LIN-32/Atonal Controls Oxygen Sensing Neuron Development in Caenorhabditis elegans

Romanos, Teresa Rojo; Pladevall-Morera, David; Langebeck-Jensen, Kasper; Hansen, Stine; Ng, Leelee; Pocock, Roger

Published in:
Scientific Reports

DOI:
10.1038/s41598-017-07876-4

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Romanos, T. R., Pladevall-Morera, D., Langebeck-Jensen, K., Hansen, S., Ng, L., & Pocock, R. (2017). LIN-32/Atonal Controls Oxygen Sensing Neuron Development in Caenorhabditis elegans. Scientific Reports, 7(1), [7294]. https://doi.org/10.1038/s41598-017-07876-4
LIN-32/Atonal Controls Oxygen Sensing Neuron Development in Caenorhabditis elegans

Teresa Rojo Romanos\textsuperscript{1,2}, David Pladevall-Morera\textsuperscript{1,2}, Kasper Langebeck-Jensen\textsuperscript{2}, Stine Hansen\textsuperscript{2}, Leelee Ng\textsuperscript{1} & Roger Pocock\textsuperscript{1,2}

Development of complex nervous systems requires precisely controlled neurogenesis. The generation and specification of neurons occur through the transcriptional and post-transcriptional control of complex regulatory networks. In vertebrates and invertebrates, the proneural basic-helix-loop-helix (bHLH) family of transcription factors has multiple functions in neurogenesis. Here, we identified the LIN-32/Atonal bHLH transcription factor as a key regulator of URXL/R oxygen-sensing neuron development in Caenorhabditis elegans. When LIN-32/Atonal expression is lost, the expression of URX specification and terminal differentiation genes is abrogated. As such, lin-32 mutant animals are unable to respond to increases in environmental oxygen. The URX neurons are generated from a branch of the cell lineage that also produces the CEPDL/R and URADL/R neurons. We found development of these neurons is also defective, suggesting that LIN-32/Atonal regulates neuronal development of the entire lineage. Finally, our results show that aspects of URX neuronal fate are partially restored in lin-32 mutant animals when the apoptosis pathway is inhibited. This suggests that, as in other organisms, LIN-32/Atonal regulates neuronal apoptosis.

The development of complex systems like the brain requires the generation of a vast array of cell types with distinct morphology and function. Such cellular diversity is achieved by transcription factors and microRNAs that regulate the generation and specification of terminal neuronal subtypes\textsuperscript{1,2}. Previous studies using model organisms have shown that a family of basic-helix-loop-helix transcription factors act as proneural regulators to control the development of neuroectodermal progenitor cells\textsuperscript{3}. Proneural genes were first identified in Drosophila in the 1980s and have subsequently been shown to have multiple functions in the developing brain, including neuronal specification and guidance\textsuperscript{4-6}. Atonal is a well-studied member of the proneural basic-helix-loop-helix transcription factor family that was originally identified in Drosophila, where it is required for correct development of sensory organ precursors\textsuperscript{7}. The Atonal homolog in mouse (Atoh1) is also expressed in the developing nervous system where it performs conserved functions in the development of the cochlea and the specification of auditory hair cells\textsuperscript{8-10}. Multiple studies have shown catastrophic apoptosis in the cochlea of Atoh1-null mouse embryos, leading to hearing loss\textsuperscript{8,10,11}. In C. elegans, the Atonal homolog LIN-32 is required for the development of multiple neuronal lineages that generate sensory rays within the male reproductive organ\textsuperscript{12,13}, mechanosensory neurons\textsuperscript{14,15}, Q neuroblasts\textsuperscript{16} and the CEPD and PDE dopaminergic neurons\textsuperscript{17}.

We study the URXL/R body cavity neurons in C. elegans as a model for neuronal development. These neurons regulate multiple aspects of animal behaviour and physiology\textsuperscript{18,19}. The cell bodies and ciliated dendrites of the URX neurons are positioned within the coelomic fluid\textsuperscript{20}, which potentially enable these neurons to receive and transmit information systemically. A major function of the URX neurons is in oxygen sensing\textsuperscript{18}. Through expression of the soluble guanylate cyclases GCY-35 and GCY-36, the URX neurons coordinate behavioral responses to sensing of environmental O$_2$ upshifts\textsuperscript{18}. In addition, a recent function for the URX neurons has been identified in the integration of O$_2$ availability and internal metabolic state\textsuperscript{19}.

The specialised functions of the URX neurons are facilitated through the expression of particular terminal differentiation genes\textsuperscript{18,19,21}. We and others previously found that expression of terminal fate reporters for URX neuron fate is regulated by the Sox transcription factor EGL-13 and the AHR-1/aryl hydrocarbon receptor\textsuperscript{12,22}.

