Loss-of-function of β-catenin bar-1 slows development and activates the Wnt pathway in Caenorhabditis elegans

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C. elegans is extensively used to study the Wnt-pathway and most of the core-signalling components are known. Four β-catenins are important gene expression regulators in Wnt-signalling. One of these, bar-1, is part of the canonical Wnt-pathway. Together with Wnt effector pop-1, bar-1 forms a transcription activation complex which regulates the transcription of downstream genes. The effects of bar-1 loss-of-function mutations on many phenotypes have been studied well. However, the effects on global gene expression are unknown. Here we report the effects of a loss-of-function mutation bar-1(ga80). By analysing the transcriptome and developmental phenotyping we show that bar-1(ga80) impairs developmental timing. This developmental difference confounds the comparison of the gene expression profile between the mutant and the reference strain. When corrected for this difference it was possible to identify genes that were directly affected by the bar-1 mutation. We show that the Wnt-pathway itself is activated, as well as transcription factors elt-3, pgm-1, msl-1 and pha-4 and their associated genes. The outcomes imply that this response compensates for the loss of functional bar-1. Altogether we show that bar-1 loss-of-function leads to delayed development possibly caused by an induction of a stress response, reflected by daf-16 activated genes.
worm strain EW15 carrying the β-catenin-loss-of-function point mutation bar-1(ga80). This mutation causes a Glu to Stop codon change at amino acid 97 of the predicted BAR-1 protein. By analysing the transcriptome and developmental phenotyping we show that bar-1(ga80) impairs developmental timing. Moreover we found that without a functioning bar-1 ~ 7,500 genes were affected. Our results further suggest that the loss of bar-1 is partially compensated by redundancy in the Wnt-signalling pathway, pointing towards a feedback mechanism between β-catenin activation and the expression levels of Wnt-signalling pathway encoding genes.

Methods

Strains. The following strains were used: EW15 (Bristol N2 strain, carrying the mutation bar-1(ga80)) and wild type Bristol N2. Upon arrival in the lab EW15 was outcrossed at least 4 times. Worms were kept in maintenance at 12°C and before recommended by. For within-array normalization of the RNA-array data the Loess Feature Extraction Software version 10.5, following manufacturers’ guidelines. For replicate. Three independent duplicates per strain were measured. The microarrays manufactured by Agilent. Input of total RNA was approximately 200 ng for each sample (as estimated), and 48 hours after synchronisation we found that the transcriptomes of worms (age of 48 h) of N2 to the bar-1(ga80) mutant strain of the same age by microarray analysis. Of the 20,887 genes that were tested on the microarray, 5,772 genes were differentially expressed (−log10(p) > 2.0 at FDR = 0.05). In bar-1(ga80), 51% was down-regulated (2,927 genes), and 49% was up-regulated (2,855 genes) compared to N2. During the initial analyses, we noticed that many of the differentially expressed genes were related to development, for example genes encoding for collagens and vitellogens. Recently we reported that genome wide gene expression can rapidly and massively change during the L4 stage. To test for the developmental difference within the L4 stage, we compared the differentially expressed genes between N2 and bar-1(ga80) with the set of genes reported by Snoek et al. (2014) (Figure 1). The differentially expressed genes between bar-1(ga80) and N2 were enriched for genes changing during L4 development (hypergeometric test, p ≤ 10−200). To exactly pinpoint the developmental delay, we used the expression levels which have a linear correlation with L4 developmental timing to estimate the developmental age of the bar-1(ga80) and N2 samples. Even though all RNA samples from both genotypes were taken at 48 hours after synchronisation we found that the bar-1(ga80) worms developed more slowly (44.4 h ± 0.9) than the N2 samples (47.7 h ± 0.8) (two-sided t-test, p = 6∗10−4) (Supplementary figure 1).

Bar-1 regulates the transcriptome of developing C. elegans. To test the role of Bar-1 in regulating the transcriptome, we compared the transcriptomes of 54 h old L4 bar-1(ga80) and N2 worms (age of 48 h) using a linear model (CDF empirical distribution function) to identify genes that were differentially expressed (FDR < 0.01). We identified a significant (−log10(p) > 3) difference between these two genotypes. The black dots represent the spots on the array, the log2 effect between N2 and bar-1(ga80) is shown versus the LOD score. The yellow x indicate spots of genes affected by developmental effects during L4 development.
we selected those genes that did not have a developmental sided t-test, p, eggs were laid in N2 and 48 hours. A and Figure 2A) indicating a developmental delay of 3.3 hours after the replicate of the developmental delay of bar-1(ga80) increased over time (0 h at 0 h, –3.3 h at 48 h and 6 h at 62 hour). This implies that the mutation affected the entire developmental period from egg to adult. Our results show that bar-1(ga80) does not affect a single developmental stage, because than the developmental difference between bar-1(ga80) and N2 would remain constant during the subsequent stages.

