3K). In contrast, all
dmisti8/i8 larvae showed increased rebound sleep compared to dmist+/+ sibling controls (Figure
3J, K, Figure S4C). Such sensitivity to sleep pressure induced by neuronal activity suggests
that dmisti8/i8 fish either have accumulated high basal levels of sleep pressure, for example as
a consequence of extended wakefulness at night, or more rapidly accumulate sleep pressure
in response to increased brain activity during waking. This result indicates that dmisti8/i8 fish
have disrupted sleep homeostasis.

In summary, long-term tracking across both dmist alleles and assessment of responses to
drug-induced sleep pressure indicates that Dmist is a sensitive modulator of sleep/wake states.
Dmist is distantly related to the Na\(^+\)/K\(^+\) pump regulator Fxyd1 (Phospholemman)

Because Dmist is a small, single pass transmembrane domain protein without any clear functional motifs, we searched for similar peptides that might provide clues for how Dmist might regulate behaviour. Using the multiple sequence alignment tool MAFFT to align the zebrafish, mouse, and human Dmist peptides (Katoh and Toh, 2010) and seeding a JackHMMR iterative search of the Uniprot database (Johnson et al., 2010) found distant homology between Dmist and Fxyd1/Phospholemman (Figure 4A), a small transmembrane domain peptide that regulates ion channels and pumps, including the Na\(^+\),K\(^+\)-ATPase pump (Crambert et al., 2002). Dmist and Fxyd1 share 27-34% amino acid homology, including an RRR motif at the C-terminal end, although Dmist lacks a canonical FXYD domain (Figure 4A). This similarity suggested that Dmist may belong to a class of small, single pass transmembrane ion pump regulators.

Using \textit{in situ} hybridisation, we found that \textit{fxyd1} is expressed in cells along the brain ventricle and choroid plexus (Figure 4C) in contrast to the neuronal expression of \textit{dmist} (Figure 2D). Despite these different expression patterns, based on their sequence similarity we reasoned that Fxyd1 and Dmist may regulate the same molecular processes that are involved in sleep. To test this hypothesis, we used CRISPR/Cas9 to generate a 28 bp deletion in the 3\(^{rd}\) exon of the zebrafish \textit{fxyd1} gene, causing a frameshift and severely truncated protein that lacks the FXYD, transmembrane, and C-terminal domains (Figure 4B). Contrary to a previous report based on morpholino knockdown (Chang et al., 2012), \textit{fxyd1}^{Δ28/Δ28} larvae were viable with no detectable defect in inflation of the brain ventricles. We therefore tested \textit{fxyd1} mutant larvae for sleep phenotypes. Like \textit{dmist} mutants, \textit{fxyd1}^{Δ28/Δ28} larvae slept less at night (Figure 4D-F). Interestingly, this sleep loss is mainly due to shorter sleep bouts (Figure 4F), indicating that \textit{fxyd1} mutants initiate sleep normally but do not properly maintain it, unlike \textit{dmist} mutants, which initiate fewer night-time sleep bouts, although in both cases there is consolidation of the
waking state at night (Figure 3I). Thus, despite the non-neuronal expression of fxyd1 in the brain, the gene most closely related to dmist also modulates sleep at night.

The brain-wide Na⁺/K⁺ pump alpha subunit Atp1a3a regulates sleep at night

Given the similarity between Dmist and Fxyd1 and their effects on night-time sleep, we hypothesized that mutations in Na⁺/K⁺ pump subunits known to interact with Fxyd1 might also affect sleep. We focused on the Na⁺/K⁺ pump alpha-3 subunit (Atp1a3), as this has been shown to directly interact with Fxyd1 in mammalian brain tissue (Feschenko et al., 2003) and mutations in ATP1A3 in humans causes several neurological and movement disorders, including rapid-onset dystonia parkinsonism (De Carvalho Aguiar et al., 2004) and alternating hemiplegia of childhood (Heinzen et al., 2014, 2012). Murine Dmist expression correlates well with Atp1a3 distribution across 5 brain cell types in mouse (Pearson correlation coefficient = 0.63). Zebrafish have two Atp1a3 paralogs, atp1a3a and atp1a3b. Similar to dmist, atp1a3a is widely expressed in the larval zebrafish brain (Figure 5C, compare to Figure 2D). While atp1a3b is also expressed in the zebrafish brain, its expression is more limited to regions of the midbrain and hindbrain (Figure 5C). To test whether these subunits are involved in regulating zebrafish sleep, we made CRISPR-targeted mutants of both atp1a3a and atp1a3b and isolated an allele with a 19 base deletion in atp1a3a and an allele with a 14 base deletion in atp1a3b. Both mutations are predicted to generate null alleles due to deletion of the start codon (Figure 5A, B). Both atp1a3aΔ19/Δ19 and atp1a3bΔ14/Δ14 mutant larvae were healthy and viable through early development, and, contrary to a previous report based on morpholino injections (Doğanli et al., 2013), neither mutant had defects in the inflation of their brain ventricles. Sleep-wake tracking experiments found that atp1a3bΔ14/Δ14 mutants were slightly more active during the day but did not have sleep phenotypes (Figure 5F, G). In contrast, mutation of atp1a3a resulted in large effects on sleep-wake behaviour. Compared to wild type
and heterozygous siblings, *atp1a3a*\(^{\Delta 19/\Delta 19}\) mutants were hyperactive throughout the day and night, and had a large reduction in sleep at night (Figure 5D, F). The night-time sleep reduction was due to a reduction in the length of sleep bouts, as *atp1a3a* mutants even had a small increase in the number of sleep bouts at night (Figure 5F).

The similar night-time reduction in sleep in *dmist* and *atp1a3a* mutants, combined with the protein homology between Dmist and Fxyd1, suggested that Dmist may be involved in regulating the Na\(^+\)/K\(^+\) pump. Consistent with this hypothesis, brains from both *dmist*\(^{i8/i8}\) and *atp1a3a*\(^{\Delta 19/\Delta 19}\) larvae had elevated intracellular sodium levels after exposure to PTZ (Figure 5G). Thus, neither *dmist* nor *atp1a3a* mutants were able to restore intracellular sodium balance after sustained neuronal activity as quickly as wild type siblings. Consistent with the night-specific alterations in sleep behaviour, we also found that baseline brain Na\(^+\) levels in *dmist* mutants were significantly elevated at night but not during the day (Figure 5H). Collectively, these data are consistent with the hypothesis that night-time sleep duration is affected by changes in Na\(^+\)/K\(^+\) pump function and that Dmist is required for maintaining this function both at night and after sustained neuronal activity.
Genetic screening discovers \textit{dmist}, a novel sleep-regulatory gene

Using a reverse genetic viral screening strategy, we discovered a short-sleeping mutant, \textit{dmist}, which has a disruption in a previously uncharacterized gene encoding a small transmembrane peptide. Given that the \textit{dmist} mutant appeared within the limited number of lines that we screened, it is likely that many other sleep genes are still waiting to be discovered in future screens. In zebrafish, one promising screening strategy will be to employ CRISPR/Cas9 genome editing to systematically target candidate genes. Advances in the efficiency of this technology now makes it feasible to perform a CRISPR "F0 screen" in which the consequences of bi-allelic, gene-specific mutations are rapidly tested in the first generation, with only the most promising lines pursued in germline-transmitted mutant lines (Grunwald et al., 2019; Jao et al., 2013; Shah et al., 2015; Shankaran et al., 2017; Wu et al., 2018). CRISPR F0 screens could be scaled to systematically target the large number of candidate sleep-regulatory genes identified through human GWAS studies and sequencing of human patients suffering from insomnia and neuropsychiatric disorders (Allebrandt et al., 2013; Jansen et al., 2019; Lek et al., 2016; Palagini et al., 2019).