\textsuperscript{1}Development and Stem Cells Program, Monash Biomedicine Discovery Institute and Department of Anatomy and Developmental Biology, Monash University, Melbourne, Victoria, 3800, Australia.  \textsuperscript{2}Biotech Research and Innovation Centre, University of Copenhagen, Ole Maaløes Vej 5, Copenhagen, Denmark. Correspondence and requests for materials should be addressed to R.P. (email: roger.pocock@monash.edu)
Here, we find that LIN-32/Atonal is also required for the correct development of the URX O$_2$-sensing neurons. In lin-32 mutant animals, reporters for URX-expressed transcription factors, including EGL-13, and terminal differentiation genes are abrogated. As a result, lin-32 mutant animals are unable to respond to O$_2$ upshifts. We found that LIN-32 is also required for the expression of terminal fate reporters for other neurons generated from the URX sublineage (URADL/R and CEPDL/R), indicating that neuronal development within the entire sublineage is defective. Our subsequent investigation found that aspects of URX neuronal fate are restored when apoptosis is perturbed, suggesting that LIN-32 either acts to inhibit apoptosis in this lineage or that the misspecified state of these neurons promotes cell death. As such, our study has revealed a previously unappreciated function of LIN-32/Atonal in regulating O$_2$-sensing neuron development in the brain.

**Results**

**LIN-32/Atonal is required for URX neuron development.** In order to identify regulators required for the development of the URX O$_2$-sensing neurons, we performed a classical EMS forward genetic mutagenesis screen using the URX fluorescent reporter strain ynlIs22[flp-8::GFP]. This reporter is consistently expressed in the URXL/R neurons in addition to the AUAL/R and PVM neurons. We isolated a mutant allele, rp1, which exhibits highly penetrant loss of GFP expression in both the URX and PVM neurons (Fig. 1A). We used single nucleotide polymorphism (SNP) mapping to identify the molecular lesion responsible for this defect. This analysis narrowed down the location of the lesion close to genetic position –17 on the X chromosome. Mutations in the lin-32 gene, which encodes a bHLH transcription factor and is located close to this region (~16.05), had previously been described to have defects in PVSM specification. Therefore, we manually sequenced the lin-32 locus and identified a base-pair change C$\rightarrow$T in the rp1 mutant, which converts a glutamine (Q) at position 126 to a premature amber STOP codon (Fig. 1B). To confirm that loss of LIN-32 causes defects in URX development, we crossed the flp-8::GFP fluorescent reporter with the previously described lin-32(tm1446) allele, a 511 bp deletion that removes the coding region of the bHLH domain, and is a presumed null allele. Loss of flp-8::GFP expression in the URX neurons was phenocopied in the lin-32(tm1446) mutant strain showing that LIN-32 is important for URX development (Fig. 1C). To confirm that loss of LIN-32 is causative, flp-8::GFP expression was restored in the URX neurons of lin-32(tm1446) mutant animals through expression of an integrated lin-32::GFP rescuing translational transgene (ecls10) (Fig. 1C).

To better understand the function of lin-32 in URX specification, we used fluorescent reporter strains to monitor expression of a battery of terminal genes expressed in the URX neurons (Fig. 2A,B). We analyzed the expression of the terminally-expressed guanylate cyclases GCY-35 and GCY-36, and the Phe-Met-Arg-Phe-NH$_2$ (BAG-mediated response) and from 10% to 21% O$_2$ (URX-mediated response). In contrast to wild type, we found that LIN-32 is required for the expression of terminal fate reporters for other neurons generated from the AB lineage and are sisters of the dopaminergic CEPDL/R neurons (Fig. 3A). We asked whether regulatory elements evoking terminal fate are abrogated. As a result, lin-32 mutant animals are unable to respond to O$_2$ upshifts, whereas the ability to respond to O$_2$ downshifts was unaffected (Fig. 2C). These data indicate that the O$_2$-sensing function of the URX neurons is abrogated in lin-32(tm1446) mutant animals, likely due to the loss of guanylate cyclase expression.

**lin-32 mutants are defective in oxygen sensing.** The URX neurons are required for sensing and coordinating responses to fluctuations of O$_2$. These responses are controlled by the guanylate cyclases GCY-35 and GCY-36. Since LIN-32 is required for the expression of these O$_2$-sensing guanylate cyclases, in addition to the URX specification transcription factors, we hypothesized that lin-32 mutant animals would be defective in O$_2$ sensing. We therefore used a well-established behavioral paradigm to examine the importance of LIN-32 in O$_2$ sensing. We tracked animals in a chamber without food, in an air-flow that switched between 21% to 10% O$_2$. We crossed a URAD neuron reporter, ynlIs22[flp-8::GFP], into the lin-32(tm1446) mutant strain showing that LIN-32 is important for URX development (Fig. 1C). We found that loss of LIN-32 is causative, flp-8::GFP expression was restored in the URX neurons of lin-32(tm1446) mutant animals through expression of an integrated lin-32::GFP rescuing translational transgene (ecls10) (Fig. 1C).

