Published in final edited form as:

Science. 2011 January 21; 331(6015): 304–308. doi:10.1126/science.1199082.

Direct conversion of *C. elegans* germ cells into specific neuron types

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Abstract

The ability of transcription factors to directly reprogram the identity of cell types is usually restricted and is defined by cellular context. We show here that through ectopic expression of single *C. elegans* transcription factors, the identity of mitotic germ cells can be directly converted into that of specific neuron types (glutamatergic, cholinergic or GABAergic). This reprogramming event requires the removal of the histone chaperone LIN-53/RbAp48, a component of several histone remodeling and modifying complexes, and this removal can be mimicked by chemical inhibition of histone deacetylases. Our findings illustrate the ability of germ cells to be directly converted into individual, terminally differentiated neuron types and demonstrate that a specific chromatin factor provides a barrier for cellular reprogramming.

There is much interest in developing methods to direct cell differentiation to a specific cell fate. However, transcription factors required to induce the identity of specific cell types in a multicellular organism are remarkably ineffective in imposing their fate-inducing activity when ectopically expressed, resulting in the generally accepted paradigm that transcription factors can exert their activities only in specific cellular contexts (1, 2). Classic examples for such context dependency are the cell-type restricted ability of MyoD to induce muscle cell features (3), the region-restricted ability of ectopically expressed Pax-6/eyeless to induce ectopic eyes (4) or, as a more recent example, the restricted ability of a cocktail of transcription factors to directly reprogram the identity of pancreatic cell types (5). Our understanding of the mechanistic basis of the context dependency of transcription factor activity is limited. Overcoming such context dependency would have major implications for a variety of different applications. For example, the generation of specific cell type through transcription factor-mediated reprogramming strategies may allow the establishment of *in vitro* disease models for basic research or the provision of source material for cellular replacement therapies.

We sought to establish a system in which we can study the mechanistic basis of the context-dependency of transcription factor activity. To this end, we employed a genetic approach in the nematode *C. elegans*, using the Zn finger transcription factor CHE-1, which is required to induce the identity of a specific class of gustatory neurons, called ASE neurons (6, 7). CHE-1 exerts this activity through binding directly to a *cis*-regulatory motif (termed the "ASE motif") present in many ASE-specific terminal differentiation genes, such as chemoreceptors, signaling proteins, neurotransmitter transporters, receptors and others (6). Like CHE-1, several other invertebrate and vertebrate transcription factors are known to also co-regulate many terminal features of differentiated neurons in such a manner and have been

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Material and Methods are in the Supplementary Online Material

termed "terminal selectors" (8). To test whether CHE-1 is not only required but also sufficient to induce ASE fate, we ectopically expressed CHE-1 throughout the entire animal in either larval or adult stages, using an inducible heat-shock promoter. Such misexpression results in broad ectopic expression of an artificial reporter of CHE-1 transcription factor activity, which is composed of a multimerized ASE motif ("8× ASE motif reporter") (6) (Fig. 1). This indicates that, in principle, CHE-1 can exert its biochemical activity of DNA binding and transcriptional activation without spatial or temporal constraints. In contrast, postembryonic ectopic expression of *che-1* during larval or in adult stages is able to induce markers for terminal ASE fate (the *gcy-5* chemoreceptor and the *ceh-36* homeobox gene; see **Table S1** for list of markers) only in a small number of head sensory neurons but nowhere else in the animal (Fig. 1; **Table S2**). This result illustrates the context-dependency of the ASE-fate inducing activity of CHE-1.

To test the hypothesis that the mechanistic basis for such context-dependency lies in inhibitory, perhaps chromatin-based mechanisms that may prevent CHE-1 from reprogramming the identity of other cells, we screened through an RNAi library that targets all genes in the *C.elegans* genome with predicted roles in chromatin regulation, based on the presence of characteristic protein domains (9) (**Table S3**). We found that RNAi-mediated knock down of *lin-53*, the *C.elegans* ortholog of the phylogenetically conserved, WD40 domain-containing Retinoblastoma (Rb)-binding protein RbAp46/48 (10), permits ectopically expressed CHE-1 to induce the ASE neuronal fate markers *gcy-5* and *ceh-36* in a large number of normally non-neuronal cells in the midbody region of larval and adult animals (Fig. 2). Up to 52% of animals (n=227) show this effect (**Table S4**) and this effect can be observed using distinct, non-overlapping dsRNA clones that target *lin-53* and six out of six tested transgenic *che-1* lines (see Supplementary Methods).