\textbf{Dmist is related to the Na\textsuperscript{+}/K\textsuperscript{+} pump regulator Fxyd1}

The small Dmist transmembrane protein is highly conserved across vertebrates, expressed in neurons, and important for maintaining normal sleep levels. How can such a small, single pass transmembrane protein lacking any clear functional domains modulate the function of neurons and ultimately animal behaviour? The recognition that Dmist has sequence homology (~35\% amino acid similarity) and structural homology (e.g. signal peptide and single pass transmembrane domains; a conserved ‘RRR’ motif) to the Na\textsuperscript{+},K\textsuperscript{+}-ATPase pump regulator Fxyd1 (Phospholemman) offers some important clues.
Fxyd1/Phospholemman is a member of the FXYD protein family, of which there are seven mammalian members (Sweadner and Rael, 2000). Each of the FXYD proteins is small, contains a characteristic FXYD domain, and has a single transmembrane domain. FXYD family members interact with alpha subunits of the Na\(^+\),K\(^+\) ATPase to regulate the function of this pump, with individual family members expressed in different tissues to modulate Na\(^+\),K\(^+\) ATPase activity depending on the physiological needs of the tissue (Geering et al., 2003). In cardiac muscle, FXYD1 is thought to act as a hub through which various signalling cascades, such as PKA, PKC, or nitric oxide, can activate or inhibit Na\(^+\) pump activity (Pavlovic et al., 2013). For example, FXYD1 is critical for mediating the increased Na\(^+\) pump activity observed after β-receptor stimulation via cAMP-PKA signalling (Despa et al., 2008). Much less is known about FXYD1’s role in non-cardiac tissue, although it is expressed in neurons in the mammalian cerebellum, the choroid plexus, and ependymal cells, where it interacts with all three alpha subunits of the Na\(^+\),K\(^+\) ATPase (Feschenko et al., 2003).

In zebrafish, we also found that fxyd1 is expressed in cells around the ventricles and in the choroid plexus (Figure 4C), in contrast to dmist which is expressed in neurons throughout the brain. Despite the different expression patterns, mutation of each gene resulted in a similar reduction of sleep at night. However, unlike dmist mutants, which have fewer sleep bouts (i.e. initiate sleep less) and an increase in waking locomotor activity, fxyd1 mutants have shorter sleep bouts (i.e. cannot maintain sleep) on average and do not have a locomotor activity phenotype. Just as the various FXYD family members modulate the Na\(^+\)/K\(^+\) pump in different tissue- and context-specific ways, this phenotypic variation between fxyd1 and dmist mutants could be due to the different fxyd1 and dmist expression patterns, modulation kinetics of pump/channel dynamics, or interaction with alternative accessory proteins or signal transduction cascades. Nevertheless, the similar timing and magnitude of sleep reduction,
combined with the structural similarity of Fxyd1 and Dmist, suggest that they may regulate similar sleep-related processes.

Dmist, the sodium pump, and sleep

The similarity between Dmist and FXYD1 led us to directly manipulate the Na\(^{+}\),K\(^{+}\) ATPase to test its importance in sleep. The Na\(^{+}\),K\(^{+}\)-ATPase is the major regulator of intracellular Na\(^{+}\) in all cells and, by actively exchanging two imported K\(^{+}\) ions for three exported Na\(^{+}\) ions, is essential for determining cellular resting membrane potential (reviewed in Clausen et al., 2017). The Na\(^{+}\),K\(^{+}\)-ATPase is made up of a catalytic alpha subunit (4 known isoforms, ATP1A1-4), a supporting beta subunit (3 isoforms, ATP1B1-3), and a regulatory gamma subunit (the FXYD proteins). The alpha1 and alpha3 subunits are the predominant catalytic subunits in neurons (alpha2 is mostly restricted to glia), although the alpha1 subunit is also used ubiquitously in all tissues (McGrail et al., 1991). By mutating zebrafish orthologs of Atp1a3, we therefore could test the neuronal-specific role of the Na\(^{+}\),K\(^{+}\)-ATPase in sleep.

Mutations in both zebrafish Atp1a3 orthologs increased waking locomotor behaviour during the day. However, only mutations in the brain-wide atp1a3a, but not in the more restricted atp1a3b, led to changes in night-time sleep. The atp1a3a mutants have a bigger sleep reduction than either dmist\(^{vir}\), dmist\(^{i8}\) or fxyd1\(^{428}\) mutants, which is expected since increased phenotypic severity is a common feature of perturbations in ion channel/pump subunits compared to their modulators (Cirelli et al., 2005; Wu et al., 2014). Autosomal dominant missense mutations leading to loss of function in ATP1A3 cause movement disorders such as rapid-onset dystonia parkinsonism and childhood alternating hemiplegia (recurrent paralysis on one side) in humans (Canfield et al., 2002; Heinzen et al., 2014), while loss of function mutations in Atp1a3 result in generalised seizures and locomotor abnormalities, including...
hypermotility, in mice (Clapcote et al., 2009; Hunanyan et al., 2015; Ikeda et al., 2013; Kirshenbaum et al., 2011; Sugimoto et al., 2014). Our data also suggests that, in addition to movement abnormalities, insomnia at night is a direct behavioural consequence of atp1a3a mutation, consistent with a recent report of very high prevalence of insomnia in patients with childhood alternating hemiplegia, some of which harboured mutations in Atp1a3 (Kansagra et al., 2019). Small molecule screens aimed at ameliorating zebrafish atp1a3a mutant phenotypes may therefore be a promising approach for the rapid identification of new therapies for the management of this disease (Hoffman et al., 2016; Rihel et al., 2010).

Together, the night-specific sleep phenotypes of dmist, fxyd1, and atp1a3a mutants point to a role for the Na⁺,K⁺-ATPase in boosting sleep at night. How might the alpha3 catalytic subunit of the Na⁺/K⁺ pump regulate sleep, and how could Dmist be involved? Compared to the alpha1 subunit, Atp1a3 has a lower affinity for Na⁺ ions, and therefore plays an essential role in repolarizing neurons in response to rapid Na⁺ increases during high levels of neuronal activity (Azarias et al., 2013). We confirmed that Atp1a3a is essential in zebrafish for re-establishing proper brain intracellular Na⁺ levels following high neuronal activity induced by the GABA-receptor antagonist, PTZ. Dmist is also essential for re-establishing brain Na⁺ levels after PTZ exposure (Figure 5G) but not during the baseline day conditions (Figure 5H). Given that the intensity of global neuronal activity impacts the magnitude of subsequent sleep rebound and engagement of the Galanin sleep-homeostatic output arm (Reichert et al., 2019), this model of Dmist-Atp1a3a rebalancing Na⁺ during high neuronal firing can also explain the observed exaggerated sleep rebound in dmist mutants following a short PTZ exposure (Figure 3J) as a consequence of increased neuronal depolarization due to defective Na⁺ pump activity. Intriguingly, a meta-analysis of neuronal transcripts that increase during rodent sleep deprivation found mouse Dmist among the top responding genes (Stuart Peirson, personal communication), suggesting Dmist may play a similar role in regulating neuronal responses to
physical sleep deprivation in mammals. Whether this transcriptional change reflects functional modulation of the Na\textsuperscript{+}/K\textsuperscript{+} pump in mammalian neurons awaits experimental validation.