**lin-32 controls the development of all neurons in the URX lineage.** The URXL/R neurons are derived from the AB lineage and are sisters of the dopaminergic CEPDL/R neurons (Fig. 3A). We asked whether LIN-32 performs a specific function in the development of the URX neurons or plays a more general role in the development of other neurons closely related by lineage. To this end, we crossed lin-32(tm1446) mutant animals into the vtsIs[dat-1::GFP] fluorescent reporter strain, which is expressed in all dopaminergic neurons in C. elegans, including the CEPD neurons. We found that the expression of this reporter is abolished in the CEPD neurons of lin-32(tm1446) mutant animals (Fig. 3B), confirming a previous study. The URX and CEPD neurons are generated from the ABplaaa and ABarpapa branches of the AB lineage. The other cells generated by these lineages are four hypodermal cells (two hyp4 and two hyp6 cells), the URADL/R neurons and four additional cells that undergo apoptosis. We therefore asked whether LIN-32 is also required for the development the URAD neurons, which are generated slightly earlier and from an adjacent branch of the lineage to the URX neurons. We crossed a URAD neuron reporter, ynlIs22[flp-21::GFP], into the lin-32(tm1446) mutant strain and observed that expression in the URAD neurons was completely abolished. Therefore, LIN-32 is required for the development of all neurons in this sublineage (URX, CEPD and URAD). Previous studies have shown that in the absence of LIN-32, neurons from certain lineages exhibit a hypodermal state. We examined whether additional hypodermal cells are present in lin-32(tm1446) mutant animals using a hypodermal fluorescent reporter strain rp5Is109[dpy-7::NLS::dsRed2]. We found that the number of hypodermal cells present in the head of lin-32(tm1446) mutant animals (17–24 cells) was different from wild type (17–25 cells) in the head of L4 larvae (N > 63). This suggests that the six neurons lost from the ABplaaa and ABarpapa lineages in lin-32 mutant
animals do not acquire a hypodermal fate. Taken together, these results indicate that LIN-32 is required for the development of all neurons generated from this sublineage.

ham-1 mutants exhibit defects in URX development. LIN-32 has been shown to function in parallel with the storkhead transcription factor, HAM-1 (HSN abnormal migration), to regulate the migration and division of Q neuroblasts. Thus, we tested whether similar means of regulation exist during URX development by crossing a ham-1(n1438) strong loss-of-function mutant with two URX fluorescent reporters - kylIs417[gcy-36::GFP] and ynlIs22[flp-8::GFP]. We found that ham-1(n1438) mutant animals exhibit defects in the

Figure 1. rp1 is a lesion in lin-32 and causes URX developmental defects. (A) Quantification of rp1-induced URX defects using the flp-8::GFP reporter of URX fate. flp-8::GFP is expressed in the URX, AUA and PVM neurons. In rp1 animals, expression of flp-8::GFP is almost abolished in the URX and PVM neurons while expression in the AUA neurons is unaffected. URX-like and 2URX-like indicate that the neuronal morphology is similar to URX but the neuron is positioned incorrectly. N > 60. Statistical significance between wild-type and lin-32(rp1) animals was evaluated using a t-test. ****P < 0.0001. (B) Molecular identity of the rp1 allele and the previously described lin-32(tm1446) deletion allele. The rp1 lesion is a C to T transition that converts a glutamine (Q) to a premature amber stop codon. (C) Quantification of flp-8::GFP expression in the URX neurons in wild-type, lin-32(tm1446) mutant and lin-32(tm1446); ezIs10[lin-32::GFP + unc-119(+)] rescued animals. N > 60. Statistical significance between wild-type and lin-32(tm1446) animals was evaluated using one-way ANOVA analysis. ****P < 0.0001. Note the high number of 1URX-like and 2URX-like animals in rescued animals. These are neurons that exhibit a URX morphology but are mispositioned. We found that the lin-32::GFP rescuing transgene can cause URX mispositioning defects in wild-type animals (not shown) suggesting that dosage of LIN-32 is important for neuron position.
expression of both markers in the URX neurons (Fig. 4A). When we analyzed the \( \text{lin-32} \) \( \text{tm1446} \); \( \text{ham-1} \) \( \text{n1438} \) double mutant we observed a similar phenotype to the \( \text{lin-32} \) single mutant (Fig. 4A). Due to the high penetrant loss of URX marker expression in \( \text{lin-32} \) mutant animals, we cannot conclude whether \( \text{ham-1} \) functions in a parallel or in the same pathway as \( \text{lin-32} \). However, we have found that \( \text{ham-1} \) is important for the development of the URX neurons.

\( \text{pig-1} \) and \( \text{hlh-2} \) do not regulate the development of the URX neurons. It has been shown that HAM-1 promotes the expression of the serine/threonine kinase PIG-1/MELK to regulate the Q.a asymmetric division. We therefore asked whether \( \text{pig-1} \) mutant animals exhibit similar defects in the URX neurons as the \( \text{ham-1} \) mutant. We crossed the \( \text{pig-1} \) \( \text{gm344} \) null mutant with two fluorescent reporters for the URX neurons - \( \text{kyIs417} \) [\( \text{gcy-36::GFP} \)] and \( \text{ynIs22} \) [\( \text{flp-8::GFP} \)] (Fig. 4B). We observed no detectable change in URX reporter expression in \( \text{pig-1} \) \( \text{gm344} \) mutant animals, indicating that PIG-1 is not required for proper development of the URX neurons (Fig. 4B). Furthermore, the \( \text{pig-1} \) \( \text{gm344} \); \( \text{lin-32} \) \( \text{tm1446} \) double mutant animals exhibit the \( \text{lin-32} \)
single mutant URX phenotype. These results suggest that HAM-1 regulates URX development independently of PIG-1.

