

HLH-30/TFEB is necessary for chromatin reorganization and maintenance of cell quiescence during starvation in *C. elegans*

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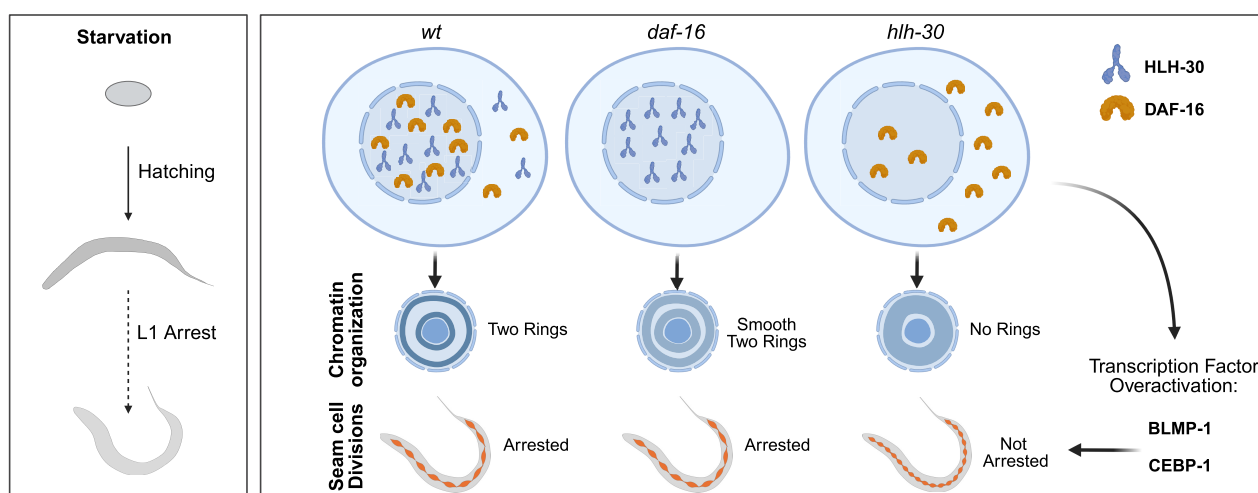
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Abstract

Cellular quiescence is a low metabolic, non-proliferative state critical for tissue maintenance and regenerative capacity, with broad implications for aging and age-related diseases. In *Caenorhabditis elegans*, L1 developmental arrest upon hatching in the absence of food provides a robust *in vivo* model to study quiescence. Here, we investigate the roles of the transcription factors HLH-30/TFEB and DAF-16/FOXO during L1 arrest. We show that HLH-30 and DAF-16 collaborate to ensure survival under starvation, with reciprocal regulation of their subcellular localization and transcriptional activity. HLH-30 exerts broad transcriptional control during L1 arrest, modulating genes involved in chromosome organization and cell cycle progression. Profiling of chromatin spatial distribution reveals that HLH-30 is required for fasting-induced 3D chromatin reorganization. Loss of HLH-30 disrupts seam cell cycle arrest and causes the overactivation of the transcription factors BLMP-1 and CEBP-1, which contribute to premature initiation of developmental programs under starvation. Our findings uncover previously unrecognized functions of HLH-30 in genome architecture and quiescence regulation, highlighting conserved mechanisms of transcriptional control during nutrient deprivation with implications for aging and disease.

Graphical abstract



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Introduction

Cell quiescence is a reversible non-proliferative state maintained by cell cycle inhibitors. Despite the lack of proliferation, quiescence is a metabolically active process involving protective mechanisms to ensure survival and enable re-entry into the cell cycle (reviewed in Murley *et al.* [1]). This process is crucial for various cell types, particularly for preserving adult stem cell pools, thereby contributing to tissue homeostasis and regeneration [2]. Adult stem cells can reside in tissues for decades, but their survival and reactivation capacity diminish over time [3–4]. Aged stem cells accumulate reactive oxygen species (ROS), DNA damage, protein aggregates, and show mitochondrial malfunction [5]. These cellular damage impairs the reactivation of quiescent cells, reducing regenerative capacity as we age [6–8].

To study cell quiescence *in vivo*, we use the developmental arrest of *Caenorhabditis elegans* L1 larvae as a model [6, 9, 10]. During *C. elegans* late embryogenesis, blast cells arrest prior to hatching [11]. When embryos hatch in the absence of food, the newly hatched larvae enter a state known as L1 arrest, characterized by decreased metabolic activity and the entry of blast cells into quiescence. This arrest occurs at the G1 phase in somatic cells [11] and at the G2 phase in germ cells [12]. Furthermore, starvation at the L1 stage triggers large-scale chromatin reorganization [13]. These larvae can survive several weeks without nutrition and exhibit enhanced stress resistance (reviewed in Baugh LR [14]). This state is fully reversible; upon re-exposure to food, larvae resume postembryonic development and cells return to a proliferative state.

During L1 arrest, larvae exhibit aging markers such as increased ROS, protein aggregation, and mitochondrial fragmentation [6]. After prolonged L1 arrest, aged quiescent larvae experience delays in restarting proliferation due to a latency phase in the division of stem cell-like blast cells (seam cells) [15]. Both the increase of aging markers and the delayed return to proliferation resemble the deepening of quiescence and delayed cell cycle re-entry undergone by mammalian cells [9].

The insulin/IGF-1-like signaling (IIS) pathway is a primary regulator of L1 arrest. The transcription factor (TF) DAF-16, the *C. elegans* ortholog of mammalian FOXO3A (Forkhead box O3), is activated following the reduction of IIS under nutrient deprivation [16–19]. DAF-16 acts at the beginning of L1 arrest by activating stress response pathways (reviewed in Baugh LR [14]). Its loss compromises survival and recovery from L1 starvation [20]. DAF-16 has also been implicated in maintaining the quiescence of seam cells during the arrest [21].

TOR signaling also plays a role during the L1 arrest. Inhibition of TOR complex 1 (TORC1) by AAK-2/AMPK and DAF-18/PTEN is necessary to maintain the quiescence of the germline cells in arrested L1 larvae [22]. TORC1 is also required for the activation of progenitor cell divisions in the presence of ethanol and amino acids [23]. For instance, expressing constitutively active versions of the TOR regulators RAGC-1 and RHEB-1 in the hypodermis triggers P-cell migration in a *let-363/TOR*- and *daf-15/RAPTOR*-dependent manner [23]. Recent evidence also indicates that TOR inhibition is essential for the chromatin reorganization observed during fasting [13].

The TF HLH-30, the ortholog of mammalian TFEB (Transcription Factor EB), is regulated by TOR signaling and plays a vital role in metabolic regulation during L1 starvation

[24–27]. Mutant animals lacking HLH-30 exhibit severe defects upon L1 starvation, becoming unable to resume development after 30 h of starvation and showing complete loss of survival after 5 days [26]. Interestingly, depending on the physiological context, DAF-16/FOXO and HLH-30/TFEB function synergistically or have opposing effects [28], but their combined effect had never been tested during L1 arrest.

We investigated the roles of the TFs DAF-16 and HLH-30 during L1 arrest by analyzing single and double mutants. Our findings reveal a reciprocal influence between these factors, demonstrated through changes in subcellular localization and global gene expression patterns. Notably, we uncovered previously unknown functions of HLH-30 in regulating L1 arrest, including the regulation of gene expression programs associated with chromosome organization and cell cycle progression. An analysis of spatial chromatin distribution further showed that HLH-30 is essential for fasting-induced chromatin reorganization. Moreover, *hlh-30* mutants exhibited impaired seam cell cycle arrest under starvation, a defect partially attributed to the overactivation of the TFs BLMP-1 and CEBP-1. Overall, our study highlights novel roles for HLH-30 in maintaining cell quiescence and shaping genome architecture. This research sheds light on conserved mechanisms of transcriptional regulation during cellular quiescence and nutrient deprivation—processes that are fundamental to aging, metabolic disorders, and cancer.

Materials and methods

Caenorhabditis elegans strains and growth conditions

Nematodes were grown according to standard methods, maintaining them on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50-1 bacteria at 20°C. All strains used in this study are listed in [Supplementary Table S1](#).

Generation of starved L1 larvae

To induce L1 larval arrest by starvation, embryos were isolated from gravid adults using an alkaline hypochlorite solution. The embryos were then resuspended in M9 minimum medium at a density of 20 embryos per microliter and were incubated at 20°C with gentle shaking, allowing them to hatch. In the absence of food, the newly hatched larvae arrested development at the L1 stage. We counted the days of arrest from the moment of embryo preparation, that is, day 1 corresponds to 24 h after embryo preparation.

Generation of fed L1 larvae

For the fed L1 samples, we isolated embryos as detailed above but resuspended them in S-basal containing *E. coli* OP50-1 at 10 g/l. In this condition, larvae initiate postembryonic development after hatching, which occurs ~12 h after embryo preparation. Thus, after 24 h, fed samples are ~12 h into postembryonic development.

RPB-2 and DAF-15 auxin-mediated degradation

To induce the degradation of the RNA polymerase II subunit RPB-2 and the TORC1 component DAF-15, we used strains carrying endogenously tagged versions of the genes with an auxin-inducible degron (AID), *cer135[rpb-2::GFP*

(*delta*)*piRNA::AID::3xFLAG*], and *daf-15::mNG::AID*, respectively. A single copy of TIR1 transgene *ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119 (+)]II* was present in these strains to allow the induction of degradation in the presence of auxin. To trigger protein degradation, 1 mM of the auxin analog 1-naphthaleneacetic acid (NAA) was added to the M9 suspension immediately following embryo preparation.