Closer examination of the animals reveals that *gcy-5* and *ceh-36* induction is observed in cells located within the germ line (Fig. 2). Differential interference contrast (DIC) microscopy reveals that germ cells of *che-1^{heat-shock}; lin-53(RNAi)* animals lose their characteristic, fried egg-shaped nuclear and nucleolar morphology and rather adopt a larger, speckled neuronal nuclear morphology (Fig. 2). Moreover, these cells grow cellular extensions resembling axo/dendritic projections (Fig. 2; **Suppl. Movies 1–3**). These neuron-like cells indeed originate from the germline as they are not observed following ectopic *che-1* expression and *lin-53* removal in a *glp-4* mutant background (**Fig. S1**), in which no germline is formed (11). Genetic removal of sperm (*fem-3lf* mutants; (12)) or oocyte (*fem-3gf* mutants (13)) or prevention of entry into meiosis (*glp-1gf* mutants; (14)) does not affect neuron induction, whereas severe reduction of the mitotic pool (*glp-1lf* mutants (14)) does, suggesting that it is the mitotic germ cells that become neuron-like (**Fig. S1**). This is further supported by the position of the converted cells relative to mitotic and meiotic markers (**Fig. S2**) and the observation that second and third larval stage animals which contain mostly mitotic, but no meiotic cells show germ cell to neuron conversion upon ectopic *che-1* expression and *lin-53* knockdown (**Table S4**). The conversion to neuron-like cells is efficient and fast; up to around 60 germ cells (out of a total of around 200 mitotic germ cells) undergo neuronal induction (**Fig. S3**) and morphological changes and marker induction first occur 6 hours after *che-1* induction (Table S5). For comparison, the induction of the 8× ASE motif, an indicator of CHE-1 transcriptional activity occurs 4 hours after *che-1* induction in the gonad. The induction of the 8 × ASE motif in the gonad of wild-type animals, as well as antibody staining conducted in both wild-type and *lin-53(RNAi)* animals, rules out the possibility that *lin-53(RNAi)* merely results in germline derepression of the *che-1* transgene (we also note that *lin-53(RNAi)* does not result in germline derepression of a previously described transgenic array, *let-858::gfp*, known to be derepressed after loss of several different chromatin factors (15)).

We assessed the nature of these *che-1*-induced, neuron-like cells with a number of fate markers. Through antibody staining of a marker that labels specific germ cell structures, the P-granules, we confirmed that this germ cell feature is indeed lost upon ectopic *che-1* expression and *lin-53* knockdown (**Fig.S4**). Moreover, the reprogrammed cells express six out of six tested pan-neuronal reporter genes: the *rab-3/Rab3* and *snb-1/synaptobrevin* genes, which encode presynaptic proteins normally exclusively expressed in all cell of the nervous system, the pan-neuronal axonal regulators *unc-33/CRMP* and *unc-119* and the pan-neuronal signaling factor *rgef-1* (Fig.3A; see **Table S1** for transgenic reporters). Antibody staining against endogenous proteins also shows ectopic expression of presynaptic synaptobrevin/SNB-1 and RIM/UNC-10 proteins; those proteins appear to cluster in presynaptic specialization in the axonal extensions of the reprogrammed germ cells (Fig.3B; **Fig.S5**). The reprogrammed cells also express a ciliated marker gene, *osm-6*, a member of the intraflagellar transport particle, normally expressed exclusively in all ciliated sensory neurons, including ASE (Fig.4A). GFP-tagged OSM-6 protein also appears to cluster in particles in the induced neurons (Fig.4A) suggesting that these cells express and cluster intraflagellar transport particles. All tested components of the gene battery that combinatorially define ASE identity are also expressed in the induced neurons. That is, aside from the above mentioned chemoreceptor *gcy-5* and the transcription factor *ceh-36* (shown again in Fig.4A), the putative chemoreceptor *gcy-7*, which is normally exclusively expressed in ASE, and the vesicular glutamate transporter *eat-4*, normally expressed in ASE and a restricted number of additional head ganglia neurons, are also expressed in the reprogrammed germ cells (Fig.4A). Induction of *eat-4* demonstrates that the reprogrammed cells are glutamatergic. The reprogrammed ASE-like cells do not express a battery of markers that are normally expressed in other neuron types, such as dopaminergic, serotonergic, cholinergic and GABAergic markers, among others (Fig.4B). This argues that the reprogrammed cells are not merely generic and/or mis-specified neurons but closely resemble normally differentiated ASE neurons. Taken together, animals ectopically expressing *che-1* and lacking *lin-53* not only contain their normal set of 2 ASE gustatory neurons in the head, but contain a gonad filled with dozens of ASE-like neurons.