We also found that Dmist is required for proper maintenance of brain intracellular Na\textsuperscript{+} levels selectively at night but not during the baseline day, mirroring the timing of sleep disruption in dmist\textsuperscript{i8/i8} animals. This suggests that the decreased night-time sleep of dmist mutants is due to a specific requirement for Dmist modulation of the Na\textsuperscript{+}/K\textsuperscript{+} pump at night. However, we cannot exclude the possibility that Dmist's function is required in only a subset of critical sleep/wake regulatory neurons during the day that then influence behaviour at night, such as the wake-active, sleep-homeostatic regulating serotonergic neurons of the dorsal raphe (Oikonomou et al., 2019). Another possibility is that disruption of proper establishment of the Na\textsuperscript{+} electrochemical gradient in dmist mutant neurons leads to dysfunction of various neurotransmitter reuptake transporters, including those for glycine, GABA, glutamate, serotonin, dopamine, and norepinephrine, which rely on energy from the Na\textsuperscript{+} gradient to function (Kristensen et al., 2011). A third possibility is that Dmist and the Na\textsuperscript{+},K\textsuperscript{+}-ATPase regulate sleep not by modulation of neuronal activity per se but rather via modulation of extracellular ion concentrations. Recent work has demonstrated that interstitial ions fluctuate across the sleep/wake cycle in mouse; for example, extracellular K\textsuperscript{+} is high during wakefulness (Ding et al., 2016). Moreover, cerebrospinal fluid containing the ion concentrations found during wakefulness directly applied to the brain can locally shift neuronal activity into wake-like states. Given that the Na\textsuperscript{+},K\textsuperscript{+}-ATPase actively exchanges Na\textsuperscript{+} ions for K\textsuperscript{+}, the high intracellular Na\textsuperscript{+} levels we observe in atp1a3a and dmist mutants is likely accompanied by high extracellular K\textsuperscript{+}. Although we can only speculate at this time, a model in which extracellular ions that accumulate during wakefulness and then directly signal onto sleep-regulatory neurons could provide a direct link between Na\textsuperscript{+},K\textsuperscript{+} ATPase activity, neuronal firing, and sleep homeostasis.
In conclusion, through a genetic screening strategy in zebrafish, we have identified a novel brain expressed gene that encodes a small transmembrane protein regulator of night-time sleep and wake behaviours. Future work will be required to uncover the precise signalling dynamics by which Dmist regulates the Na\(^+\),K\(^+\)-ATPase and sleep.

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MATERIALS AND METHODS

Zebrafish husbandry

All zebrafish lines were housed on a 14hr:10hr light:dark schedule in dechlorinated water at 27.5°C and routine husbandry was performed by the UCL Zebrafish Facility. Embryos were collected from spontaneous spawning and staged according to Kimmel et al. 1995.

Embryos and larvae were raised on a 14hr:10hr light:dark schedule in 10cm Petri dishes at a density of 50 embryos per 10cm Petri dish. Embryo water (~pH7.3, temperature 28.5°C, conductivity ~423.7uS with methylene blue) was changed daily and animals over 4 days post fertilisation were euthanized by overdose of MS-222 (300 mg/l) or 15% 2-Phenoyethanol (77699 SIGMA-ALDRICH) at the end of experiments.

Raising of genetically altered zebrafish and all experimental procedures were performed under project licence 70/7612 and PA8D4D0E5 awarded to JR under the UK Animals (Scientific Procedures) Act 1986 guidelines.

Table 1. Zebrafish lines

| Strain designation | Gene identifier | Additional Information |
|--------------------|-----------------|------------------------|
| 10543/dmist^vir    | ENSDARG00000095754 | Line maintained at J Rihel lab |
| dmist^9             | ENSDARG00000095754 | Line maintained at J Rihel lab |
| fxyd1Δ28           | ENSDARG00000099014 | Line maintained at J Rihel lab |
| atp1a3aΔ19          | ENSDARG0000018259 | Line maintained at J Rihel lab |
| atp1a3bΔ14          | ENSDARG00000104139 | Line maintained at J Rihel lab |
The *dmist*<sup>vir</sup> allele was generated in wild type line T/AB-5 (Varshney et al., 2013) and outcrossed to Harvard AB. The *dmist*<sup>i8</sup>, *fxyd1 Δ28*, *atp1a3a Δ19*, and *atp1a3b Δ14* alleles were generated and maintained at UCL on an AB/TL background. Both *dmist*<sup>i8</sup> and *dmist*<sup>vir</sup> were outcrossed to the AB strain at UCL for 3 generations.

**Larval Tracking**

At 4 days post fertilisation (dpf), zebrafish larvae were placed into individual wells of a 96-square well plate (WHA7701-1651 Sigma-Aldrich) filled with 650 μl of embryo water per well and tracked for 3 days under a 14:10 light:dark schedule (lights on-09:00, lights off-23:00) using automated videotracking in ViewPoint ZebraBoxes (Viewpoint Life Sciences). The 96-well plate was under constant illumination with infrared LEDs, and white LEDs simulated the light:dark schedule. Videography (with one-third inch Dragonfly2 PointGrey monochrome camera, frame rate: 25-30 Hz; fixed-angle megapixel lens, Computar M5018-MP) of individual larval activity was recorded in quantization mode to detect movement by background subtraction between frames in individual wells with 60 second integration time bins. Parameters used for detection were calibrated according to the sensitivity of individual boxes but were in the following range: detection threshold, 15-20; burst, 50 pixels; freeze, 3-4 pixels. Embryo water in the wells was topped up daily with fresh water, and ambient room temperature was maintained at approximately 26°C. Output data was sorted, parsed and analysed by custom Perl and Matlab scripts (MATLAB R2016 version 9.1, The MathWorks), as in Rihel et al. 2010.

Oxygen-permeable lids (Applied Biosystems 4311971) were applied over the top of the 96-well plate when performing experiments in constant darkness, and the larvae were left undisturbed for the duration of the experiment to avoid light exposure.

At the end of the experiment, all larvae were visually checked for health before euthanasia and transfer to individual wells of a 96-well PCR plate for DNA extraction and genotyping.
Behavioural analysis

Sleep parameters were calculated as in Rihel et al. 2010. Behavioural summaries across multiple experiments were determined by using the Matlab fitlme function to fit a linear mixed effects model for each parameter with genotype as a fixed effect and independent experiment as a random effect, then representing the effect size as a % change from the wild type value. Before fitting the linear mixed effects model, the parameters sleep, sleep length, and waking activity were log normalized by calculating the log of 1+ the parameter value for each larva.