In other neuronal lineages, LIN-32 functions together with the bHLH transcription factor HLH-2/E/daugh-terless12. Furthermore, it has been shown that LIN-32 and HLH-2 physically interact in vitro12. We therefore asked whether the ubiquitously expressed $\text{hlh}^{-2}$ also functions with $\text{lin}^{-32}$ to regulate URX development. To this end, we crossed the $\text{hlh}^{-2}(\text{tm1768})$ mutant with two fluorescent reporters for the URX neurons - $\text{kyIs417}[\text{gcy}^{-36}::\text{GFP}]$ and $\text{ynIs22}[\text{flp}^{-8}::\text{GFP}]$ (Fig. 4C). We found that the $\text{hlh}^{-2}(\text{tm1768})$ mutation does not affect the expression of these URX reporters. Taken together, these data suggest that LIN-32 acts independently of HLH-2 in the regulation of URX development.

Suppression of apoptosis partially restores URX fate in lin-32 null mutants. We have shown that in addition to the URXL/R neurons, LIN-32 regulates the expression of neuronal reporters for the two other neuronal subtypes generated from the same lineage (URADL/R and CEPDL/R) (Fig. 3). Furthermore, LIN-32 is required for the expression of two URX-fate-determining transcription factors EGL-13/Sox and UNC-86/POU. This indicates that LIN-32 functions upstream of these factors during URX development and is perhaps required for URX generation or survival. Due to the previously identified function for Atonal in Drosophila tumor forma-
tion34, we speculated that LIN-32 might regulate the programmed cell death (apoptosis) program of the URX neurons.

In C. elegans, the CED-3 caspase is essential for almost all apoptotic cell deaths35. Therefore, we analyzed the effect of $\text{ced}^{-3}$ loss on the URX developmental defects of lin-32 mutant animals (Fig. 5A). We crossed $\text{lin}^{-32}(\text{tm1446});\text{flp}^{-8}::\text{GFP}$ animals with two independent $\text{ced}^{-3}$ apoptosis defective mutants - $\text{ced}^{-3}(\text{n717})$ and $\text{ced}^{-3}(\text{n2452})$36,37. We found that loss of $\text{ced}^{-3}$ function partially restored the expression of $\text{flp}^{-8}::\text{GFP}$ in the URX neurons of $\text{lin}^{-32}(\text{tm1446})$ mutant animals (Fig. 5A). Loss of $\text{ced}^{-3}$ was however unable to restore the expression of $\text{flp}^{-8}::\text{GFP}$ in animals lacking the URX neuron specification transcription factors, EGL-13/Sox and AHR-1/aryl hydrocarbon receptor (Fig. 5A), supporting a specific role of LIN-32 in apoptotic control. To ask whether...
other aspects of URX fate are restored in lin-32(tm1446) animals when apoptosis is suppressed, we examined two additional reporters - gcy-35:mCherry and gcy-36::GFP (Fig. 5B,C). We found that the expression of gcy-36::GFP, but not gcy-35:mCherry, was also partially restored in lin-32; ced-3 double mutant animals, however at a lower degree than the flp-8::GFP reporter (Fig. 5A–C). This may suggest that the transcriptional requirements for driving terminal differentiation genes within the surviving URX neurons of lin-32; ced-3 mutant animals are not fully intact, that additional apoptotic pathways may be inhibited, or that LIN-32 may also regulate gene expression of certain terminal fate markers.

Figure 4. The role of HAM-1, PIG-1 and HLH-2 in URX specification. (A) HAM-1 regulates URX differentiation. Quantification of gcy-36::GFP (left) and flp-8::GFP (right) expression in wild type, lin-32(tm1446), ham-1(n1438) and lin-32(tm1446); ham-1(n1438) mutant animals. N > 70. (B) PIG-1 is not required for URX specification. Quantification of gcy-36::GFP (left) and flp-8::GFP (right) expression in wild type, lin-32(tm1446), pig-1(gm344) and lin-32(tm1446); pig-1(gm344) mutant animals. N > 70. (C) HLH-2 is not required for URX specification. Quantification of gcy-36::GFP (left) and flp-8::GFP (right) expression in wild type, lin-32(tm1446), hlh-2(tm1768) and lin-32(tm1446); hlh-2(tm1768) mutant animals. N = 90. Statistical significance between wild-type and mutant strains was evaluated using one-way ANOVA analysis. ****P < 0.0001; *P < 0.01; n.s. not significantly different from control.
Discussion
This study identified the LIN-32/Atonal proneural transcription factor as a regulator of the URX neuronal lineage. We found that the expression of reporters for URX terminal fate and of URX specification genes were severely affected when LIN-32 is mutated. This disruption of URX development has an effect on organismal function, as lin-32 mutant animals are unable to coordinate URX-mediated responses to changes in environmental O₂. We further showed that loss of apoptotic caspase function promotes survival of the URX neurons in lin-32 mutant animals. Taken together, our data suggest that the URX neurons, or URX precursors, undergo apoptosis in lin-32 mutant animals. This finding is consistent with the function of Atonal homologs during apoptosis in other organisms.