Analysis incorporating developmental effects. To exclude the effects of the developmental delay of bar-1(ga80) from other effects of bar-1(ga80) on gene expression, we included the transcriptional effects during L4 development in the analysis (Figure 3). Here we found 7,557 (FDR = 0.05) genes to be affected by the bar-1(ga80) mutation either with or without a development effect. Of these genes, 3,920 were up-regulated and 3,637 were down-regulated in the bar-1(ga80) mutant (Supplementary figure C).

As developmental effects were very strong and affected many genes,2,3,4,5,6 we selected those genes that did not have a developmental effect (P < 0.05) or an effect which was opposite of what one would expect in a relatively slower developed bar-1(ga80) mutant (Supplemental figure C). We also selected on effect size (>0.5 or <−0.5) which resulted in 710 down- and 425 up-regulated genes compared to N2 (FDR = 0.05; Supplement Table 1).

**BAR-1 strongly affects collagens and hedge-hog signalling.** The set of genes down-regulated in bar-1(ga80) compared to N2 (Supplement Table 1) contains many non-annotated genes. These genes could complement the genes with known functions, but could also constitute new functions. Furthermore genes like mai-1, daf-4, pha-11, sta-2, pk-2, pes-8, cnp-2, hmit-1.1, hmit-1.2, gcy-32, nlp-23 and fkb-5 have a strongly reduced expression in bar-1(ga80). These genes might function together with the strongly down-regulated groups of genes, like collagens (rol-1, bli-2, bli-1, dpy-3, lon-3), col-type genes (col-175, -38, -71, -120, -40, -49, -138, -110, -97, -79, -70) or other cuticle related components (cutl-18, cutl-28, mlt-18, mlt-12, nas-27 and gly-1). Components of hedge-hog (hh) signalling were also much lower expressed in bar-1(ga80) such as: the warthog genes wnt-6 and wnt-4; groundhog-like genes grl-15, grl-5 and grl-14; hedgehog-like genes, hog-1, grl-2, grl-1 and grl-12. This shows that hh-signalling is affected by bar-1(ga80) mutation. Taken together, BAR-1 activity is most likely required for activation of collagens and other cuticle genes as well as genes involved in hedge-hog signalling.

**Expression of Wnt-signalling components.** The expression of most Wnt-signalling components changed during development and were
affected by bar-1(ga80) mutation (Figure 3) (core-Wnt pathway genes selected by*). All four β-catenins were differentially expressed in bar-1(ga80). Expression of bar-1 was lower whereas wrm-1, hmp-1 and sys-1 all showed a slight increase in expression (see 4 upper blocks in the first column of Figure 3). In N2, these three β-catenins showed increased expression levels during L4 development whereas expression of bar-1 hardly changed throughout the L4 stage in N2 (p = 0.042; see 4 upper blocks in the third column of Figure 3).

Of the five Wnt genes in C. elegans, only mom-2 was higher expressed in bar-1(ga80). For the other Wnt genes no significant effect was found. Of the Wnt genes, only mom-2 expression increased during L4 development, whereas expression of cwn-1, cwn-2 and lin-44 decreased (p < 0.001 in all cases). Egl-20 was not differentially expressed throughout L4 and was also not affected by the bar-1 mutation.

The members of the destruction complex showed no or only minor expression differences between bar-1(ga80) and N2 during development. One of the dishevelled genes, dsh-1, was affected by the bar-1 mutation. This gene was higher expressed in bar-1(ga80). It was also the only gene of the three dishevelled genes that did not show a change in expression.

The Wnt receptors cfr-2, mig-1 and lin-18 (ryk/derailed) were higher expressed in bar-1(ga80), and their expression did not change during development. The two other Wnt-receptors were slightly affected by development. Expression of mom-5 increased during L4 development whereas lin-17 expression decreased. Of the other genes, sfrp-1, an extracellular active Wnt-inhibitor, had a lower expression in the bar-1 mutant compared to N2. The expression of sfrp-1 decreased during L4 development in N2. Even though bar-1(ga80) showed a developmental delay, the expression of sfrp-1 decreased compared to N2. Some of the transcription factors known to be involved in the Wnt-pathway, like EOR-1* and EGL-27* (or associated with the Wnt pathway, like SKN-1*), were up-regulated, but their targets were not enriched for in the differentially expressed genes set (hypergeometric test, p > 0.1).