Subcellular localization of HLH-30 and DAF-16

To determine the subcellular localization of HLH-30 and DAF-16, strains expressing the fluorescently tagged versions of these proteins (HLH-30::GFP and DAF-16::GFP) were used. At the indicated time points, 18 µl of the suspension of L1-arrested larvae were collected and incubated with 2 µl of 10 mM Levamisole (yielding a 1 mM final concentration). After precipitation, the localization of these reporters was evaluated by semi-quantitative visual inspection in a population of at least 100 animals per sample. Animals were classified into three distinct categories based on the distribution of the fluorescent signal: (i) Cytoplasmic, characterized by diffuse fluorescence within the cytoplasm and little to no discernible enrichment in the nuclei (<10% of visible nuclei); (ii) Intermediate, defined by a noticeable nuclear accumulation restricted to a subset of cells (~10%–70% of visible nuclei); and (iii) Nuclear, exhibiting strong and distinct fluorescence enrichment in the majority of the nuclei throughout the body (>70% of visible nuclei). Based on these data, the percentage of animals with each localization pattern was calculated. To prevent artificial translocation of HLH-30 and DAF-16, a reduced concentration of Levamisole (1 mM) was used as previously described [29–33]. Similarly, room temperature was maintained at 20°C during visualization to avoid potential temperature-induced effects on HLH-30 or DAF-16 activation. A minimum of three independent biological replicates for each time point were analyzed for each condition.

Survival and recovery assays

To evaluate survival of starved L1 larvae, we pipetted 5 µl of day 3-arrested larvae onto a drop of M9 on a glass slide and scored them directly under a dissecting scope. We counted the number of live larvae and divided it by the total number of larvae observed to calculate the percentage of survival. To measure recovery capacity of the starved L1 larvae, we pipetted 5 ml of day 1-starved larvae to NGM plates with OP50-1. We counted the number of larvae and then incubated the plates at 20°C. After 2 days, we counted the number of larvae that had resumed development and divided by the number of larvae counted after plating to calculate the percentage of larvae that recovered. At least three independent biological replicates were performed.

Total RNA sample preparation and mRNA sequencing

We performed two complete experiments, each including biological replicates. Experiment 1 aims to evaluate the contribution of the TFs DAF-16 and HLH-30 to gene expression during L1 starvation. Samples consisted of L1 larvae from the wild-type strain and the mutants *daf-16 (mu86)*, *hlh-30 (tm1978)*, and *daf-16 (mu86); hlh-30 (tm1978)*, collected after 1 day of L1 starvation in M9 buffer. We performed three independent biological replicates of this experiment. Experiment 2 aims to evaluate the impact of L1 starvation on gene

expression. Samples of L1 larvae of the wild-type strain, either starved or fed, also collected on day 1 (24 h from embryo isolation). We performed two independent biological replicates of this experiment. For RNA extraction, we collected at least 140 000 L1 larvae per sample. Total RNA was extracted using the NZYTotal RNA Isolation Kit (NZYtech, MB13402). A total of 150 ng of RNA per sample was used to prepare libraries following the Illumina Stranded mRNA Prep Ligation, compatible with the Illumina sequencing system. The quality of the libraries was assessed for adequate size distribution and integrity using the TapeStation DNA High Sensitive D1000 assay, and sufficient concentration using Qubit™ DNA HS Assay fluorimetry. Samples were sequenced using the Illumina NextSeq 500 High Output platform.

RNA-seq functional analysis and visualization

Quality control and cleaning of the raw reads was performed with FASTP package (v0.22.0). Clean reads were then aligned to the *C. elegans* reference genome (https://ftp.ensembl.org/pub/release-110/gff3/caenorhabditis_elegans/) using the STAR package (v2.7.10b). Raw counts for individual genes were quantified with the featureCounts command of the SUBREAD (v2.0.3) package. To normalize the counts, we applied TMM (trimmed mean of M values) normalization with edgeR (v3.34.1) package within the R environment. Differentially expressed genes (DEGs) were analyzed in the mutant strains in comparison to the wild type. Pairwise comparisons were performed between each of the mutants and the wild-type strain using DESeq2 (v1.42.0) [34]. The genes of the three conditions with an adjusted *P*-value < .05 (Wald test), regardless of the Fold Change between samples, were selected for further analysis.

Functional enrichment analysis of DEGs was performed in R using the clusterProfiler package (v4.14.6) [35], using Gene Ontology (GO). The input consisted of a filtered list of DEGs (adjusted *P*-value < .05 from differential expression analysis) (Supplementary File S2).

Ensembl gene IDs were converted to Entrez IDs prior to enrichment. GO enrichment analysis was performed using the enrichGO() function, specifying the Biological Process ontology (ont = “BP”) with pvalueCutoff = 0.01 and pAdjustMethod = “BH” (Benjamini–Hochberg correction). Only terms with *P*.adjust < .01 were retained for downstream analysis. To reduce redundancy among enriched GO terms, semantic similarity was calculated using the Wang method implemented in the GOSemSim package. Redundant terms were filtered using a similarity threshold of 0.8, retaining representative terms from clusters of highly similar GO categories. For visualization, enriched GO terms were ranked by adjusted *P*-values and the top 20 categories (showCategory = 20) were displayed using scatter plots generated with ggplot2 (v3.5.2) [36]. Gene–term interaction networks were generated using cnetplot(), retaining the top 12 GO terms per condition (showCategory = 12), with nodes colored according to log2 fold changes (foldChange parameter). Gene expression patterns related to the mitotic cell cycle and chromosome organization were displayed as a heat map generated with the ComplexHeatmap package (v2.22.0) [37], following Z-score normalization across samples. Data preprocessing, including file import and filtering, was performed with readxl (v1.4.5) and dplyr (v1.1.4).

Identification of potential HLH-30 target genes in L4 larval and late embryonic stages

To identify genes that might be regulated by direct binding of HLH-30, we analyzed publicly available HLH-30 ChIP-seq datasets generated by the modENCODE/ENCODE consortium. The dataset for the L4 larval stage corresponded to ENCFF793PTZ, while the one for the late embryonic stage corresponded to ENCFF574KYZ. Both datasets were aligned to the *C. elegans* reference genome WBcel235 (ce11).

Gene annotations were obtained from the ParaSite WBPS19 canonical gene set in WormBase (*Caenorhabditis elegans* PRJNA13758), using the file *caenorhabditis_elegans.PRJNA13758.WBPS19.canonical_geneset.gtf*. From this annotation, the transcription start site (TSS) coordinates were extracted for all annotated genes. Genes were classified as direct targets of HLH-30 when a peak was identified upstream of the annotated TSS. These genes were annotated as “TRUE,” while genes without a peak were annotated as “FALSE” (Supplementary File S2).

Analysis of chromatin spatial distribution

Microscopy was performed using a live-cell imaging system (Confocal Spinning Disk Microscope) from VisiTron Systems GmbH, equipped as follows: Nikon Eclipse Ti2 microscope with a Plan Apo λ 100 $\times$ /1.45 oil objective, Yokogawa CSU-W1 confocal scanner unit, VS-Homogenizer, Electron Multiplying CCD camera (Andor iXon Series), and VisiView software for image acquisition. For the analysis of chromatin profiling in intestinal nuclei, the protocol previously described [13] was followed. In brief, live imaging was conducted on 2% agarose pads supplemented with 0.15% sodium azide to immobilize the worms. All images were acquired with 50 stacks and a z-spacing of 200 nm. The nuclei of the mid-intestine were manually segmented in 2D, based on HIS-72::GFP, and the nucleolar center annotated using Cell-ACDC [38]. To extract the intensity profiles, we proceeded as described in Al-Refaie *et al.* [13]. A total of 78 cells per strain were analyzed from three independent biological replicates.

Fluorescence reporter analysis

For analyzing fluorescence reporters, unless otherwise specified, at the indicated time points, 50 μ l of the suspension of L1-arrested larvae were transferred to a clean tube and centrifuged for 1 min at 850 $\times$ g. Then, 40 μ l of the supernatant was removed, and Levamisole was added to the sample at 10 mM to paralyze the animals. The sample was then transferred to a glass slide, covered with a coverslip, and the GFP signal was visualized using a Leica scope M205 FCA with GFP excitation/emission filters. *Plin-4::YFP* signal from each larva was normalized to the average signal from the wild-type strain in each replicate.

Analysis of seam cell divisions

For analyzing seam cell divisions, we used strains carrying the *Pscm::NLS-GFP* construct, which is specifically expressed in seam cells. We counted the fraction of animals presenting one or more divided cells in the V1–V4 seam cell lineage. At least 50 larvae per strain and replicate were analyzed for each sample.

To evaluate seam cell divisions in fed larvae, embryos were resuspended in S-basal medium containing *E. coli* OP50-1

at 10 g/l. The number of animals presenting divisions was counted at the indicated time points after egg preparation.

Analysis of *cki-1* activation

For the analysis of *cki-1* activation, strains carrying the fluorescent reporter *cki-1::GFP* were used. Images of at least 80 larvae were acquired using fixed intensity and exposure parameters. Using Fiji/ImageJ, images were binarized. After binarization, the number of seam cells displaying signal per larva was quantified. Three independent biological replicates were performed. The GFP signal was visualized using a Leica scope M205 FCA with GFP excitation/emission filters.

Analysis of PCN-1 induction

For the analysis of *pcn-1* activation, strains carrying the translational reporter *Ppcn-1::pcn-1::GFP* were used. We counted the fraction of animals in a population of at least 50 larvae showing each category of PCN-1 induction (full, mid, or no induction) as follows. Full induction corresponds to animals showing GFP signal in H and V1–V4 seam cells, mid induction corresponds to larvae showing GFP signal in at least one V1–V4 seam cell, and the fraction of animals not showing GFP signal was categorized as larvae without PCN-1 induction. We performed five independent biological replicates. The GFP signal was visualized using a Leica scope M205 FCA with GFP excitation/emission filters.