Figure 5. URX cell fate is restored by inhibiting apoptosis. (A) Quantification of flp-8::GFP expression in wild type, lin-32(tm1446), ced-3(n717), ced-3(n2452), lin-32(tm1446); ced-3(n717), lin-32(tm1446); ced-3(n2452) and ahr-1(a93); ced-3(n717); egl-13(ku194) mutant animals. The lin-32(tm1446); ced-3 double mutants partially restore flp-8::GFP expression in the URX neurons. In contrast, in a URX specification factor mutant background the expression of flp-8::GFP cannot be restored by loss of ced-3. N > 85. Note that compound loss of ahr-1(a93) and egl-13(ku194) causes ~95% loss of flp-8::GFP expression. (B) Quantification of gcy-35::mCherry expression in wild type, lin-32(tm1446), ced-3(n717), ced-3(n2452); lin-32(tm1446); ced-3(n717) and lin-32(tm1446); ced-3(n2452) mutant animals. N > 80. (C) Quantification of gcy-36::GFP expression in wild type, lin-32(tm1446), ced-3(n717), ced-3(n2452), lin-32(tm1446); ced-3(n717) and lin-32(tm1446); ced-3(n2452) mutant animals. N > 90. Statistical significance between wild-type and mutant strains was evaluated using one-way ANOVA analysis. ****P < 0.0001; ***P < 0.0005; **P < 0.005; n.s. not significantly different from control.
In other neuronal systems in *C. elegans*, such as the development of the male tail, the bHLH transcription factors LIN-32 and HLH-2 function together as a heterodimer. We found however that HLH-2 is not required for URX development, suggesting that LIN-32 acts independently of HLH-2 in this regard, potentially in conjunction with another bHLH transcription factor. Another study showed that the HAM-1 stork-head transcription factor acts in parallel to LIN-32 to regulate Q neuroblast division. We found that *ham-1* mutants also fail to correctly express reporters of URX neuron fate, identifying a new function for HAM-1.

The severe defects in the development of the URX neurons, and other neurons in the same lineage, of *lin-32* mutant animals suggested that either the entire sublineage is not generated or that neurons of this sublineage become hypodermal cells. However, we found that the hypodermal cell number in the head region of *lin-32* mutant animals was not different to wild type. We therefore hypothesized that either precursors of or the URX neurons themselves undergo inappropriate apoptosis. In support of this, we found that removal of CED-3 caspase partially restored expression of URX fate markers in the *lin-32* mutant but not *ahr-1; egl-13* double mutant animals. This is probably because AHR-1 and EGL-13 regulate gene expression in the URX neurons, whereas, LIN-32 also regulates apoptosis. Previous work has postulated that cells contain antagonistic protective and killing factors and that shifts in the balance of such factors can regulate programmed cell death decisions. Therefore, LIN-32 may be required for the regulation of this balance.

Studies from other organisms support a role for Atonal transcription factors in the regulation of apoptosis. In *Drosophila melanogaster*, Atonal regulates apoptosis and proliferation, and overexpression of Atonal in the eye of the fly results in increased levels of caspase-3. As such, Atonal in *Drosophila* promotes apoptosis in the context of the eye. Similarly, genetic studies using mouse and human colorectal cancer cells indicate a tumor suppressor function of Atoh1. Here, loss of Atoh1 prevented JNK-mediated apoptosis, leading to tumor progression. Both these studies therefore support a function for Atonal in the promotion of apoptosis. In contrast, studies of cochlear development in the mouse have shown that Atoh1 is required to prevent apoptosis. This study showed that loss of Atoh1 leads to impaired hearing due to inappropriate apoptosis of hair cells. Our findings suggest that in *C. elegans* LIN-32/Atonal negatively regulates apoptosis in the URX lineage. The contrasting functions for Atonal homologs in these studies in apoptotic control probably reflect the distinct cellular contexts within which Atonal acts. The identification of direct transcriptional Atonal target genes in these models will provide a better understanding of how this conserved transcription factor regulates cell survival. Intriguingly, Atoh1 is expressed in the dorsal hindbrain of mammals and loss of Atoh1 results in respiratory failure in mice before birth. Therefore, our finding that LIN-32/Atonal is required for correct development of *O2*-sensing neurons in *C. elegans* may have revealed a conserved function for Atonal homologs.

**Methods**

**Mutant and transgenic reporter strains.** Worms were cultured using standard conditions on NGM agar plates and maintained at 20 °C. A complete list of strains used for this study is detailed in Table S1.