Circadian period for every larva was calculated using the Matlab findpeaks function on the activity (delta-pixels) timeseries data with a minimum peak distance of 18 hours (1080 minutes). N-way ANOVA was calculated to evaluate differences between groups.

Code and data are available at https://github.com/ilbarlow/Dmist.

Adult tracking

Fish from a dmist8/+ x dmist8/+ cross were raised in a mixed gender tank to adulthood. Zebrafish adults (aged 3-4 months) were randomly selected and tracked on a 14:10 light:dark cycle (180 lux at water surface, lit from above) for three days as in (Chiu et al., 2016). In brief, fish were placed into uncovered plastic chambers (7x12x8.5 cm; WxLxH) with small holes for water exchange, and these were placed in a circulating water tank (46x54 cm with 4.5 cm water height). This setup was supplied with fish water from the home aquarium heated to 28°C and pumped from a 45L reservoir at a flow rate of 1.3 L/min. Infrared light (60 Degree, 54 LED Video Camera Red Infrared Illuminator Lamp, SourcingMap, with the ambient light detector covered) was continuously supplied from below. Fish were tracked at 15 Hz using Viewpoint Life Sciences ZebraBox tracking software in tracking mode, with a background threshold of 40, inactive cut-off of 1.3 cm/sec, and a small movement cut-off of 8 cm/sec. Each track was visually inspected for errors at one-minute resolution across the entire session and analysed using custom Matlab scripts (MATLAB R2016 version 9.1, The Mathworks, Inc). During the
tracking, the experimenter was blinded to genotype, which was determined by fin-clip after the experiment. Females and males were originally analysed separately; since no significant gender effect was found (two-way ANOVA, genotypeXgender), data from both genders were pooled for the final analysis.

Genotyping

Prior to genotyping, adult fish were anaesthetised in 30 μg/ml MS-222, fin-clipped by cutting a small section of the caudal fin, and then allowed to recover in fresh fish water. For pooled experiments, 3dpf larvae from heterozygous in-crosses were fin-clipped as in Wilkinson et al., 2013 and allowed to recover in a square 96-well plate to keep larvae separate prior to pooling larvae of the same genotype. Genomic DNA was extracted from adult fin clips and larvae by boiling for 30 minutes in 50 μl 1X base solution (0.025 M KOH, 0.2 mM EDTA). Once cooled, an equal volume (50 μl) of neutralisation buffer (0.04 M Tris-HCl) was then added and undiluted genomic DNA used for genotyping.

The dmist\textsuperscript{vir} genotype was detected by PCR (standard conditions) using a cocktail of three primers (0.36 mM final concentration each primer) to detect the wild type allele and viral insertion (see Table 2) so that genotypes could be assigned according to size of bands detected (dmist\textsuperscript{vir/vir} 800bp; dmist\textsuperscript{vir/+} 508bp and 800bp; dmist\textsuperscript{+/+} 508bp).

The dmist\textsuperscript{8} genotype was assigned by KASP genotyping using allele-specific primers (dmist\textsuperscript{8} allele 5'-GATCTCCCT[GCAGAAAGAT]CTTTCTGCA-3' = FAM, dmist\textsuperscript{8} allele 5'-GATCTCCCT[CACCG]CTTTCTGCA-3' = HEX; KASP mastermix KBS-1016-011) and assay were prepared and analysed according to manufacturer’s protocol (LGC genomics).

The atp1a3a\textsuperscript{419} genotype was assigned by KASP genotyping using allele-specific primers (atp1a3a\textsuperscript{419} allele 5'-
GACAGACTGAAGAAACAGCGACTGACGGCTC[CAAAATGGGGGTAAGAGTC]-3' = FAM,

\[ \text{atp1a3a}^+ \text{ allele 5'}-\text{GACAGACTGAAGAAACAGCGACTGACGGCTC}-3' \] = HEX).

The \( \text{atp1a3b}^{Δ14} \) genotype was assigned by PCR using MiSeq_atp1a3b primers (Table 2), with the \( \text{atp1a3b}^{Δ14} \) allele running 14bp faster than the \( \text{atp1a3b}^+ \) allele.

\( \text{fxyd1}^{Δ28} \) was assigned by KASP genotyping using allele-specific primers (\( \text{fxyd1}^{Δ28} \) allele 5'-GAAGGTGGAGTCAACGGATTTAATAAACTTTATTGTGCTTTTGTAGTTGT[A]-3' = HEX,

\( \text{fxyd1}^+ \) allele 5'-GAAGGTGAGTCAACGGATTTAATAAACTTTATTGTGCTTTTGTAGTTGT[G]-3' = FAM) or PCR using MiSeq_fxyd1 primers (see Table 2) followed by digestion with the restriction enzyme Drdl, which yields bands at 138bp and 133bp for \( \text{fxyd1}^{+/+} \); 138bp, 133bp and 271bp for \( \text{fxyd1}^{+/Δ28} \), and 243bp for \( \text{fxyd1}^{Δ28} \).

3'RACE

FirstChoice RLM-RACE kit (Ambion AM1700) was used to amplify the 5' and 3' ends from cDNA obtained from 4dpf larvae raised on a 14:10 LD cycle and C57BL/6 E13.5 mouse embryos obtained from Parnavalas lab (UCL). 5' and 3'RACE primers were designed according the manufacturer’s guidelines (Table 2) and the manufacturer’s protocol was followed. Clones were sequenced by Sanger sequencing.

In situ hybridisation

Probes were designed to target the 3'UTR and entire open reading frame (ORF) of \( \text{dmist}\_\text{Dr} \) transcript using primers that amplified the target region from zebrafish cDNA under standard PCR conditions (expected size 1325bp; Table 2). The PCR product was cloned into pSC vector (Strataclone PCR cloning kit Agilent 240205-12) and verified by Sanger sequencing. Antisense probe was generated by cleavage of pSC-dmist plasmid with XbaI and in vitro transcribed with T3 polymerase (Promega P2083) using 1 µg DNA template according to standard in vitro
transcription protocol (see full protocol at dx.doi.org/10.17504/protocols.io.ba4pigvn). RNA probe was extracted and purified using ZYMO RNA concentrator kit (Zymo #R1013).

Whole mount in situ hybridisation was performed according to (Thisse and Thisse, 2008) with the following adaptations. Embryos <5dpf were dechorionated and fixed at the appropriate stage in 4% paraformaldehyde (PFA) overnight at 4°C. 5dpf larvae were fixed in 4% PFA/4% sucrose overnight at 4°C and then washed 3x5mins in PBS prior to dissecting out the brain. Fixed embryos were washed 3x5mins in PBS, progressively dehydrated into 100% methanol (MeOH) and stored at -20°C overnight. Prior to pre-hybridisation embryos were bleached for 30 mins in the dark (0.05% formamide, 0.5X SSC, 6% H2O2) and then fixed in 4% PFA for 30 mins at room temperature. To image, the embryos were progressively rehydrated into 0.1% PBTw, progressively sunk in to 80% glycerol, and imaged on Nikon compound microscope (Nikon Eclipse Ni, Leica MC190HD camera).