Analysis of transcription factor activation and age estimates

To estimate TF activation, we used CelEsT (github.com/IBMB-MFP/CelEsT-app) [39]. We used the raw count (Supplementary File S2) from the mRNA-seq experiments to calculate the TF activation scores and *P*-values and represented them in Volcano plots. Within the app, also starting from raw counts, we used the RAPToR (Real Age Prediction from Transcriptome staging On Reference) package to estimate the age of each sample according to their expression data [40].

Statistical analysis

For statistics, we have used the averages of independent biological replicates to avoid the inflated *N* value from using individual animals [41]. A complete report of biological replicates and, when relevant, number of larvae per sample can be found at Supplementary Table S2. We used an unpaired two-tailed *t*-test to compare the means of two groups. For the rest of the results, we used one-way ANOVA to compare more than two groups. After one-way ANOVA, we either performed Dunnett’s multiple comparison to compare the average of each genotype to the average of the wild type, or Tukey’s multiple comparison to compare the average of each genotype to all the other genotypes. The type of analysis applied to each panel is detailed in the figure legends. For clarity, some plots show only relevant comparisons. In all cases, * means $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. Graphs and statistics were performed on GraphPad Prism 10. The complete statistics report can be found in Supplementary File S1.

Results

HLH-30 and DAF-16 collaborate to allow survival to L1 arrest

To investigate a possible collaborative role of DAF-16 and HLH-30 during L1 starvation, we first analyzed the subcellular localization of DAF-16 and HLH-30 using the fluorescent reporters *ot971[daf-16::gfp]* and *sqIs17[hlh-30p::hlh-30::gfp]* (Fig. 1A–C). As previously shown with a DAF-16 multicopy reporter [42–43], DAF-16::GFP showed high levels of nuclear localization at the beginning of L1 starvation, and these levels decreased over time in starvation (Fig. 1B). In *hlh-30* mutants, DAF-16 nuclear localization was significantly reduced when compared to the wild-type background on days 1 and 4 (Fig. 1B). Localization at later stages of arrest is not assessable due to the limited survival of the mutants. In contrast, HLH-30::GFP exhibited ~50% nuclear localization on day 1 of L1 arrest, which declined by day 4 before increasing again at later stages of arrest (Fig. 1C). In the *daf-16* mutant background, HLH-30 showed higher nuclear localization than in the wild type (Fig. 1C). These data show that DAF-16 and HLH-30 subcellular localization depend on each other and suggest that a collaboration between both TFs could occur at the beginning of L1 starvation, when both TFs are found in the nucleus. Indeed, previous analysis of direct and indirect DAF-16 and HLH-30 targets over L1 arrest revealed that the transcriptional response controlled by these TFs is mounted early during L1 starvation [44].

We then evaluated the impact of DAF-16 and HLH-30 on survival and in the capacity to recover from L1 starvation. Since the mutant *hlh-30 (tm1978)* shows impaired survival and recovery even after short periods of arrest [26], we focused on measuring survival after 3 days and recovery after 1 day of arrest (Fig. 1D). As expected, after this short period of starvation, the *daf-16 (mu86)* was indistinguishable from the wild type, but *hlh-30 (tm1978)* mutants showed strong defects in survival and recovery. Despite the apparent dispensability of DAF-16 to survive short arrest, the analysis of the double mutant showed that the *daf-16 (mu86)* mutation aggravated the defects of *hlh-30 (tm1978)* (Fig. 1E and F). These results suggest that there might be some degree of redundancy between DAF-16 and HLH-30 at the beginning of L1 arrest. We then checked the survival and recovery upon DAF-16 overactivation by testing the mutants in a *daf-2 (e1370)* mutant background (Supplementary Fig. S1B and C). DAF-2 is the sole insulin receptor in *C. elegans*, and responsible for inhibiting DAF-16 activation (Supplementary Fig. S1A). At short times of L1 arrest, the *daf-2* mutation improved the survival and recovery of the *hlh-30* mutant in a DAF-16-dependent manner (Supplementary Fig. S1B and C), suggesting that DAF-16 can take over some HLH-30 functions.

HLH-30 exerts pervasive transcriptional control at L1 arrest

To understand the roles of DAF-16 and HLH-30 at the initial stages of L1 arrest, we performed mRNA sequencing of the wild type, *daf-16 (mu86)*, *hlh-30 (tm1978)*, and the *daf-16;hlh-30* double mutant on day 1 of starvation. The initial evaluation of expression dynamics by principal-component analysis showed that the biological replicates clustered together. Furthermore, the *daf-16* mutant is positioned close to the wild-type strain along PC1, in contrast to *hlh-30* and the double mutant (Fig. 2A). This suggests that the effect of the

hlh-30 mutation on the double mutant is stronger than that of the *daf-16* mutation. Indeed, we found a greater transcriptional deregulation in the *hlh-30* and double mutants than in the *daf-16* mutant strain. Specifically, in comparison to the wild type, we found 2975 differentially regulated genes in the *daf-16* mutant, 7899 genes in the *hlh-30* mutant, and 10 188 genes in the double *daf-16;hlh-30* mutant (Fig. 2B, Supplementary Fig. S2A–C, and Supplementary File S2). Most of the deregulated genes in the single mutants were also found in the double mutant (72% for the *daf-16* mutant and 90% for the *hlh-30* mutant). The high level of gene deregulation and the strong overlap between the *hlh-30* and the double mutants suggest a high similarity between these two conditions (Fig. 2B).

Among the DEGs, we observed that well-known specific DAF-16 and HLH-30 targets were deregulated exclusively in the samples corresponding to the *daf-16* and *hlh-30* single mutants, respectively (Supplementary Fig. S2D and E). This observation validated our experimental approach. We also detected changes in gene expression that suggest different types of collaboration between DAF-16 and HLH-30. Some genes required both DAF-16 and HLH-30 for full activation (Supplementary Fig. S2F), pointing to a collaborative function of these TFs. In contrast, other genes showed reduced expression only in the double mutant (Supplementary Fig. S2G), suggesting a redundant role between DAF-16 and HLH-30 on day 1 of L1 arrest.

To identify biological processes associated with transcriptional activation, we performed functional enrichment analysis on the gene lists of DEGs with $\text{Padj} < .05$, for each mutant strain. This was achieved through the implementation of the clusterProfiler package (v4.14.6) [35]. The annotation source used was the Gene Ontology (GO) terms for Biological Processes. The top 20 most significant categories were selected for representation. Results for the *daf-16* mutant showed a significant enrichment for categories related to defense response, cellular catabolism, and lipid metabolism (Fig. 2C and Supplementary Fig. S3A). Strikingly, although HLH-30 had been described as a master regulator of lysosome biogenesis and autophagy [24, 45], the categories with greater representation for the *hlh-30* and the double *daf-16;hlh-30* mutants were related to two groups: cell division and defense response (Fig. 2C and Supplementary Fig. S3B and C). The categories grouped within defense response are shared by all three mutants, which could reveal cooperation between these two TFs, as previously reported in a different context [28]. However, the enrichment in cell division categories appears to be specific to the absence of HLH-30 (Fig. 2C–D), suggesting a role for this protein in the control of this biological process at the onset of L1 arrest. Among the biological processes specifically affected in the *hlh-30* and the double *daf-16;hlh-30* mutants was also chromosome organization (Fig. 2C–D and Supplementary File S2). This suggests that HLH-30 might regulate chromatin on a large scale, which could explain the large impact of the *hlh-30* mutation on gene transcription (Fig. 2B). To identify potential direct target genes of HLH-30 within these two categories, we analyzed publicly available HLH-30 ChIP-seq datasets generated by the modENCODE/ENCODE consortium [46, 47] during the L4 and late embryonic stages (Supplementary File S2). Approximately 31% of the genes belonging to the “Mitotic Cell Cycle” category were identified as putative targets at the L4 stage, compared to 15% at the late embryonic stage. Within the “Chromosome Organi-