**Forward genetic screen.** In the forward genetic screen, the URX reporter strain yIs22[flp-8::GFP] was mutagenized with EMS (ethyl methanesulphonate) according to standard protocols. Potential URX cell fate mutants in the F2 population were manually isolated based on loss of GFP signal under a dissecting fluorescence stereomicroscope.

**Mapping of *rp1*.** The genomic lesion of *rp1* was identified using single-nucleotide polymorphism (SNP) mapping. Hawaiian (CB4856) males were crossed into *C. elegans* strains, such as the development of the male tail, the bHLH transcription factors LIN-32 and HLH-2 function together as a heterodimer. We found however that HLH-2 is not required for URX development, suggesting that LIN-32 acts independently of HLH-2 in this regard, potentially in conjunction with another bHLH transcription factor. Another study showed that the HAM-1 stork-head transcription factor acts in parallel to LIN-32 to regulate Q neuroblast division. We found that *ham-1* mutants also fail to correctly express reporters of URX neuron fate, identifying a new function for HAM-1.

The severe defects in the development of the URX neurons, and other neurons in the same lineage, of *lin-32* mutant animals suggested that either the entire sublineage is not generated or that neurons of this sublineage become hypodermal cells. However, we found that the hypodermal cell number in the head region of *lin-32* mutant animals was not different to wild type. We therefore hypothesized that either precursors of or the URX neurons themselves undergo inappropriate apoptosis. In support of this, we found that removal of CED-3 caspase partially restored expression of URX fate markers in the *lin-32* mutant but not *ahr-1; egl-13* double mutant animals. This is probably because AHR-1 and EGL-13 regulate gene expression in the URX neurons, whereas, LIN-32 also regulates apoptosis. Previous work has postulated that cells contain antagonistic protective and killing factors and that shifts in the balance of such factors can regulate programmed cell death decisions. Therefore, LIN-32 may be required for the regulation of this balance.

Studies from other organisms support a role for Atonal transcription factors in the regulation of apoptosis. In *Drosophila melanogaster*, Atonal regulates apoptosis and proliferation, and overexpression of Atonal in the eye of the fly results in increased levels of caspase-3. As such, Atonal in *Drosophila* promotes apoptosis in the context of the eye. Similarly, genetic studies using mouse and human colorectal cancer cells indicate a tumor suppressor function of Atoh1. Here, loss of Atoh1 prevented JNK-mediated apoptosis, leading to tumor progression. Both these studies therefore support a function for Atonal in the promotion of apoptosis. In contrast, studies of cochlear development in the mouse have shown that Atoh1 is required to prevent apoptosis. This study showed that loss of Atoh1 leads to impaired hearing due to inappropriate apoptosis of hair cells. Our findings suggest that in *C. elegans* LIN-32/Atonal negatively regulates apoptosis in the URX lineage. The contrasting functions for Atonal homologs in these studies in apoptotic control probably reflect the distinct cellular contexts within which Atonal acts. The identification of direct transcriptional Atonal target genes in these models will provide a better understanding of how this conserved transcription factor regulates cell survival. Intriguingly, Atoh1 is expressed in the dorsal hindbrain of mammals and loss of Atoh1 results in respiratory failure in mice before birth. Therefore, our finding that LIN-32/Atonal is required for correct development of *O2*-sensing neurons in *C. elegans* may have revealed a conserved function for Atonal homologs.

**Methods**

**Mutant and transgenic reporter strains.** Worms were cultured using standard conditions on NGM agar plates and maintained at 20 °C. A complete list of strains used for this study is detailed in Table S1.

**Forward genetic screen.** In the forward genetic screen, the URX reporter strain yIs22[flp-8::GFP] was mutagenized with EMS (ethyl methanesulphonate) according to standard protocols. Potential URX cell fate mutants in the F2 population were manually isolated based on loss of GFP signal under a dissecting fluorescence stereomicroscope.

**Mapping of *rp1*.** The genomic lesion of *rp1* was identified using single-nucleotide polymorphism (SNP) mapping. Hawaiian (CB4856) males were crossed into *rp1* mutants and the mutant phenotype (URX loss) was recovered in the F2 generation. The progeny from 10 F2’s were examined by SNP mapping and the causative lesion was linked to the genetic positions of -17 and -8 on the X chromosome. Subsequent single worm SNP mapping showed strong linkage to -17. The inability of *rp1* males to mate and the loss of PVM GFP revealed a mutation at position 376 of the *rp1* cDNA, leading to a premature STOP codon (Q12STOP). A complete list of strains used for this study is detailed in Table S1.

**Fluorescence microscopy.** L4/young adult animals were analyzed for neuronal cell fate defects by mounting them on a glass slide with a 5% agarose pad, using 20 mM NaN₃ as an anesthetic. Images were taken using an upright fluorescence microscope (Zeiss, AXIO Imager M2) and ZEN software (version 2.0).