RT-qPCR

Larvae from heterozygous in-crosses (dmist<sup>8/+</sup> or dmist<sup>vir/+</sup>) were genotyped at 3dpf and allowed to recover fully before euthanizing at 5dpf. RNA was extracted from three 5dpf embryos of each genotype by snap freezing in liquid nitrogen and TRIzol RNA extraction (Ambion 15596026) with the following modifications to the manufacturer’s protocol: 400 μl total TRIzol reagent used to homogenise larvae using a pellet pestle homogenizer, and 5 μg glycogen (Invitrogen Cat No. 10814010; 20 μg/μl) was added to the RNA solution after chloroform extraction to aid precipitation of the RNA. The cDNA library was synthesised from high quality RNA (Agilent AffinityScript qPCR cDNA synthesis kit 600559), diluted 1:10, and gene-specific primers (Table 2) were used for amplification of target genes with SYBR green mastermix in BioRad CFX Real-Time qPCR instrument. The expression levels were normalised to the housekeeping gene EF1alpha (primers in Table 2) and analysed using custom Matlab scripts (MATLAB v9.2 2017, The Mathworks 2017).
Sodium Green Assay

Cell permanent Sodium Green tetraacetate (Invitrogen, S6901) was prepared fresh from frozen stock by dissolving in DMSO to 1 mM then diluting in fish water to a final concentration of 10 µM. About 50 larvae (5-7 dpf) from \textit{atp1a3a}^{\text{atf19/+}} or \textit{dmist}^{i8/+} in-crosses were placed in wells of a 6 well plate, then most fish water was removed and replaced with 3 mL of the 10 µM Sodium Green solution for two hours. During exposure, the plate was covered in foil and placed in a 28°C incubator. For PTZ experiments, larvae were also exposed to 10 mM PTZ (diluted from 1mM stock dissolved in water) for two hours. For timepoints at night (ZT17-19), larvae were handled and collected under red light. After soaking in Sodium Green, larvae were washed 3X with fish water, anaesthetised with MS-2222, and fixed in 4% PFA/4% sucrose overnight at 4°C. After 3X wash in PBS, larval brains were dissected and placed in 200 µL PBS in a 48 well plate, and the matched bodies were used for genotyping (see Genotyping). Brains were imaged using an upright MVX10 MacroView microscope with an MC PLAPO 1x objective (both OLYMPUS) with a mercury lamp for fluorescent excitation at 488 nm (OLYMPUS, U-HGLGPS). Images of roughly the same focal plane (dorsal/ventral view) were taken with an XM10 OLYMPUS camera by a single exposure following minimal light exposure (to avoid bleaching). Mean fluorescent intensity was calculated from ROIs placed on the optic tectum/midbrain using ImageJ and normalized to the average fluorescence intensity for each imaging session.

Protein Alignments

Cross-species \textit{dmist} homologues were identified by reciprocal BLASTp of C-terminal region of \textit{Dmist}_\textit{Dr} in vertebrate genomes. Translations of candidate transcript open reading frames were then aligned with \textit{Dmist}_\textit{Dr} using ClustalOmega to calculate the percentage identity matrix (\url{www.ebi.ac.uk/Tools/msa/clustalo/}) and visualised with the tool Multiple Align Show (\url{www.biinformatics.org/sms/multi_align.html}).
To identify Dmist orthologues, Dmist peptides were aligned with the multiple sequence alignment tool MAFFT (Katoh and Toh, 2010) and seeded into a JackHMMR iterative search of the Uniprot database (Johnson et al., 2010). Protein-protein alignments of Dmist to Fxyd1 were then performed using ClustalOmega and visualized with the tool Multiple Align Show.

**CRISPR/Cas9 gene targeting**

CRISPR targets were designed and synthesised according to Gagnon et al., 2014 using ChopChop (Montague et al. 2014; http://chopchop.cbu.uib.no/; see Table 2 for sequences) to identify target sites. 100 pg sgRNA and 300 pg Cas9 mRNA (pT3TS-nCas9n) were injected into the yolk of 1-cell stage AB-TL embryos obtained from natural spawning. F0 fish were screened by high resolution melt (HRM) analysis using gene-specific primers (Table 2) with Precision melt supermix (Biorad 1725112) according to the manufacturer’s protocol in a BioRad CFX RT-PCR thermocycler. Positive founders identified in HRM analysis were then sequenced by Illumina MiSeq sequencing using gene specific primers with adapters (Table 2) according to the manufacturer’s protocol.

**Molecular cloning**

GFP was fused to the Dmist_Dr open reading frame (ORF) by Gateway cloning (Kwan et al., 2007). Gene-specific primers were designed to amplify a PCR product that was recombined with middle donor vector (Table 2; Invitrogen Gateway pDONR221 Cat No. 12536017, Invitrogen Gateway BP Clonase II Cat No. 11789020) to generate a middle entry clone (pME-Dmist). pME-Dmist was recombined with 5’ (p5E-CMV/SP6) and 3’ (p3E-GFPpA) entry clones and destination vector (pDestTol2pA2) using Gateway Technology (Invitrogen LR Clonase II Plus enzyme Cat No. 12538200) following the manufacturer’s protocol.

A 3bp mutation was introduced into the *CMV:dreammist-GFPpA* by inverse PCR using specific primers (Table 2) and KOD high fidelity hot start polymerase (Millipore 71085). The template was degraded by DpnI digest and circular PCR product was transformed into
OneShot TOP10 chemically competent E.coli (Invitrogen C4040). Both CMV:dreammist-GFPpA and CMV:dreammistA22W-GFPpA constructs were checked by Sanger sequencing.

For labelling the plasma membrane, mRNA was in vitro transcribed from pCS2-myr-Cherry linearised with Not1, in vitro transcribed with SP6 mMessage mMACHINE (Ambion AM1340), purified and quantified with a QuBit spectrophotometer, and injected at 0.04 μg/μL.

Microinjection and imaging

For Dmist-GFP and DmistA22W-GFP live imaging, embryos from an AB-TL in-cross were injected with 1 nL of plasmid (7 ng/μL). After developing to 90% epiboly, the embryos were placed on a glass coverslip and observed on an inverted confocal microscope (SPinv, Leica) with a 40X objective.

RNAseq

Larvae from heterozygous in-crosses (dmist^{B/+} x dmist^{B/+} and dmist^{vir/+} x dmist^{vir/+}) were raised to adulthood, genotyped and then homozygous and wild type siblings kept separate. Homozygous and wild-type sibling fish were then in-crossed so that first cousins were directly compared. RNA was extracted from 30 6dpf larvae using the same protocol as for RT-qPCR and sent for RNAseq analysis at the UCL Institute of Child Health with a sequencing depth of 75 million reads per sample. Differential analysis of transcript count level between groups was performed as in Love et al., 2014 and additional analysis was performed using custom Matlab scripts (MATLAB v9.2 2017, The Mathworks 2017).

Mouse RNAseq analysis

The dataset was downloaded from (https://web.stanford.edu/group/barres_lab/brain_rnaseq.html; Zhang et al., 2014) and hierarchical clustering (average linkage) and Pearson correlation calculation analysis were performed using custom Matlab scripts (MATLAB v9.2 2017, The Mathworks 2017).
Experimental Design and Statistical Analyses

Data was tested for normality using the Kolmogorov-Smirnov test. If data were normally distributed, N-way ANOVA (alpha=0.05) was used with correction for multiple comparisons using Tukey’s test. If non-parametric, the Kruskal-Wallis test was used with correction for multiple comparisons using Dunn-Sidak (alpha=0.05). Outliers were removed by Grubb’s test (threshold p<0.01). Data were grouped by genotype and gender for adult experiments and grouped by genotype and day of experiment for larval experiments.