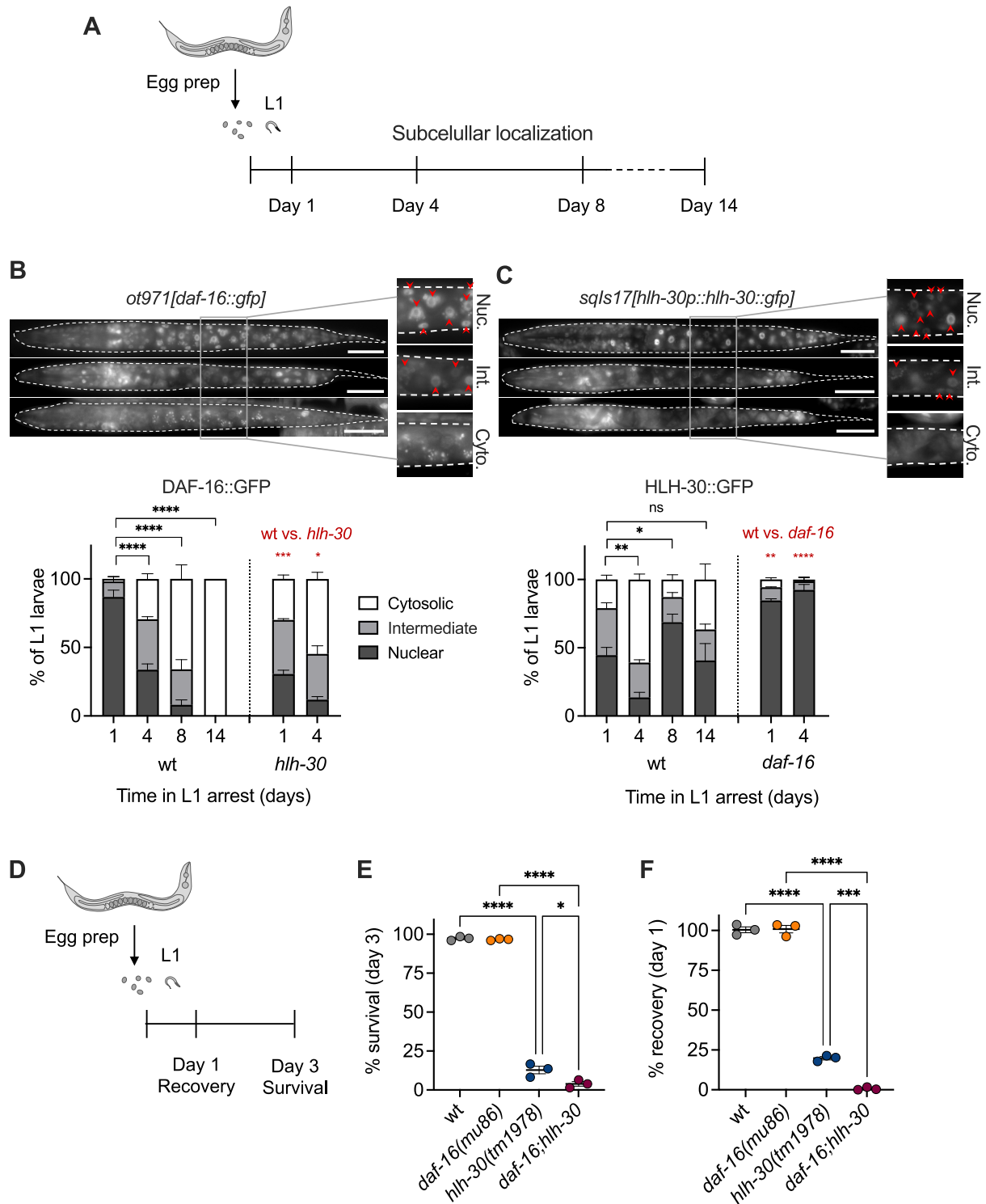


Figure 1. DAF-16 and HLH-30 play a role in the first hours of starvation. **(A)** Time points for the analysis of the subcellular localization of DAF-16::GFP and HLH-30::GFP. Representative images and quantification of the subcellular localization on day 1 of L1 arrest of DAF-16::GFP **(B)** and HLH-30::GFP **(C)**. Red arrowheads mark nuclear localization of the TFs in a section of the worms. Each larva in a population was categorized as showing cytosolic, intermediate, or nuclear localization of the corresponding TF. Error bars represent SEM. We first compared the averages of nuclear localization by one-way ANOVA followed by Dunnett's multiple comparisons to test the differences between day 1 and longer times of arrest in the wild-type background (marked in black). We used *t*-test to compare the nuclear fraction on day 1 or 4 of the mutant samples to the corresponding days of the wild type (red symbols). Scale bar: 20 μ m. **(D)** Time points for analysis of recovery and survival of the wild type, the *daf-16(mu86)*, and *hlh-30(tm1978)* mutant, and the corresponding double mutant. **(E)** Percentage of L1 larvae alive after 3 days of starvation for the wild type, the *daf-16(mu86)*, and *hlh-30(tm1978)* mutant, and the corresponding double mutant. **(F)** Percentage of L1 larvae that resume development upon refeeding after 1 day of starvation, for the wild type, the *daf-16(mu86)*, and *hlh-30(tm1978)* mutant, and the corresponding double mutant. Dots represent results from independent experiments, and the lines mark the mean ($\pm$ SEM). We performed one-way ANOVA followed by Tukey's multiple comparisons to compare all samples to each other. Only relevant comparisons are shown. The complete statistics report can be found in [Supplementary File S1](#).

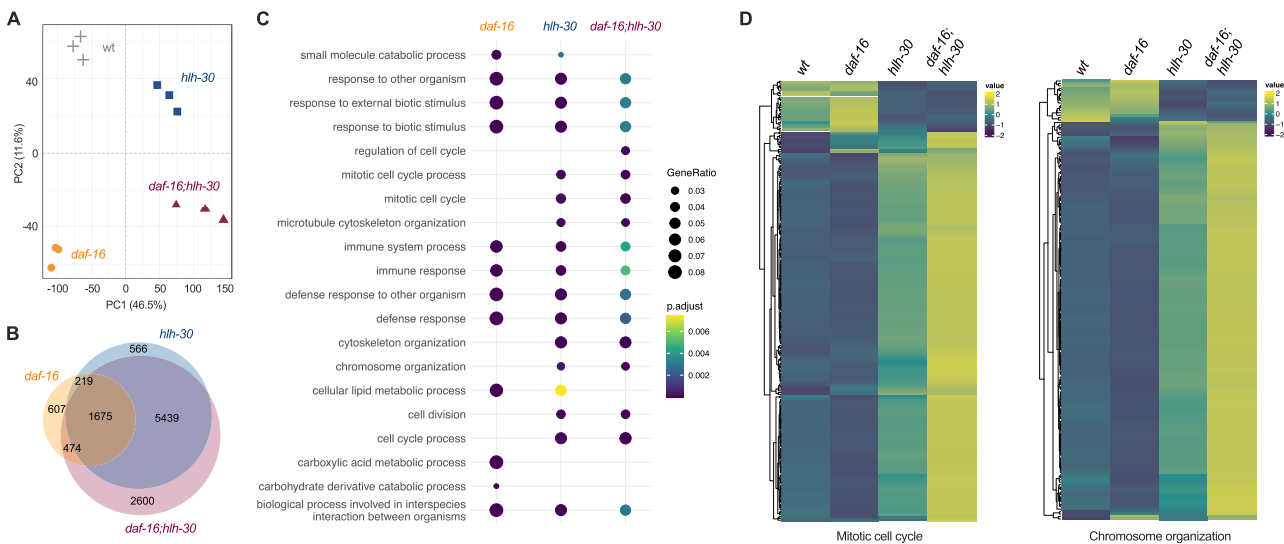


Figure 2. DAF-16 and HLH-30 impact transcription during L1 starvation. **(A)** Principal-component analysis of mRNA-seq data for all the four strains and three independent biological replicates. PC1 and PC2 account for 46.5% and 11.6% of the total variance, respectively. **(B)** Venn diagram showing the number of DEGs for each of the mutants. **(C)** Representation of the top 20 most significant biological processes affected across all samples. **(D)** Heatmaps showing the expression of genes within the categories “Mitotic cell cycle” (256 genes) and “Chromosome organization” (185 genes) in the wild type, the *daf-16*(*mu86*) and *hlh-30*(*tm1978*) mutants, and the corresponding double mutant.

zation” category, we identified 29% and 9% of the genes as potential targets at the L4 and late embryonic stages, respectively.

HLH-30 is necessary for fasting-induced chromatin reorganization

Interestingly, fasting at the L1 stage provokes a large-scale spatial reorganization of chromatin in *C. elegans* intestinal cells, which can be reversed upon nutrient supplementation. In fed L1 larvae, chromatin is distributed more diffusely, with its intensity increasing gradually toward the nuclear envelope without forming discrete structures. When larvae are starved, however, the genome undergoes rapid, large-scale spatial reorganization into two highly defined concentric rings. This reorganization induced by fasting requires inhibition of the mTORC1 pathway. Indeed, *let-363*/TOR RNAi treatment or depletion of DAF-15/Raptor leads to fasted-like chromatin organization in fed animals [13]. However, the elements that control chromatin reorganization downstream of mTORC1 remain unknown. Although HLH-30 activation upon mTORC1 inhibition has been previously reported [24, 25], this regulation has not yet been tested in L1-arrested larvae. Since *C. elegans* mTORC1 mutants are not viable, we used an AID system to promote DAF-15/Raptor degradation. Consistently, after 1 day of arrest, we observed increased nuclear localization of HLH-30 upon DAF-15 degradation (Supplementary Fig. S4A), confirming that mTORC1 restrains HLH-30 nuclear translocation also during L1 arrest. We then aimed to investigate a possible role of HLH-30 in chromatin reorganization during L1 starvation.

We analyzed chromatin architecture in intestinal cells by performing live imaging of HIS-72/H3.3 fused to GFP, as previously done [13] for the wild type, *daf-16* (*mu86*), *hlh-30* (*tm1978*), and the double mutant *daf-16;hlh-30* larvae on day 1 of L1 arrest (Fig. 3A). In wild-type animals, we observed the previously reported reorganization of the intestinal genome into two concentric chromatin rings [13] (Fig. 3A–C).

In contrast, all mutant conditions displayed altered chromatin organization, albeit to varying degrees, with *daf-16* mutants deviating less from the fasting-induced wild-type pattern than *hlh-30* mutants (Fig. 3A–C and Supplementary Fig. S4B–D). Interestingly, the chromatin profile of the *daf-16;hlh-30* double mutant did not show an additive effect of the two mutations, as the profiles of *hlh-30* and *daf-16;hlh-30* were nearly identical, with a correlation coefficient of 0.9988 (Supplementary Fig. S4B). Together, these results indicate that although both DAF-16 and HLH-30 contribute to fasting-induced chromatin reorganization, they likely act through overlapping mechanisms, with HLH-30 playing the more prominent role.

HLH-30 is necessary to maintain cell quiescence during L1 arrest

Other interesting categories enriched in *hlh-30* and *daf-16;hlh-30* mutants were those related to cell cycle and cell division. This result was particularly relevant given that the HLH-30 ortholog TFEB has diverse roles in cancer, functioning both as an oncogene and as a tumor suppressor, in lysosome-dependent and -independent manners [48]. Furthermore, cell cycle arrest at the G1 phase is a relevant process during L1 starvation in *C. elegans*. The *hlh-30* mutant shows deregulation of multiple cell cycle-related genes (Fig. 2D), suggesting that HLH-30 could play a role in the regulation of cell quiescence during L1 starvation. However, a role for HLH-30 in cell cycle quiescence had previously been ruled out based on the analysis on M mesoblast cell divisions during L1 arrest [26].