**Behavioral assays.** Oxygen sensing behavioral assays were conducted as previously described. Wild type and *rp1* mutant animals were starved for 1 h and then transferred to 14 cm NGM plates containing a 56 × 56 mm arena of Whatman filter paper soaked in 20 mM CuCl₂. Between 80 and 120 animals were used in a single experiment and each experimental condition was repeated four to six times. A custom-made transparent gas mixing units (Vögtlin Instruments) and controlled by LabView software. Recordings were illuminated with flat red LED lights and made at three frames per second on a 4-megapixel CCD camera (Jai), using Streampix software (Norpix). For movie analysis, MatLab-based image processing and tracking scripts were used.
as previously described. The resulting trajectories were used to calculate instantaneous speed during continuous forward movements (1-sec binning).

**Statistical analysis.** Statistical analyses were performed in Graphpad Prism 7 using t-test or one-way ANOVA where applicable. Differences with a P-value < 0.05 were considered significant.

**References**

1. Johnston, R. J. & Hobert, O. A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. *Nature* **426**, 845–849 (2003).

2. Hobert, O. Regulation of terminal differentiation programs in the nervous system. *Annu Rev Cell Dev Biol* **27**, 681–696, doi:10.1146/annurev-cellbio-092910-154226 (2011).

3. Powell, L. M. & Jarman, A. P. Context dependence of proneural bHLH proteins. *Curr Opin Genet Dev* **18**, 411–417, doi:10.1016/j.gde.2008.07.012 (2008).

4. Romani, S., Campuzano, S. & Modolell, J. The achaete-scute complex is expressed in neurogenic regions of Drosophila embryos. *EMBO J* **6**, 2085–2092 (1987).

5. Cabrera, C. V., Martinez-Arias, A. & Bate, M. The expression of three members of the achaete-scute gene complex correlates with neuroblast segregation in Drosophila. *Cell* **50**, 425–433 (1987).

6. Bertrand, N., Castro, D. S. & Guillemot, F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* **3**, 517–530, doi:10.1038/nrn874 (2002).

7. Jarman, A. P., Grau, Y., Jan, L. Y. & Jan, Y. N. achaete is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* **73**, 1307–1321 (1993).

8. Bermingham, N. A. et al. Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837–1841 (1999).

9. Ben-Arie, N. et al. Functional conservation of atonal and Math in the CNS and PNS. *Development* **127**, 1039–1048 (2000).

10. Cai, T., Seymour, M. L., Zhang, H., Pereira, F. A. & Groves, A. K. Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. *J Neurosci* **33**, 10110–10122, doi:10.1523/JNEUROSCI.5606-12.2013 (2013).

11. Chen, P., Johnson, J. E., Zogbi, H. Y. & Segel, N. The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell determination. *Development* **129**, 2417–2426 (2002).

12. Portman, D. S. & Emmons, S. W. The basic helix-loop-helix transcription factors LIN-32 and HLH-2 function together in multiple steps of a *C. elegans* neuronal sublineage. *Development* **127**, 5415–5426 (2000).

13. Zhao, C. & Emmons, S. W. A transcription factor controlling development of peripheral sense organs in *C. elegans*. *Nature* **373**, 74–78 (1995).

14. Hedgecock, E. M., Culotti, J. G., Hall, D. H. & Stern, B. D. Genetics of cell and axon migrations in Caenorhabditis elegans. *Development* **100**, 365–382 (1987).

15. Chalfie, M. & Au, M. Genetic control of differentiation of the Caenorhabditis elegans touch receptor neurons. *Science* **243**, 1027–1033 (1989).

16. Zhu, Z. et al. A proneural gene controls C. elegans neuroblast asymmetric division and migration. *FEBS Lett* **588**, 1136–1143, doi:10.1016/j.febslet.2014.02.036 (2014).

17. Doitsidou, M., Flames, N., Lee, A. C., Boyanov, A. & Hobert, O. Automated screening for mutants affecting dopaminergic-neuron specification in *C. elegans*. *Nat Methods* **5**, 869–872, doi:10.1038/nmeth.1250 (2008).

18. Zimmer, M. et al. Neurons detect increases and decreases in oxygen levels using distinct guanylate cyclases. *Neuron* **61**, 865–879, doi:10.1016/j.neuron.2009.02.013 (2009).

19. Wilham, E. et al. *C. elegans* Body Cavity Neurons Are Homeostatic Sensors that Integrate Fluctuations in Oxygen Availability and Internal Nutrient Reserves. *Cell Rep.*, doi:10.1016/j.celrep.2016.01.052 (2016).

20. White, J. G., Southgate, E., Thomson, J. N. & Brenner, S. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society of London B. Biological Sciences* **314**, 1–340 (1986).

21. Gramstrup Petersen, J. et al. EGL-13/SoxD specifies distinct O2 and CO2 sensory neuron fates in Caenorhabditis elegans. *PLoS Genet* **9**, e1003511, doi:10.1371/journal.pgen.1003511 (2013).

22. Qin, H. & Powell-Coffman, J. A. The Caenorhabditis elegans aryl hydrocarbon receptor, AHR-1, regulates neuronal development. *Dev Biol* **270**, 64–75 (2004).

23. Kim, K. & Li, C. Expression and regulation of an FMRFamide-related neuropeptide gene family in Caenorhabditis elegans. *J Comp Neurol* **475**, 540–550, doi:10.1002/cne.20725 (2004).

24. Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H. & Plasterk, R. H. Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. *Nat Genet* **28**, 160–164 (2001).

25. Miller, R. M. & Portman, D. S. The Wnt/beta-catenin asymmetry pathway patterns the atonal ortholog lin-32 to diversify cell fate in a Caenorhabditis elegans sensory lineage. *J Neurosci* **31**, 13281–13291, doi:10.1523/JNEUROSCI.6540-10.2011 (2011).

26. Ross, J. M., Kalis, A. K., Murphy, M. W. & Zarkower, D. The DM domain protein MAB-3 promotes sex-specific neurogenesis in C. elegans by regulating bHLH proteins. *Dev Cell* **8**, 881–892, doi:10.1016/j.devcel.2005.03.017 (2005).

27. Rojo Romanos, T., Petersen, J. G., Rivero, A. R. & Pocock, R. A novel role for the zinc-finger transcription factor EGL-46 in the differentiation of gas-sensing neurons in Caenorhabditis elegans. *Genetics* **199**, 157–163, doi:10.1534/genetics.114.172049 (2015).

28. Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol* **100**, 64–119, doi:0012-1666(83)90201-4 [pii] (1983).

29. Naas, R., Miller, D. M. & Blakely, R. D. *c elegans*: a novel pharmacogenetic model to study Parkinson's disease. *Parkinsonism Relat Disord* **7**, 185–191 (2001).

30. Guenther, C. & Garriga, G. Asymmetric distribution of the *C. elegans* HAM-1 protein in neuroblasts enables daughter cells to adopt distinct fates. *Development* **122**, 3509–3518 (1996).

31. Frank, C. A., Hawkins, N. C., Guenther, C., Horvitz, H. R. & Garriga, G. *C. elegans* HAM-1 positions the cleavage plane and regulates apoptosis in asymmetric neuroblast divisions. *Dev Biol* **284**, 301–310, doi:10.1016/j.ydbio.2005.05.026 (2005).

32. Cordes, S., Frank, C. A. & Garriga, G. The *C. elegans* MELK ortholog PIG-1 regulates cell size asymmetry and daughter cell fate in asymmetric neuroblast divisions. *Development* **133**, 2747–2756, doi:10.1242/dev.02447 (2006).

33. Feng, G. et al. Developmental stage-dependent transcriptional regulatory pathways control neuroblast lineage progression. *Development* **140**, 3838–3847, doi:10.1242/dev.098723 (2013).

34. Bossuyt, W. et al. The atonal proneural transcription factor links differentiation and tumor formation in Drosophila. *Plos Biol* **7**, e1000140, doi:10.1371/journal.pbio.1000140 (2009).

35. Ellis, H. M. & Horvitz, H. R. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**, 817–829 (1986).

36. Shaham, S., Reddien, P. W., Davies, B. R. & Horvitz, H. R. Mutational analysis of the Caenorhabditis elegans cell-death gene ced-3. *Genetics* **153**, 1655–1671 (1999).

37. Shabam, S. & Horvitz, H. R. Developing Caenorhabditis elegans neurons may contain both cell-death protective and killer activities. *Genes Dev* **10**, 578–591 (1996).
38. Jarman, A. P., Sun, Y., Jan, L. Y. & Jan, Y. N. Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. Development 121, 199–2030 (1995).
39. Bossuyt, W. et al. Atonal homolog 1 is a tumor suppressor gene. Plos Biol 7, e39, doi:10.1371/journal.pbio.1000039 (2009).
40. Rose, M. F. et al. Math1 is essential for the development of hindbrain neurons critical for perinatal breathing. Neuron 64, 341–354, doi:10.1016/j.neuron.2009.10.023 (2009).
41. Brenner, S. The genetics of Caenorhabditis elegans. Genetics 77, 71–94 (1974).
42. Du, H. & Chalfie, M. Genes Regulating Touch Cell Development in Caenorhabditis elegans. Genetics 158, 197–207 (2001).
43. Ramot, D., Johnson, B. E., Berry, T. L., Carnell, L. & Goodman, M. B. The Parallel Worm Tracker: A Platform for Measuring Average Speed and Drug-Induced Paralysis in Nematodes. Plos One 3, doi:10.1371/journal.pone.0002208 (2008).
44. Tsunozaki, M., Chalasani, S. H. & Bargmann, C. I. A Behavioral switch: cGMP and PKC signaling in olfactory neurons reverses odor preference in C. elegans. Neuron 59, 959–971, doi:10.1016/j.neuron.2008.07.038 (2008).

Acknowledgements
We thank members of Pocock laboratory for comments on the manuscript. Some strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440), and by Shohei Mitani at the National Bioresource Project (Japan). This work was supported by a grant from the European Research Council (ERC Starting Grant number 260807), Monash University Biomedicine Discovery Fellowship and veski innovation fellowship: VIF 23 to R.P.

Author Contributions
T.R.R., D.P.-M., K.L.-J., S.L.H., L.N. and R.P. performed the experiments, T.R.R. and R.P. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-07876-4

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017