All code is available at https://github.com/ilbarlow/Dmist.
### Table 2. Primer Sequences

| Oligo Name | Sequence (5' -> 3') | Annealing temperature (°C) | Application |
|------------|---------------------|-----------------------------|-------------|
| 1 dmist_vir_fw | CACAGGGATGTGATGCCGGTTAAC | 55 | pmIstVr genotyping |
| 2 dmist_vir_rev | GTACGACATACGCTCACAATCACGTC | 55 | pmIstVr genotyping |
| 3 vir_fw | CACAGGGATGTGATGCCGGTTAAC | 55 | pmIstVr genotyping |
| 4 dmist_Ds_5RACE_fw | CTGTCGCGAGGGTTTGAAG | 55-65 | pmDStRACE |
| 5 dmist_Ds_5RACE_rev_outer | AATGGTCACTCAGCGGTGTC | 55-65 | pmDStRACE |
| 6 dmist_Ds_5RACE_rev_inner | AATTGCACATCGCGGTGTC | 55-65 | pmDStRACE |
| 7 dmist_Ds_3RACE_fw | GAACTGCAGGTGGATTTA | 55-65 | pmDStRACE |
| 8 dmist_Ds_3RACE_rev_outer | GGTATAGCGCTATTGTCGTCCA | 55-65 | pmDStRACE |
| 9 minst_Mn_5RACE_outer | GCCTGTCGCGAGGGTTTGAAG | 55-65 | pmMn3RACE |
| 10 minst_Mn_5RACE_inner | GTGTCGCGAGGGTTTGAAG | 55-65 | pmMn3RACE |
| 11 dmist_Ds-fw | TTTTGCGCAACTGTCACAGC | 56 | pmDSt probe |
| 12 dmist_Ds_rev | CGATTGCTATATGATTTCGCGCACTG | 56 | pmDSt probe |
| 13 pCPR_dmist_fw | CACAGGGATGTGATGCCGGTTAAC | 60 | RT-qPCR |
| 14 pCPR_dmist_rev | TGCGACATACGCTCACAATCACGTC | 60 | RT-qPCR |
| 15 pCPR_andr13a_fw | GAACTGCAGGTGGATTTA | 60 | RT-qPCR |
| 16 pCPR_andr13a_rev | GAACTGCAGGTGGATTTA | 60 | RT-qPCR |
| 17 pCPR_sick4a-fw | ACATGGTGGCGAGGGTTTGAAG | 60 | RT-qPCR |
| 18 pCPR_sick4brev | TCCACGCAATTGATGCGATACGTCG | 60 | RT-qPCR |
| 19 elfalpha_fw | GTCCTGCGAGGGTTTGAAG | 60 | RT-qPCR |
| 20 elfbeta-fw | GTCCTGCGAGGGTTTGAAG | 60 | RT-qPCR |
| 21 Stratmin_sRNA | ATTAGCAGCTACATAGCTT | n/a | CRSPR |
| 22 Tapa13a_sRNA | ATTAGCAGCTACATAGCTT | n/a | CRSPR |
| 23 Tapa4b_sRNA | ATTAGCAGCTACATAGCTT | n/a | CRSPR |
| 24 HRM_dmist_fw | GCCACATCTTACGACAGCGCA | 59 | HRM |
| 25 HRM_dmist_rev | GCCACATCTTACGACAGCGCA | 59 | HRM |
| 26 HMRN酯_hfw | GAGACAGCTTGATGATGCGATACGTCG | 55 | HRM |
| 27 HMRN酯_hrev | GAGACAGCTTGATGATGCGATACGTCG | 55 | HRM |
| 28 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 29 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 30 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 31 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 32 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 33 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 34 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 35 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 36 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 37 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 38 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 39 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 40 HMRN酯_hfw | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 41 HMRN酯_hrev | AAATCGACCTACGAGCGATACGTCG | 58 | HRM |
| 42 attB3-dreamist FORWARD primer | GTTACGATATGATGATGCGATACGTCG | 55-60 | Gateway |
| 43 Dmst-GPPA23Fw | GCTTTCATGCTCGAGCGATACGTCG | 66 | DmS |
| 44 Dmst-GPPA23Rev | GCTTTCATGCTCGAGCGATACGTCG | 66 | DmS |
**Figure Legends**

**Figure 1. A viral insertion mini-screen identifies a short-sleeping mutant, dreammist.**

A-B) Representative 48hr mean sleep (A) and waking activity (B) traces of progeny from *dmist*<sup>vir/+</sup> in-cross from the initial screened clutch. White blocks show day (lights on) and grey blocks show night (lights off). The ribbon around each trace indicates standard error of the mean.

C-F) Analysis of sleep/wake architecture of *dmist*<sup>vir/vir</sup> in the original screen during the day (white panel) and night (grey panel). C) Quantification of total sleep across two days and nights shows decreased day and night sleep in *dmist*<sup>vir/vir</sup>. Analysis of sleep architecture reveals fewer sleep bouts during the day (D) and shorter sleep bouts at night (E) in *dmist*<sup>vir/vir</sup> compared with sibling controls. F) Daytime waking activity is also increased in *dmist*<sup>vir/vir</sup> (*dmist*<sup>+/+</sup> n=38, *dmist*<sup>vir/+</sup> n=84, *dmist*<sup>vir/vir</sup> n=19). The black lines show the mean ± SEM, except in E, which labels the median. *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA, Tukey’s post hoc test.

G) Combining N=5 independent tracking experiments using a linear mixed effects model with genotype as a fixed effect and experiment as a random effect reveals *dmist*<sup>vir/vir</sup> larvae have decreased total sleep and changes to sleep architecture during both the day and night compared to *dmist*<sup>+/+</sup> siblings. Plotted are the genotype effect sizes (95% confidence interval) on each parameter relative to wild type. Shading indicates day (white) and night (grey), and p-values are assigned by an F-test on the fixed effects coefficients from the linear mixed effects model. *p<0.05, **p<0.01, ***p<0.001, ns p>0.05
**Figure 2.** *dmist* encodes a conserved vertebrate single pass transmembrane protein.

A) *dmist* mutants harbour a viral insertion in the 1st intron of *si:key0234h16.7. *dmist* is syntenic with *ankrd13* and GIT in mouse, human, and zebrafish.

B) Quantitative RT-qPCR of *dmist* (red) show reduced expression of *dmist* and not the 5’ and 3’ flanking zebrafish genes, *slc6a4b* (cyan) and *ankrd13a* (blue), in *dmist*vir/vir larvae compared to *dmist*vir/+ and *dmist*+/+ siblings. **p<0.01, *p<0.05; one-way ANOVA, Tukey’s post-hoc test.** Data shows mean ± SEM normalized to the wild type mean.