Here, we decided to analyze the state of seam cells during starvation, as the *C. elegans* hypodermis conveys nutritional information to control cell quiescence [23]. During postembryonic development of fed larvae, seam cells divide before the M mesoblast. We used a double reporter to follow the divisions of seam (*Pscm::gfp*) and M cells (*Pblh-8::gfp*) in the wild type, and the *daf-16* (*mu86*), *hlh-30* (*tm1978*), and dou-

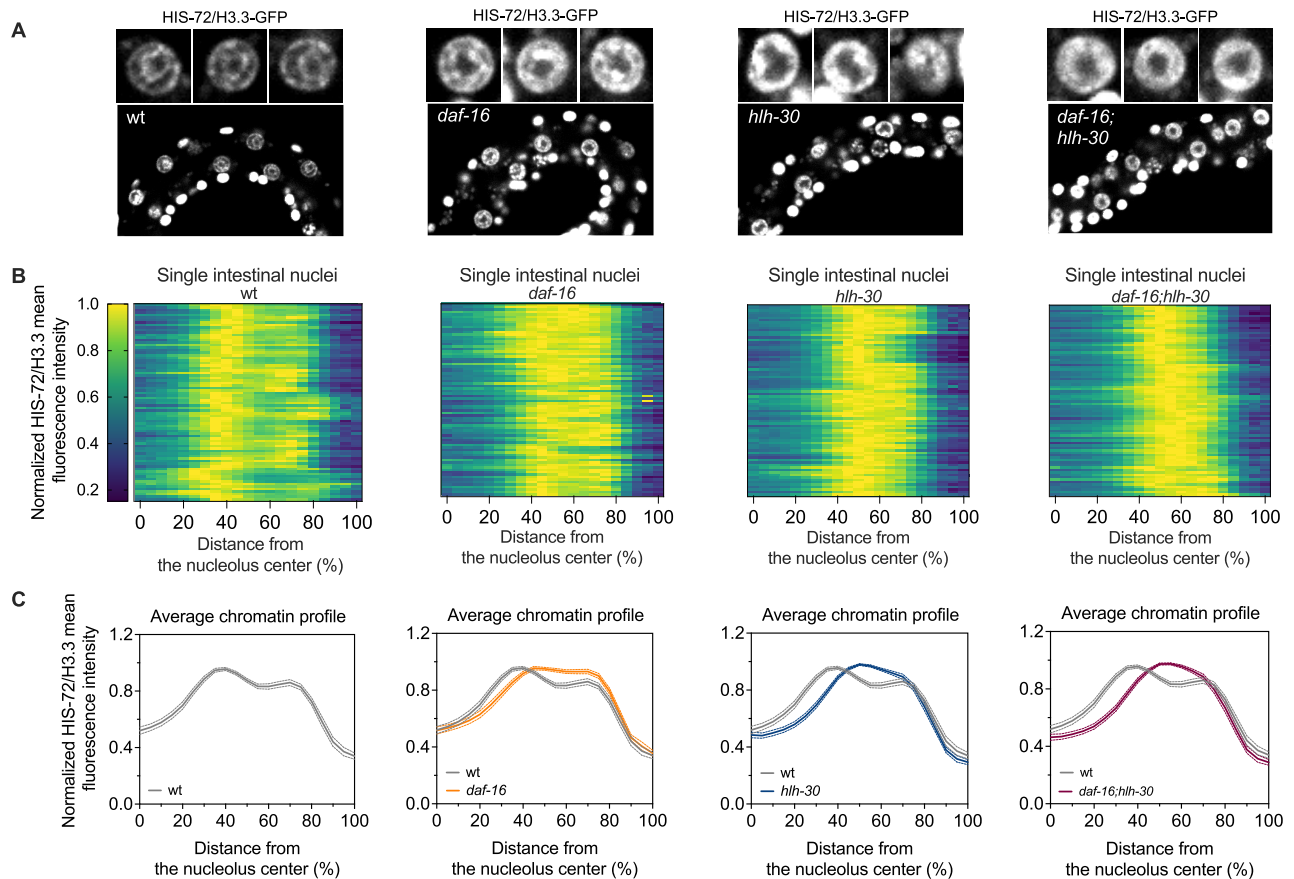


Figure 3. The *hlh-30*(*tm1978*) mutation impairs chromatin reorganization upon starvation. **(A)** Representative pictures of starved L1 larvae expressing the HIS-72/H3.3-GFP in a wild-type background, the *daf-16*(*mu86*), *hlh-30*(*tm1978*) mutants, and the corresponding double mutant. Top: representative intestinal nuclei. Bottom: representative section of L1 larvae. **(B)** Heat maps showing HIS-72/H3.3-GFP fluorescence intensity in individual intestinal nuclei, with each row corresponding to one nucleus, as a function of the distance from the center of the nucleolus, which is centrally located in the intestine. Each plot includes a total of 78 nuclei from larvae tested in three independent experiments. **(C)** Averaged fluorescence intensity of the nuclei shown in panel (B). The dashed lines represent the 95% confidence interval of the mean profile. Average profiles were compared to estimate the statistical significance of differences between the mutants and the wild type, and the *P*-values are provided in Supplementary Fig. S4D.

ble mutants. We calculated the fraction of starved larvae with divisions after 1 and 2 days of L1 starvation (Fig. 4A). For M cells, we did not observe any divisions on day 1 or day 2 in any of the strains (not shown). When analyzing seam cells, the *daf-16* mutant did not show a significant level of divisions compared to the wild type on day 1 or day 2. In contrast, after 2 days of starvation, the *hlh-30* mutant showed a higher percentage of larvae with seam cell divisions compared to the wild type and the *daf-16* mutant. In the case of the *daf-16* (*mu86*);*hlh-30* (*tm1978*) double mutant, the percentage of larvae with seam cell divisions was significantly different even after 1 day of L1 starvation (Fig. 4B).

Previous research demonstrated that DAF-16 is necessary for cell cycle arrest during L1 starvation [21], but only in the presence of ethanol in the arrest media [23]. These findings suggested that ethanol may suppress other factors that function redundantly with DAF-16 to maintain cell cycle arrest under starvation conditions. Given our evidence supporting a critical role for HLH-30 in this process, we investigated whether ethanol affects its subcellular localization. Notably, ethanol addition significantly reduced the nuclear localization of HLH-30 during L1 starvation (Supplementary Fig. S5A). This suggests that ethanol-mediated deactivation of HLH-30

may enhance the relative importance of DAF-16 in sustaining cell cycle arrest during nutrient deprivation, explaining why the role of HLH-30 in cell cycle arrest had passed unnoticed.

To confirm the impact of HLH-30 on cell cycle regulators, we checked the expression of the cyclin-dependent kinase inhibitor CKI-1/CIP/KIP/p27, which increases over time during L1 starvation [11, 15]. We tested the induction of *cki-1* in the wild type and mutants by quantifying the number of seam cells with induced *Pcki-1::gfp* per larva (Fig. 4C). The mutants *hlh-30* and *daf-16;hlh-30* showed a significant reduction in the number of seam cells (per larva) with induced *Pcki-1::gfp*, compared to the wild type and the *daf-16* mutant (Fig. 4D). Finally, we tested the abundance of the S-phase marker PCN-1 using a translational reporter of PCN-1 (Fig. 4E). In all strains, the signal from the reporter was evident on Z2/Z3 PGCs, as these cells arrest at the G2 phase of the cycle during L1 starvation. The GFP signal was absent from other cells in the wild-type strain and showed slight activation levels in the *daf-16* mutant on day 1. The *hlh-30* and *daf-16* (*mu86*);*hlh-30* (*tm1978*) mutants, however, showed significantly higher levels of activation of this marker in seam cells (Fig. 4F and Supplementary Fig. S5B). *pcn-1* RNA is also more abundant in *hlh-30* and *daf-16;hlh-30* mutants (Supplementary Fig. S5C).