To summarize, the up-regulation of the other three β-catenins in bar-1(ga80) probably compensates for the loss of a functional BAR-1 (Supplement figure C). Intriguingly, not only the β-catenins were higher expressed, but also the Wnt-receptors. Furthermore, the sfrp-1 gene was down-regulated. Thus, a lack of bar-1 also affects the Wnt-signalling pathway upstream, which could point to a feedback-mechanism. As the Wnt encoding gene mom-2, the Wnt-receptors cfr-2, mig-1 and lin-18 and the dishevelled gene dsh-1 were also up-regulated, our results imply that the Wnt- signalling pathway itself was activated following the knock-down of bar-1.

Natural genetic variation in Wnt-pathway genes. All genes part of, or associated with, the Wnt-pathway (Figure 3) are polymorphic across many other C. elegans wild type strains39–41. Between the two most frequently studied wild types N2 and CB4856 these polymorphisms lead to an amino acid change in almost 50% of the proteins (Supplement text 1). Furthermore enrichments of expression Quantitative Trait Loci (eQTL) of genes with affected transcript levels by bar-1(ga80) suggest that polymorphic loci between CB4856 and N2 downstream of or modulated by bar-1 and Wnt-signalling might be present (Supplement text 1). This indicates that the Wnt-signalling pathway is genetically buffered42 and the associated genes are possibly co-evolving.

Biological processes affected by bar-1(ga80). To investigate which processes were affected by the bar-1(ga80) mutation, we tested enrichment of mutation-affected genes in GO-, KEGG-, Anatomy-, Wormbook-, Gene class- and Protein domain annotations. To distinguish between bar-1 and developmental effects we excluded all the bar-1(ga80) affected genes with a developmental effect from the set of genes used for enrichment analysis (Supplementary Table 2). The results of the complete set of bar-1(ga80) affected genes including those with a developmental effect can also be found in Supplementary Table 2.

Genes lower expressed in bar-1(ga80) are enriched with genes involved in cuticle constituents (p < 1*10^-7), proteolysis (p < 1*10^-9) and the proteasome core complex (p < 1*10^-9). Whereas the proteolysis and proteasome core complex genes overlapped, they did not overlap with the cuticle constituent genes. Furthermore protein degradation related enrichments were reflected in the multiple categories tested, implying that protein degradation/turn-over might be reduced. Thus, bar-1(ga80) affects protein degradation, possibly reflecting the transition of the cell from one state into another.

The group of genes expressed higher in bar-1(ga80) consisted of a more diverse set of genes. These genes were especially related to transcriptional regulation, as shown by an enrichment of the GO-terms regulation of transcription (p < 1*10^-7), sequence specific DNA binding (p < 1*10^-7), transcription factor activity (p < 1*10^-4) and nucleus (p < 1*10^-7). Some indications were found that the Ras-pathway was affected because transcription factors known to be linked to the Ras-pathway were up-regulated, like the RAS inhibitors MDL-1 and LIN-15B. The activation of the Ras pathway is further shown by the strong up regulation of cav-1 in the bar-1(ga80) mutant.

Furthermore, also neuron-related terms were represented, as shown by enrichments of the GO-term axon (p < 1*10^-4), synapse (p < 1*10^-4) and in the anatomy terms where the three most significantly enriched groups were neuronal (p < 1*10^-10). The enrichment of these neuronal genes can point in the direction of the aberrant neuron migration that is observed in bar-1(ga80)*, the mutation might affect neuropeptide signalling.

bar-1(ga80) transcription patterns suggest DAF-16 activation. Since enrichment in transcriptional regulation was detected, we used the modENCODE30,31 set of ChIP-seq determined binding sites to search for enrichment of binding sites for transcription factors. We found that the genes higher expressed in bar-1(ga80) were enriched for binding-sites of transcription factors PHA-4, MDL-1, ELT-3 and PQM-1 (hypergeometric test, p < 1*10^-4). These transcription factors were up-regulated in the bar-1(ga80) mutant, except for elt-3 (Figure 4A). Together with the enrichment found for the binding sites, this indicates that the absence of the β-catenin BAR-1 results in an activation of transcription factors, possibly as a compensatory response. The four transcription factors for which enrichments have been found share binding sites for many of the genes. Over 50% of the up-regulated genes in the transcriptional network were associated with more than one of these four transcription factors (Figure 4B). Furthermore, PQM-1 and MDL-1 also bind near the transcription starting site of PHA-4 and ELT-3 (Supplementary figure D). This indicates that it is likely that PQM-1 or MDL-1 is involved in the transcriptional activation observed in bar-1(ga80).