C) *dmist_Dr* contains an open reading frame encoding a 70 amino acid protein that is conserved across vertebrates. All identified homologues have a predicted signal peptide sequence (magenta line), signal peptide cleavage site (magenta circle), and predicted transmembrane domain (grey), with additional highly conserved C-terminal small peptide motifs (blue lines). Identical amino acids in all species are shown in black; similar amino acids (80-99% conserved across species) are shown in grey.

D) *In situ* hybridisation using *dmist* antisense probe reveals *dmist* is maternally deposited as it is detected at the 2-cell stage. At 24hpf expression is restricted to regions containing neuronal precursors, and at 5dpf expression is widespread throughout the brain. Tel, telencephalon; Dien, diencephalon; R1-6, rhombomeres 1-6. Scale bars= 0.5 mm (2 cell and 24 hpf), 0.1 mm (5 dpf).

E-F) Representative confocal image of 90% epiboly embryo co-injected at the 1-cell stage with mRNA encoding either C-terminal tagged Dmist-GFP (E, green) or DmistA22W-GFP (F, green) and membrane-RFP (magenta). Scale bar= 25 μm.
Figure 3. CRISPR-generated *dmist* mutants sleep less and are hyperactive at night.

A) CRISPR/Cas9 targeting of the first exon in *dmist* resulted in an 8bp insertion (*dmist*<sup>i8</sup>) (grey line) within the coding sequence leading to an early stop codon (red line with *).

B) Predicted Dmist<sup>i8</sup> peptide sequence lacks most of the N-terminal signal peptide sequence (magenta) and the full C-terminus.

C-D) Representative 48 hr traces of sleep (C) and waking activity (D) for *dmist*<sup>i8/i8</sup> fish compared to *dmist*<sup>i8/+</sup> and *dmist*<sup>+/+</sup> siblings shows decreased sleep and increased waking activity at night. Mean ±SEM is shown. n=number of animals.

E-H) Analysis of sleep/wake architecture of the experiment depicted in (C, D) indicates that *dmist*<sup>i8/i8</sup> larvae sleep less at night (E) due to fewer sleep bouts (F). Sleep bout length is unchanged (G). Waking activity was also increased in *dmist*<sup>i8/i8</sup> fish (H). The black line represents the mean ± SEM except for G, which is the median. *p<0.05, **p<0.01, ***p<0.001; One-way ANOVA, Tukey’s post hoc test.

I) Combining 5 independent experiments with a linear mixed effects model reveals *dmist*<sup>i8/i8</sup> fish sleep less due to fewer sleep bouts, and also show increased waking activity at night. Plotted are the genotype effect sizes (95% confidence interval) on each parameter relative to wild type. Shading indicates day (white) and night (grey). P-values are assigned by an F-test on the fixed effects coefficients from the linear mixed effects model. *p<0.05, **p<0.01, ***p<0.001, ns p>0.05

J) *dmist*<sup>i8/i8</sup> larvae have increased rebound sleep compared to wild type siblings following exposure to 5mM PTZ. Representative sleep traces of *dmist*<sup>+/+</sup> (no drug, water vehicle controls in black; PTZ exposed in blue) and *dmist*<sup>i8/i8</sup> (no drug in purple; PTZ exposed in red) following 1 hr exposure to 5mM PTZ (black bar) in the morning. Data are mean ±SEM. *dmist*<sup>i8/+</sup> animals are not plotted for clarity but are included in panel K.
K) Rebound sleep after exposure to 5mM PTZ, calculated from the experiment in J. Each dot represents a single larva, grey lines show mean ± SEM. As shown in Figure S4C, normalizing the PTZ responses to the mean control baselines for each genotype shows that dmist<sup>i8/i8</sup> fish have a significantly increased response relative to their sibling controls (p<0.05, one-way ANOVA).

L-M) Adult dmist<sup>i8/i8</sup> fish are more active at night. 48-hour centroid tracking shows dmist<sup>i8/i8</sup> adults have a higher mean swim speed compared to their wild type siblings (L). Mean speed at night is quantified in M (cross shows mean ± SEM). *p<0.05, one-way ANOVA.
Figure 4. Mutation of the dmist related gene fxyd1 causes reduced sleep at night.

A) Schematic of zebrafish Dmist and Fxyd1 protein domains and alignments comparing human, mouse, and zebrafish Dmist and FXYD1 protein sequences. Black and grey shading indicate amino acid identity and similarity, respectively. The FXYD domain is highlighted with a red line and the RRR motif in the C-terminus is indicated with a blue line.

B) CRISPR-Cas9 targeting of the 3' exon of fxyd1 created a 28 bp deletion predicted to encode a truncated protein. The start codon is marked by a blue line. Guide RNA target sequence and PAM sequence are shown as grey bars.

C) In situ hybridisation of fxyd1 at 24hpf (whole animal) and 5dpf brain (ventral view). Anterior is to the left. Scale bar = 0.5 mm (24hpf); 0.1 mm (5 dpf).

D-E) Representative single tracking experiment showing fxyd1Δ28 mutants have decreased night-time sleep (D) but normal waking activity (E).

F) Combining 5 independent experiments with a linear mixed effects model reveals fxyd1Δ28/Δ28 larvae sleep significantly less at night due to a shorter sleep bout length compared to fxyd1+/+ siblings. Plotted are the genotype effect sizes (95% confidence interval) on each parameter relative to wild type. Shading indicates day (white) and night (grey). P-values are assigned by an F-test on the fixed effects coefficients from the linear mixed effects model.

*p<0.05, **p<0.01, ***p<0.001, ns p>0.05
Figure 5. Mutations in the Na+/K+ pump alpha subunit *atp1a3a* reduces sleep at night

A) CRISPR-Cas9 targeting of the *atp1a3a* resulted in a 19 bp deletion that eliminates the start codon (blue) and splice junction.

B) CRISPR-Cas9 targeting of *atp1a3b* resulted in a 14 bp deletion that eliminates the start codon (blue).

C) *In situ* hybridisation of *atp1a3a* and *atp1a3b* at 24hpf (whole animal) and 5dpf brain (ventral view). Anterior is to the left. Scale bar = 0.5 mm (24 hpf); 0.1 mm (5 dpf).

D) Representative single tracking experiment showing *atp1a3aΔ19/Δ19* fish are hyperactive throughout the day-night cycle and have decreased sleep at night.

E) Representative single tracking experiment showing *atp1a3bΔ14/Δ14* mutants have increased daytime waking activity but normal sleep patterns.