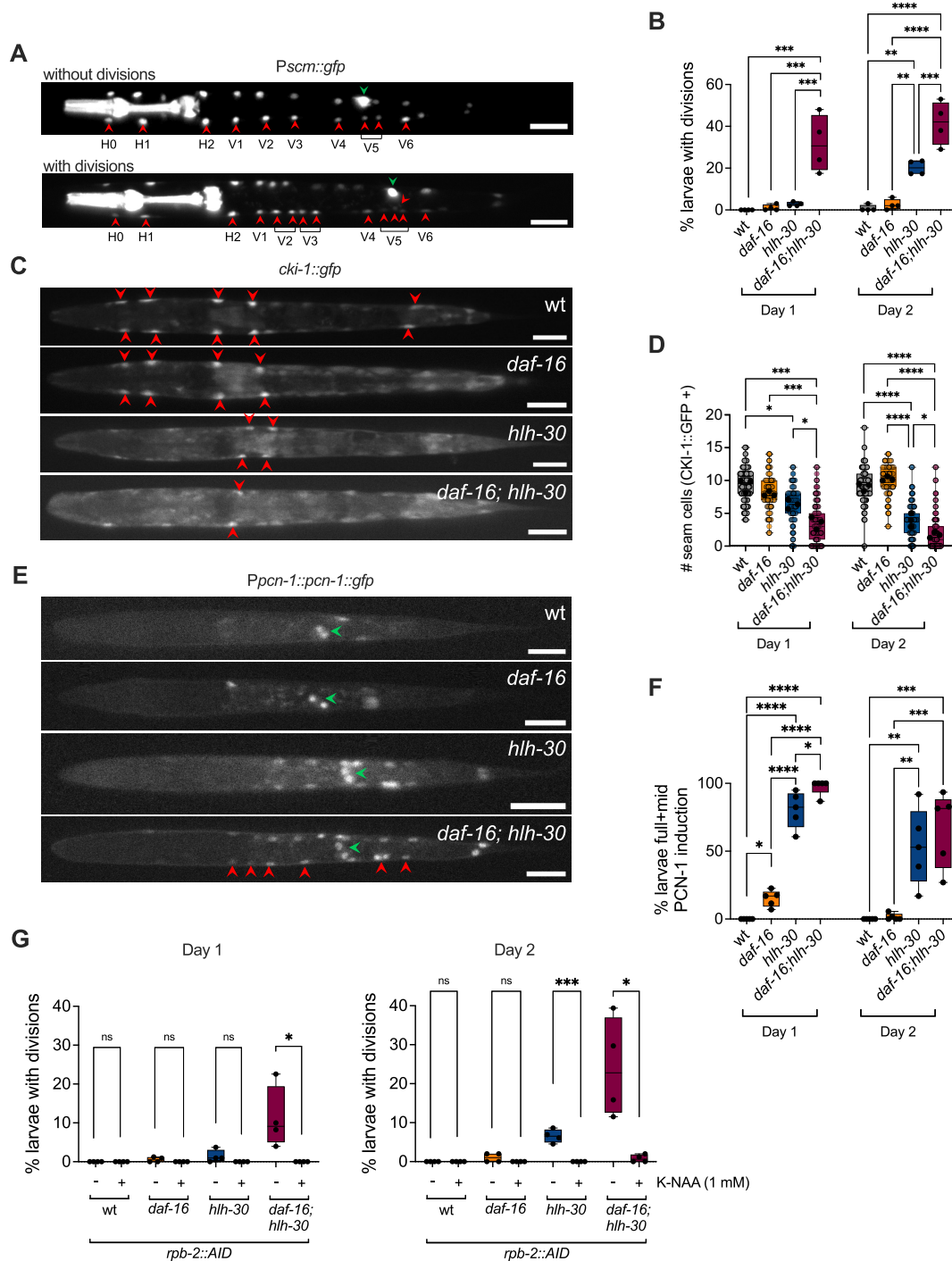


Figure 4. HLH-30 is a major regulator of cell cycle arrest during starvation. **(A)** Representative images of larvae without or with seam cell divisions. Red arrowheads mark the seam cells and the green one marks the M cell. **(B)** Percentage of larvae with seam cell divisions after 1 or 2 days of L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*. Each dot represents the values for one independent experiment. **(C)** Representative images of *cki-1* expression induction during L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*. Red arrowheads mark seam cells displaying CKI-1::GFP signal. **(D)** Number of seam cell per larva with induced *cki-1* (CKI-1::GFP+) after 1 or 2 days of L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*. Colored circles show values for individual larvae and black dots show the average for each experiment. **(E)** Representative images of *pcn-1* expression during L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*. Red arrowheads point to PCN-1::GFP expression in seam cells and green arrowheads to expression in Z2/Z3. **(F)** Fraction of larvae with mid plus high levels of PCN-1 induction after 1 or 2 days of L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*. **(G)** Percentage of larvae with seam cell divisions after 1 or 2 days of L1 starvation, for the wild type, *daf-16(mu86)*, *hlh-30(tm1978)*, and *daf-16;hlh-30*, in control conditions (–) or treated with the auxin analog NAA (+) for degradation of the RNA Polymerase II subunit *rpb-2*. Each dot represents the values for one independent experiment. In panels (B), (D), and (F), we performed one-way ANOVA followed by Tukey's multiple comparisons. Only significant differences are shown. In panel (G), we used *t*-test to evaluate the effect of *rpb-2* degradation on each genetic background. All plots are box and whiskers representing the independent replicates, marked with dots, except for panel (D), where the box and whiskers show the values for individual larvae and the averages per experiment are marked by dots. The complete statistics report can be found in [Supplementary File S1](#). Scale bar: 20 μ m.

All these results are consistent with defective inhibition of the cell cycle in *hlh-30* mutants.

To further prove that deregulation of mRNA transcription underlies aberrant cell cycle activity in *hlh-30* mutants, we tested whether RNA polymerase II activity is required for these inappropriate divisions. We tested the effect of degradation of the polymerase II subunit RPB-2 on cell division. For specific RPB-2 degradation, we used a strain with a tag for AID at the endogenous *rpb-2* locus [49] and checked the fraction of L1 with divisions after 1 and 2 days of starvation. The aberrant seam cell divisions in the *hlh-30* and *daf-16;hlh-30* mutants during starvation could be rescued by degradation of the RNA polymerase II subunit *rpb-2* (Fig. 4G). Together, these findings reveal a previously unappreciated role for HLH-30 in maintaining cell cycle quiescence during L1 starvation, acting in parallel to DAF-16 and potentially through modulation of RNA polymerase II activity.

DAF-16 and HLH-30 reciprocally impact their function in regulation of gene expression

To find candidates for the effect of HLH-30 on cell cycle progression, we turned again to the mRNA-seq results. The elevated number of differentially regulated genes in the *hlh-30* mutant makes it complex to tackle this question. Considering that changes in the activity of a single TF can provoke the differential expression of hundreds of genes, we rather analyzed TF overactivation in the mutants. For this task, we used CeEsT, a unified gene regulatory network built to estimate the activity of 487 distinct *C. elegans* TFs based on differential gene expression [39]. As a result, we obtained a score for the activity of each TF in each of the mutants, relative to the wild type. These scores represent inferred TF activity based on the expression patterns of downstream target genes and do not directly measure TF expression or protein abundance. In the *daf-16* mutant, the top overactivated TF is HLH-30 (Fig. 5A and [Supplementary File S2](#)), suggesting that in the absence of DAF-16, HLH-30 plays a more prominent role in L1 starvation. This result suggests that the increased HLH-30 nuclear localization in the *daf-16* mutant (Fig. 1C) confers a functional difference, as it impacts expression of HLH-30-dependent genes. Other TFs overactivated in the *daf-16* mutant are CEY-1, SMA-9, MADF-10, and TLP-1 (Fig. 5A and [Supplementary File S2](#)). Focusing on the TFs with reduced activity relative to the wild type, besides the obvious DAF-16 itself, we find EFL-1, PHA-4, FKH-9, and HIF-1, among others.

Mutation of *hlh-30* reduced the activation of DAF-16 (*P*-value .036) relative to the wild type (Fig. 5B and [Supplementary File S2](#)), a result that also agrees with our observation at the level of subcellular localization (Fig. 1B). In the *hlh-30* mutant, the top overactivated TFs are CEBP-1, ZIP-2, BLMP-1, ZIP-4, and MEP-1. The TFs with reduced activity in this mutant include HLH-30, MDL-1, LET-526, DVE-1, and NHR-11. In the double mutant, the lists of overactivated and downactivated TFs largely overlapped with those of the *hlh-30* mutant (Fig. 5C and [Supplementary File S2](#)).

We also investigated the effect of ethanol on TF activation in the *daf-16* mutant. We used an available dataset of DEGs between the *daf-16* mutant and the wild type under L1 starvation in S-basal with 0.1% ethanol [50]. Under these conditions, the *daf-16* mutant does not exhibit the HLH-30 overactivation that we observed in complete starvation

([Supplementary Fig. S6A](#)). Additionally, we calculated the correlation between TF activity scores in the *daf-16* mutant with ethanol and those in the single and double mutants without ethanol, across all possible combinations. We found that the *daf-16* mutant without ethanol showed a mild negative correlation with *hlh-30*, likely due to the overactivation of HLH-30-dependent genes in the *daf-16* mutant, and no correlation with the *daf-16;hlh-30* double mutant ([Supplementary Fig. S6B](#)). In contrast, the *daf-16* mutant exposed to ethanol displayed a significant positive correlation with both *hlh-30* and *daf-16;hlh-30* ([Supplementary Fig. S6B](#)). This result suggests that the reduced nuclear localization of HLH-30 in the presence of ethanol ([Supplementary Fig. S5A](#)) has functional consequences, specifically dampening the transcriptional activation of HLH-30 downstream regulated genes. In other words, ethanol masks the role of HLH-30 during L1 arrest by deactivating this TF. Instead, in complete starvation these two TFs collaborate and reciprocally impact their activation.

Overactivation of BLMP-1 and CEBP-1 in *hlh-30* mutants contribute to cell-quiescence loss under starvation

Considering the impaired seam cell arrest of the *hlh-30* and *daf-16;hlh-30* mutants, we decided to compare TF activation in these mutants with that of developing L1 larvae. We performed another mRNA-seq experiment to assess gene expression and TF activation in developing (fed) L1 compared to arrested (starved) L1. When we compared TF activation in the mutant samples among each other, and to that of the fed sample, we observed that *hlh-30* and *daf-16;hlh-30* mutants showed a significant positive correlation with the fed condition ([Supplementary Fig. S6C](#)). Additionally, *hlh-30* and *daf-16;hlh-30* showed the strongest correlation between each other. However, the relative TF activation of the *daf-16* mutant did not correlate with that of the fed sample and showed a weak negative correlation with the *hlh-30* mutant ([Supplementary Fig. S6B and C](#)). This result suggests that the *hlh-30* mutation confers transcriptional features that are similar to those of fed developing larvae.