Three of these transcription factors: PQM-1, MDL-1, and ELT-3, have been associated with the insulin/IGF-1 signalling pathway and longevity. However, for ELT-3 this relation is debated in more recent literature47. Furthermore, PQM-1 is also identified as a promoter of growth, development and reproduction. PQM-1 has an antagonistic interaction with DAF-16, where nuclear translocation of PQM-1 (promoted by DAF-2) results in depletion of DAF-16 from the nucleus (and vice-versa). Tepper et al. identified genes regulated by PQM-1 (referred to as class II genes), and genes regulated by DAF-16 (referred to as class I genes). It is also shown that some of the class I genes are also regulated by PQM-115. We tested expression of these genes in the bar-1(ga80) versus N2 and found that the class I genes were enriched for in the up-regulated genes (hypergeometric test, p < 1*10^-23) and slightly but significantly
out of 425 up-regulated genes, hypergeometric test, p-value is not provided. We showed that the point mutation (L4) of the strain EW15 carrying the bar-1 gene leads to induction of a stress response reflecting DAF-16 activation. This is further shown by the modest up-regulation of the other β-catenins, wtm-1, hmp-2 and sys-1 which all have the potential to substitute for bar-1 in transcriptional activation.

Analysis of the genes affected by bar-1(ga80) showed that genes up-regulated in bar-1(ga80) are enriched for transcription factor- and histone-binding sites and for processes like chromosome rearrangement, chromatin factors and neurogenesis. The down-regulated genes were enriched for cuticle components and signalling pathways, suggesting bar-1 directly affects these processes. We found that the transcriptional response induced in bar-1(ga80) reflects DAF-16 activation (Figure 5). This also corresponds with the developmental delay we measured. We propose that loss of bar-1 results in a compensatory/feedback response on the transcriptional level, leading to Wnt-pathway and DAF-16 activation.

Conclusion

We studied gene expression patterns during the fourth larval stage (L4) of the strain EW15 carrying the β-catenin-loss-of-function point mutation bar-1(ga80) causing a Glu to Stop codon change at amino acid 97 of the predicted BAR-1 protein. To untangle the developmental effects from the effects of the bar-1 mutation, we used a time-series dataset. We showed that bar-1(ga80) results in a slower development, as these worms take on average ~10% more time to develop than Bristol N2. Using the transcriptome to estimate the age of the worms, we found that 48 hours after synchronization, bar-1(ga80) worms are transcriptionally most similar to N2 worms at 44–45 hours after synchronization. By measuring the time that egg-deposit starts in bar-1(ga80) and Bristol N2, we confirmed this developmental delay. To our knowledge, this has not yet been reported for bar-1.

Analysis of the Wnt-pathway showed that a non-functional bar-1 causes up-regulation of Wnt-signalling components, mom-2, czf-2, mig-1, lin-18, dsh-1, mom-5 and lin-17. Together with the down-regulation of Wnt-inhibitor sfrp-1 this indicates hyper-activation of the Wnt-signalling pathway, suggesting a compensatory mechanism (Figure 5). We also analysed DAF-16 ChIP-seq data and found that the genes up-regulated in bar-1(ga80) were enriched for DAF-16 targeted genes (124 out of 425 up-regulated genes, hypergeometric test, p < 1*10^-4). Based on these results we hypothesize that loss of function of bar-1(ga80) leads to induction of a stress response reflecting DAF-16 activation, causing delayed development of the worms.

**Figure 4** Transcription factor activity and targets in up-regulated genes. (A) shows the transcript abundance of the four transcription factors enriched for targets among the up-regulated genes in bar-1(ga80). The fold-change in bar-1/N2 is shown. The levels of pqm-1, pha-4, and mdl-1 are significantly higher in bar-1(ga80). (linear model, p < 0.01), whereas this is not the case for elt-3 (linear model, p = 0.158). (B) A Venn-diagram of the up-regulated genes associated with the four enriched transcription factors. There is a high level of overlap between the associations as >50% of the targets are associated with multiple transcription factors.

**Figure 5** A model for the bar-1-lof effects. This model incorporates the findings in this paper (grey) with what is known about the Wnt-pathway regarding to BAR-1 (black) and findings reported about DAF-16 and PQM-1 (red). It is proposed that (transcriptional) activity of the β-catenin bar-1 results in a feedback loop, de-activating the Wnt-pathway. Furthermore BAR-1 activity is needed for a correct developmental program, where bar-1-lof shows indications of a DAF-16-mediated stress response. The exact level of interaction between BAR-1 and the insulin pathway remains to be elucidated.

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Author contributions

MLV.D.B., J.A.G.R., M.G.S., R.J.M.V. conducted the experiments. M.G.S., T.S., L.B.S. analysed the results, A.H., J.E.K., R.J.M.V., M.G.S. and L.B.S. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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