F) *atp1a3aΔ19/Δ19* larvae sleep less at night due to shorter sleep bouts. Plotted are the genotype effect sizes (95% confidence interval) on each parameter relative to wild type. Shading indicates day (white) and night (grey). P-values are assigned by an F-test on the fixed effects coefficients from the linear mixed effects model. *p<0.05, **p<0.01, ***p<0.001, ns p>0.05

G) Brain sodium levels are significantly elevated after exposure to PTZ in both *atp1a3aΔ19/Δ19* (N=2) and *dmis*i8/i8 (N=4) fish relative to wild type and heterozygous mutant siblings, as measured by fluorescence intensity of Sodium Green, normalized to the sample mean intensity. Crosses show mean ± SEM. *p<0.05, **p<0.01, one-way ANOVA

H) Under baseline conditions, brain sodium levels are significantly elevated in *dmis*i8/i8 fish at night but not during the day, as measured by fluorescence intensity with Sodium Green. Crosses show mean ± SEM. *p<0.05, **p<0.01, one-way ANOVA
Figure S1. A viral insertion screen for sleep-wake regulators

A) Schematic of screening strategy. Candidate genes were selected from a list of 904 mammalian genes encoding protein classes most often linked to behavioural regulation, including 1) genes previously implicated in sleep and circadian rhythms; 2) G-protein coupled receptors; 3) neuropeptide ligands; 4) channels; and 5) proteins involved in post-translational regulation, such as de-ubiquitinating enzymes (Supplemental Data 1). tBLASTN of the human protein sequences identified 1162 zebrafish orthologs (Zv6), of which 702 (60.4%) had viral inserts mapped in the ‘Zenemark’ zebrafish viral insertion library (Varshney et al., 2013). Sperm harbouring viral insertions in 25 loci were successfully used for in vitro fertilization and propagated to the F3 generation for screening. F3 larvae from single family F2 in-crosses were monitored on a 14hr:10hr light:dark cycle from 4-7dpf using videography and genotyped at the end of the experiment.

B-C) Histogram of total daytime sleep (B) and average daytime waking activity (C) normalized as standard deviations from mean (Z-score) of all the viral-insertion lines tested (including heterozygous vir/+ and homozygous vir/vir). Line 10543 (renamed dreammist) exhibited decreased daytime sleep and increased daytime activity.
**Figure S2.** *dmist*<sup>vir/vir</sup> fish are hyperactive and have normal circadian rhythms.

A) Free-running circadian period length of locomotor activity of fish that were entrained until 5 dpf in a 14hr:10hr light:dark cycle and then shifted to constant darkness, and quantified for 48 hours after the shift to darkness, shows no difference between *dmist*<sup>vir/vir</sup> larvae and their sibling controls. p>0.05, one-way ANOVA.

B-C) Representative 48hr sleep (B) and waking activity (C) traces of progeny from *dmist*<sup>vir/+</sup> in-cross following the transition from a 14hr:10hr light:dark cycle to constant dark conditions. Light and dark grey blocks show subjective day and night, respectively. Data are mean ± SEM.

D) Quantitative RT-PCR time-course before (light) and after (grey) transfer into constant dark demonstrates that *dmist* mRNA levels do not oscillate with a circadian period, unlike *per1* mRNA which does. n=3 replicates per timepoint. Expression is normalized to circadian time 3. Data are mean ± SEM.
Figure S3. *dmist* is enriched in neurons and requires the signal peptide cleavage site for membrane localisation.

A) Relative expression level of *dmist* transcript from RNA sequencing of 6dpf *dmist*<sup>vitr/vitr</sup> and *dmist*<sup>vitr</sup>/siblings. Z-score calculated by subtracting mean expression and normalising by the standard deviation across all expressed transcripts (27,243 transcripts). Data show mean ± SEM from 3 independent biological replicates. **p<0.01 Student’s t-test.

B) 3’ and 5’ RACE identify a long (1100bp) and short (215bp) 3’UTR variant in *dmist_Dr*, and a long 3’UTR (1050bp) in *Dmist_Mm*. The purple arrow indicates the ISH probe used in Figure 2D.

C) *dmist_Dr* sense probe at 24hpf shows no detectable expression.

D) Percentage identity matrix comparing *Dmist* homologues across 6 vertebrate species (100%=magenta; >70%=purple; >50%=cyan; <50%=green).

E) Hierarchical clustering of RNAseq dataset of 6 different cell types isolated from the developing (E13.5) mouse brain (Zhang et al., 2014) and post-hoc identification of *Dmist_Mm*. Data standardized by subtracting the mean expression and normalizing by the standard deviation across all expressed transcripts in each cell type (column). *Dmist_Mm* (green arrow) co-clusters with genes highly expressed in neurons (green shaded branches).

F) Pearson rank correlation of canonical cell-type markers with *Dmist_Mm* shows high co-expression with neuronal markers compared to astroglial markers. Data are mean ± SEM *p<0.05, **p<0.01; Kruskal-Wallis, Dunn-Sidak post-hoc test.

G-I) Predicted processing of Dmist to its mature form in the plasma membrane (G) and C-terminal GFP fusion to Dmist is predicted to localise to the membrane (H) The black triangle represents the cap. However, a mutation (A22W) at the signal peptide cleavage site (I) is
predicted to inhibit signal peptide cleavage and so prevent proper subcellular localisation of the mature protein.
Figure S4. CRISPR-generated dmist mutants have altered sleep and arousal phenotypes

A) Quantitative RT-PCR shows dmist<sup>i8/i8</sup> larvae have reduced dmist mRNA levels, suggesting that dmist<sup>i8</sup> transcripts undergo nonsense mediated decay. Data are mean ± SD of three biological replicates. **p<0.01; one-way ANOVA, Tukey’s post-hoc test.

B) Relative expression level of dmist transcript from RNA sequencing of 6dpf dmist<sup>i8/i8</sup> and dmist<sup>+/+</sup> siblings. Z-score calculated by subtracting mean expression and normalising by the standard deviation across all expressed transcripts. Data are mean ± SEM for 3 independent biological replicates. **p<0.01, Student’s t-test.

C) Effect size of change in sleep after 1 hour 5mM PTZ treatment (and washout) compared to vehicle treated controls (error bars show 95% confidence intervals). *p<0.05, one-way ANOVA.

D) Cumulative probability distribution of the all night-time swimming speeds in representative adult tracking experiment. The dashed lines show the half max (0.5 probability) for each curve. n indicates number of fish. *p<0.05; Kolmogorov-Smirnov test.
Figure 2

A) Schematic representation of the genomic organization of the dmist gene in Danio rerio, Mus musculus, and Homo sapiens. Gene locations and relative expression levels are shown. The dmist gene is highlighted in red.

B) Bar graphs showing relative expression levels of the dmist gene in different genotypes (+/+, vir/+; and vir/vir). The expression levels are normalized to a control gene.

C) Predicted signal peptide and transmembrane domains for the dmist gene across different species. The signal peptide and transmembrane domains are highlighted in blue and purple, respectively.

D) Images of 2-cell embryos, 24-hour post-fertilization (hpf) lateral and dorsal views, and 5-day post-fertilization (dpf) dorsal view. The dmist expression is shown in blue.

E) Immunostaining of myr-Cherry and CMV::dmist-GFP in the same cell. The merged images show the expression patterns of both constructs.

F) Immunostaining of myr-Cherry and CMV::dmistA22W-GFP in the same cell. The merged images show the expression patterns of both constructs.
Figure 3

(A) dmist_Dr dmist

(C) Sleep (mins/10 mins)

(D) Waking Activity (sec/min, 10 mins)

(E) Sleep (mins/hr)

(F) # Sleep bouts (#/hr)

(G) Sleep bout length (mins)

(H) Waking activity

(I) % Effect size relative to WT

(J) Sleep (min/10 min)

(K) Rebound Sleep (min)

(L) Mean speed (cm/sec)

(M) Mean speed (cm/sec)

* * * ns

+PTZ

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Figure 4
Figure S4