With these clues, we decided to use RAPToR (Real Age Prediction from Transcriptome staging On Reference). RAPToR uses interpolation of gene expression time-series to predict the developmental age of a sample from their expression profile [40]. With this analysis, our fed L1 sample showed an estimated age of ~12 h, consistent with samples being collected 24 h after embryo preparation (Fig. 5D). The estimated time from hatching of the starved wild type and *daf-16* mutant samples was less than 1 hour, consistent with developmental arrest after hatching. However, *hlh-30* and *daf-16;hlh-30* showed significant differences of 2 and 3 h of developmental progression, respectively, compared to the wild type (Fig. 5D).

Interestingly, we observed that HLH-30 was the most inactivated TF and BLMP-1 the highest overactivated TF in fed larvae (Fig. 5E and [Supplementary File S2](#)). BLMP-1 expression and priming activities are normally repressed during L1 arrest and expressed in animals that have been re-fed. This way, BLMP-1 acts as a pioneer TF to decompact the *lin-4* locus in response to nutrients before transcriptional activation throughout development [51]. The miRNA *lin-4* acts as a developmental switch to ensure the timely progression from L1 stage to L2 [52]. In the wild-type strain, *lin-4* transcription is not observed in starvation-arrested L1 larva

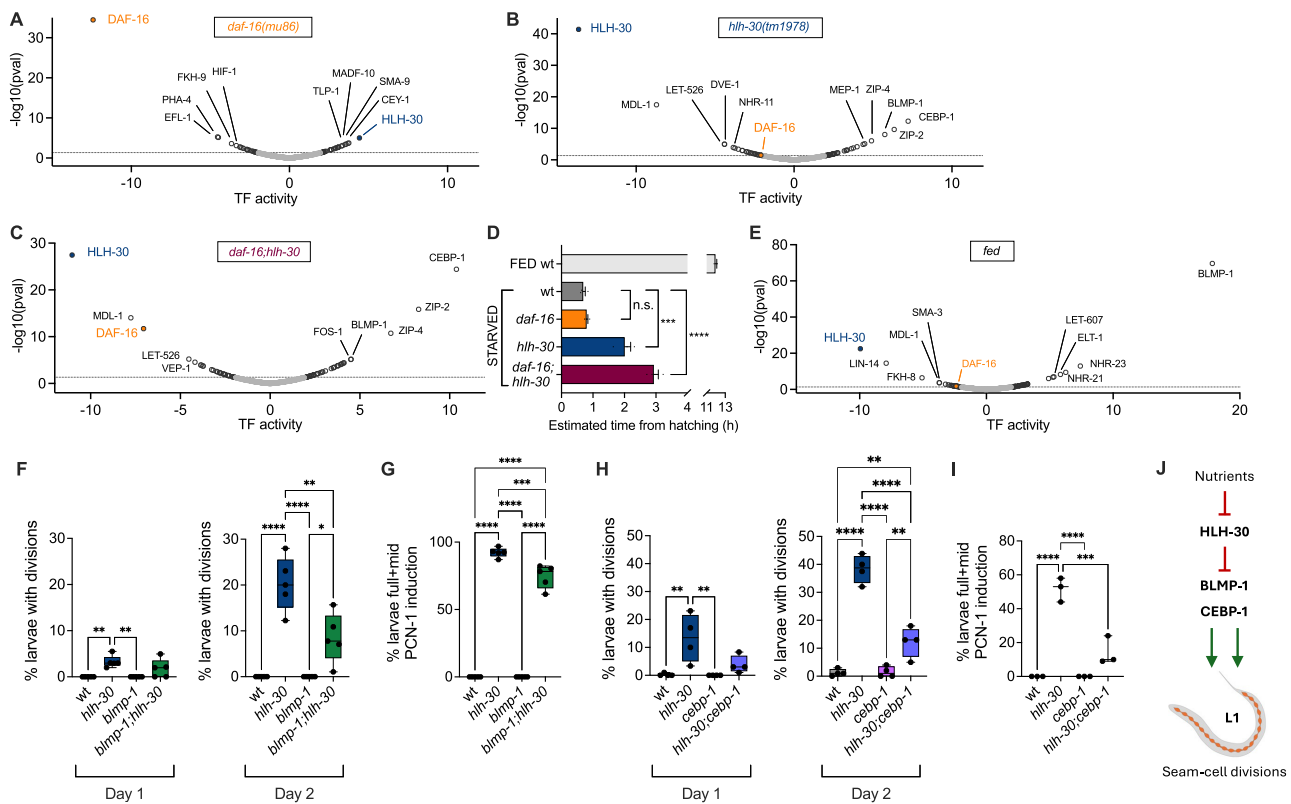


Figure 5. *hlh-30* mutation leads to BLMP-1 and CEBP-1 activation. Plots showing TF activity score relative to the wild type for day-1 starved larvae of the *daf-16* mutant (A), *hlh-30* (B), and *daf-16;hlh-30* (C). In panels (A)–(C), the dotted lines at $y = 1.333$ mark the significance threshold. TF activity changes over the threshold are represented by black circles and below are represented by gray circles. (D) Estimated age from hatching calculated by RAPToR, based on the overall gene expression for each condition. Error bars represent SEM. We compared the averages of each strain using one-way ANOVA followed by Dunnett's test to compare each sample to the wild-type strain. (E) Plot showing TF activity score for fed versus starved wild-type larvae. (F) Percentage of larvae with seam cell divisions after 1 or 2 days of L1 starvation, for the wild type, *hlh-30(tm1978)*, *blmp-1(s71)*, and *blmp-1;hlh-30* mutants. (G) Fraction of larvae with mid plus high levels of PCN-1 induction after 1 day of L1 starvation, for the same strains shown in panel (F). (H) Percentage of larvae with seam cell divisions after 1 or 2 days of L1 starvation, for the wild type, *hlh-30(tm1978)*, *cebp-1(tm2807)*, and *cebp-1;hlh-30* mutants. (I) Fraction of larvae with mid plus high levels of PCN-1 induction after 1 day of L1 starvation, for the same strains shown in panel (H). (J) Proposed model for HLH-30-mediated control of seam cell quiescence via BLMP-1 and CEBP-1 repression under different nutritional conditions. In panels (F)–(I), plots are box and whiskers representing the independent replicates, marked with black dots. For these panels, for statistical analysis, we performed one-way ANOVA followed by Tukey's multiple comparisons. Only significant differences are shown. The complete statistics report can be found in [Supplementary File S1](#).

[53]. When we checked *blmp-1* expression in the mRNA-seq data, we observed that, indeed, *blmp-1* expression was significantly increased in the *hlh-30* mutant and the double mutant, compared to the wild type and *daf-16* (Supplementary Fig. S7A). Indeed, BLMP-1 appears among the list of top five overactivated TFs in the *hlh-30* and the *daf-16;hlh-30* mutants (Fig. 5B and C). Then, we investigated the expression of the microRNA *lin-4* using a *Plin-4::yfp* reporter strain (Supplementary Fig. S7B). We observed that the levels of the miRNA *lin-4* were increased in the *hlh-30* and double mutants, being significantly different from the wild type and the *daf-16* mutant after 2 days of L1 starvation (Supplementary Fig. S7C).

All this information makes BLMP-1 an interesting candidate to mediate the arrest-defective phenotype of *hlh-30*, as the overactivation of BLMP-1 could activate the developmental program during starvation in this mutant. To test this hypothesis, we analyzed seam cell divisions and PCN-1 induction in wild type, *hlh-30*, the *blmp-1(s71)*, and *blmp-1;hlh-30* backgrounds. We observed that the *blmp-1* mutation partially rescues the increase in seam cell divisions (Fig. 5F) and in the PCN-1 fluorescent signal (Fig. 5G and

Supplementary Fig. S7D) caused by HLH-30 loss. However, the lack of BLMP-1 in the presence of food did not result in a delayed seam-cell division during the L1 developmental program (Supplementary Fig. S7E). This could mean that upon starvation, overactivation of the priming activity of BLMP-1 contributes to the loss of cell quiescence, whereas the absence of this function in fed larvae has no major impact, potentially being overcome by alternative factors that maintain normal developmental progress.

We then focused on CEBP-1, the most overactivated TF in *hlh-30* and *daf-16;hlh-30* mutants (Fig. 5B and C). CEBP-1 is the homolog of the mammalian C/EBP, a conserved basic leucine-zipper (bZIP) domain TF with a variety of roles in cell proliferation, differentiation, and stress response [54–56]. In *C. elegans*, CEBP-1 has been mainly related to axon regeneration [57]. In our mRNA-seq analysis, we observed increased expression of the *cebp-1* gene in *hlh-30* and *daf-16;hlh-30* mutants, indicating that HLH-30 exerts a repressive activity on the expression of this TF (Supplementary Fig. S7G). We therefore decided to analyze the effect of the loss-of-function mutation *cebp-1(tm2807)* on seam-cell divisions and PCN-1 induction in wild-type and *hlh-30* backgrounds. As in the

case of *blmp-1*, we also observed a partial rescue of both seam-cell divisions and PCN-1 expression (Fig. 5H and I, and [Supplementary Fig. S7H](#)). This shows for the first time a role for CEBP-1 as a positive regulator of cell proliferation in *C. elegans* larvae.

These results support a model in which HLH-30 ensures proper developmental arrest by repressing BLMP-1 and CEBP-1 activity under starvation conditions, thereby preventing premature transcriptional activation and cell cycle progression (Fig. 5J). Interestingly, when the available HLH-30 ChIP-seq datasets were analyzed, we identified HLH-30 binding sites upstream of both the *blmp-1* and *cebp-1* genes, suggesting they represent potential direct targets of HLH-30 ([Supplementary File S2 and Supplementary Fig. S8](#)).

Interestingly, mutations in *blmp-1* or *cebp-1* did not improve starvation survival or recovery from the arrest ([Supplementary Fig. S7F and I](#)). This indicates that the overactivation of these TFs only contributes to the *hlh-30*-mutant phenotype related to cell cycle arrest deficiency.

Discussion

Functional collaboration between DAF-16 and HLH-30

The TFs DAF-16/FOXO and HLH-30/TFEB frequently act together in *C. elegans*, forming a combinatorial transcriptional module that responds to various stressors, including oxidative stress, starvation, and aging. This synergy is stimulus-dependent and reflects a finely tuned evolutionary adaptation to environmental challenges [28, 58]. Both DAF-16 and HLH-30 are critical regulators that support L1 survival during starvation, sharing a substantial number of downstream regulated genes—~64% (1894 out of 2975) of the genes differentially regulated in *daf-16* mutants are also misregulated in *hlh-30* mutants (Fig. 2B).

In this study, we examined their functional interdependence during L1 arrest and observed a reciprocal influence in their activation. Notably, in the absence of DAF-16, HLH-30 becomes overactivated during L1 arrest. This conclusion is supported by both subcellular localization data and the upregulation of HLH-30-dependent genes in *daf-16* mutants (Figs 1B and 5A). Conversely, *hlh-30* mutants do not exhibit overactivation of DAF-16. Instead, they show reduced activation of this TF (Figs 1B and 5B). Interestingly, the hypomorphic *daf-2* (*e1370*) mutation enhances survival and recovery in *hlh-30* mutants in a DAF-16-dependent manner, implying that nuclear DAF-16 may partially substitute for HLH-30 function. DAF-16 and HLH-30 are known to physically interact under stress conditions in other contexts [28], and both localize to the nucleus on day 1 of L1 arrest. However, the dynamic patterns of their nuclear localization during L1 arrest (Fig. 1B and C) suggest that their interaction in this specific context warrants further investigation.

HLH-30 as a master regulator during L1 starvation

Previous investigations into the role of HLH-30 during L1 arrest have primarily focused on its function as a regulator of lysosome biogenesis. In particular, HLH-30-dependent activation of the lysosomal lipase *lip1-2* has been shown to play a role in survival under starvation conditions [26]. In this study, we reveal additional, previously unrecognized roles for HLH-30 during L1 starvation. First, HLH-30 is required

for chromatin reorganization in response to nutrient deprivation (Fig. 3A–C). Under starvation, chromatin in the intestine undergoes a distinct reorganization, forming two concentric rings at the nuclear and nucleolar periphery. This structural change is mediated by the inactivation of mTOR signaling [13] and we now demonstrate that HLH-30 is necessary for this reorganization.

Most notably, HLH-30 is also required for cell cycle arrest during L1 starvation (Fig. 4), a finding that was unexpected given that this role had previously been attributed primarily to DAF-16 [21]. We have unveiled that this discrepancy likely stems from differences in experimental conditions. In earlier studies demonstrating DAF-16's role in activating *cki-1* and promoting cell cycle arrest, the starvation medium contained ethanol. Subsequent research revealed that the failure of *daf-16* mutants to arrest cell divisions was ethanol-dependent and did not occur in ethanol-free M9 buffer [23]. This suggests that ethanol interferes with alternative compensatory mechanisms that might otherwise suppress proliferation in the absence of DAF-16. Our data further support this interpretation: we observed that ethanol reduces HLH-30 nuclear localization ([Supplementary Fig. S5A](#)) and activation of HLH-30-dependent genes ([Supplementary Fig. S6A](#)), indicating that ethanol may directly impair HLH-30 function. L1 larvae can use ethanol as a carbon source, promoting the synthesis of fatty acids and amino acids that can potentially activate the kinase activity of TORC1 [59–60]. These findings suggest that DAF-16 and HLH-30 act redundantly to mediate cell cycle arrest during L1 starvation. However, in the presence of ethanol, HLH-30 is deactivated, rendering DAF-16 essential for this process. Consistent with this, *hlh-30* mutants exhibit defective cell cycle arrest, likely due to the combined absence of HLH-30 and the reduced activation of DAF-16 in these mutants.

HLH-30 represses BLMP-1 and CEBP-1 to prevent premature developmental progression during starvation

Our data reveal that *hlh-30* mutants fail to arrest cell divisions during L1 starvation and instead exhibit a transcriptional profile resembling that of fed, developing larvae (Fig. 5D and [Supplementary Fig. S6C](#)). A defining feature of this developmental transcriptional state is the activation of BLMP-1 target genes in fed larvae (Fig. 5E), which are also markedly overexpressed in starved *hlh-30* and *daf-16;hlh-30* mutants (Fig. 5B and C). We identified a core set of deregulated TFs, where CEBP-1 is the most highly overactivated in *hlh-30*-deficient backgrounds under starvation (Fig. 5B and C). This suggests that in the absence of HLH-30, the inappropriate activation of downstream pathways compromises the starvation-induced developmental arrest program.

Notably, our findings demonstrate that the independent mutation of either *blmp-1* or *cebp-1* partially rescues the defective cell cycle arrest phenotype of *hlh-30* mutants, reducing both ectopic seam cell divisions and the premature induction of PCN-1::GFP (Fig. 5H and I), indicating that overactivation of these TFs contributes directly to the failure to suppress proliferation. Moreover, analysis of available HLH-30 ChIP-seq datasets revealed direct binding sites upstream of both the *blmp-1* and *cebp-1* loci ([Supplementary File S2](#)), supporting a model in which HLH-30 directly represses these TF genes to prevent premature developmental progression during

stress. However, it should be noted that BLMP-1 and CEBP-1 activity was inferred from transcriptomic analyses and genetic interactions. Therefore, although our results strongly support enhanced BLMP-1- and CEBP-1-dependent transcriptional activity in *hlh-30* mutants, we did not directly measure BLMP-1 or CEBP-1 protein abundance.

BLMP-1 is a conserved TF that coordinates post-embryonic developmental programs and modulates the transcriptional output of cyclically expressed genes, contributing to developmental robustness in *C. elegans* [61–62]. It is homologous to mammalian BLIMP-1 (PRDM1), a key regulator of cell fate decisions (reviewed in Bikoff *et al.* [63]). Recent work has shown that BLMP-1 facilitates transcriptional priming by promoting chromatin decompaction at target loci, thereby modulating the amplitude and duration of gene expression without altering its timing. This chromatin remodeling is regulated by nutritional inputs and becomes essential when animals resume development after nutrient-mediated arrest [51]. In *hlh-30* mutants, the overactivation of BLMP-1 targets during starvation likely reflects a failure to suppress this priming mechanism, leading to inappropriate cell cycle progression.

In mammals, C/EBP factors interact with cell cycle proteins to modulate the balance between cell proliferation and differentiation depending on the cellular microenvironment and specific isoform context [55, 56]. In *C. elegans*, CEBP-1 has been primarily studied in post-mitotic tissues, where it acts downstream of the p38/PMK-3 MAPK pathway to promote axon regeneration and neuronal stress responses [57]. However, our data uncover a novel role for this factor as a positive regulator of cell proliferation in larvae.

Interestingly, while mutating *blmp-1* or *cebp-1* mitigates the loss of cell quiescence, it does not improve the starvation survival or recovery rates of *hlh-30* mutants (Supplementary Fig. S7F and I). This allows us to conceptually uncouple the dramatic sensitivity of *hlh-30* mutants to starvation from their cell cycle arrest deficiency. Together, our results highlight a dual functionality for HLH-30 during L1 arrest: it acts through distinct downstream mechanisms to maintain starvation survival, while simultaneously safeguarding the transcriptional integrity of the L1 arrest program by directly antagonizing BLMP-1 and CEBP-1 functionalities.

Broader implications for cancer and cell senescence

Transcription factor EB (TFEB) is a central regulator of cellular homeostasis in mammals, primarily recognized for its role in controlling autophagy and lysosomal biogenesis. It activates genes involved in the degradation and recycling of cellular components, particularly under stress conditions such as nutrient deprivation or oxidative damage. Beyond these functions, TFEB contributes to metabolic regulation, neuroprotection, immune responses, and inflammation. Dysregulation of TFEB has been implicated in various diseases, including neurodegenerative disorders and several types of cancer (reviewed in Napolitano and Ballabio [64]). The rapid proliferation of cancer cells increases their demand for energy and biosynthetic materials, making enhanced autophagolysosomal activity a common feature in tumors and positioning TFEB as a promising target in cancer research. Interestingly, the oncogenic potential of TFEB appears to be more closely tied to its regulation of the cell cycle than to macroautophagy. For example, TFEB can transcriptionally upregulate CDK4/6, promoting the G1/S transition and cell division [65]. Con-

versely, TFEB also exhibits tumor-suppressive properties. We have shown that HLH-30, its *C. elegans* ortholog, is required for CKI-1 induction during starvation, and in human cell lines such as HeLa and ARPE-19, TFEB depletion significantly reduces basal p21 expression. Overexpression of TFEB, on the other hand, upregulates p21 and induces G1 arrest [66]. With respect to senescence, TFEB targets are highly expressed in quiescent cells and TFEB activation can boost lysosomal function of senescent cells to improve their reactivation in response to proliferative signals [7]. These findings underscore the context-dependent nature of TFEB's role in cancer, highlighting its dual capacity to either promote or suppress tumorigenesis. Our findings on the role of HLH-30/TFEB in cell cycle arrest underscore the role of *C. elegans* L1 starvation to understand cellular processes related to quiescence maintenance.

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Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

None declared.

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Data availability

All raw sequencing data for mRNA-seq libraries are deposited at NCBI Gene Expression Omnibus (GEO) under accession number GSE313873. The mRNA-seq processed data (raw counts, normalized TMM, DESeq lists, TF activation scores) are included as the Supplementary data (File S2). All data contained in the figures and corresponding statistical analysis are published as the Supplementary data (File S1 and S3).

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