Removal of RNA-binding gene regulatory factors has been shown to result in the formation of teratomas, i.e. cells of various origin and types, in the germline (16). The effects that we observe upon loss of *lin-53* are not a reflection of teratoma formation, since we observe no expression of various neuronal fate markers other than those of ASE fate in the gonad of *che-1^{heat-shock}; lin-53(RNAi)* animals. However, the very same neuronal markers (GABA neuron marker, cholinergic neuron marker) are expressed in the gonad of animals in which the translation regulator *gld-1*, a previously described repressor of teratoma formation (16), is knocked down (**Fig.S6**). Therefore, removal of *lin-53* does not by itself trigger alternative developmental programs but primes germ cells to be responsive to a neuronal fate inducer like *che-1*.

To test whether *lin-53* removal also permits the conversion of germ cells into other neurons types, we tested two other terminal selector genes (8), the phylogenetically conserved Pitx-type homeobox gene *unc-30*, a terminal selector required for the generation of GABAergic motor neurons in the ventral nerve cord (17, 18) and the EBF-like transcription factor *unc-3*, required for the generation of two types (A- and B-type) of cholinergic motor neurons in the ventral nerve cord (19, 20). When ectopically mis-expressed, neither *unc-30* nor *unc-3* is able to induce GABAergic or cholinergic neuron fate in the germ line, respectively. However, upon removal of *lin-53*, heat-shock induction of either *unc-30* or *unc-3* results, like *che-1* induction, in germ cells losing their characteristic morphology and instead adopting neuron-like nuclear morphology and growing axonal projections (Fig.5A,B). In the case of *unc-3*, the ectopic neurons now express a marker characteristic of cholinergic A/B-type ventral cord motor neurons (*acr-2*; Fig.5B), whereas ectopic expression of *unc-30*

results in the expression of the GABAergic marker *unc-47* (Fig.5A). In neither case do we observe any ASE marker expression to be induced (>100 animals scored); also, neither cholinergic nor GABAergic markers are induced by *che-1* (Fig.4B). We conclude that upon loss of *lin-53*, germ cells acquire the ability to be reprogrammed into distinct neuron types through the activity of neuron-type specific terminal selector transcription factors.

Does *lin-53* removal prime germ cells to only respond to factors that induce neuronal fates or can they now respond to other factors as well? To address this question, we ectopically expressed a selector gene, the *C. elegans* MyoD homolog *hlh-1* that was previously shown to be able to ectopically induce muscle fate in early embryos (21). We find that *hlh-1* is unable to convert the germ cells of *lin-53(RNAi)* animals into muscle cells (Fig.S7). These negative results need to be cautiously interpreted, but may represent a first hint toward a target selectivity of *lin-53*. That is, *lin-53* may only restrict the developmental potential specifically toward a neuronal developmental program while other factors may serve to prevent the induction of other, non-neuronal differentiation programs.

LIN-53, which is ubiquitously expressed (10), is one of several, phylogenetically conserved histone chaperones which are thought to assist in the recruitment of various distinct types of histone modifiers or remodelers, including histone methyltransferases, histone acetylases and histone deacetylases to histone H3 and histone H4 (22, 23). LIN-53 orthologs in various species have been found to be integral components of at least 6 different protein complexes, each displaying diverse biochemical and biological roles - the NURD and NURF nucleosome remodeling complexes, the CAF-1 chromatin assembly factor complex, the Sin3A transcriptional repressor complex, the PRC2 histone methyltransferase complex and the HAT1 histone acetyltransferase complex (22, 23) (Table S6). RNAi of representative members of each of these complexes either did not phenocopy the *lin-53(RNAi)*-induced reprogramming ability of ectopic CHE-1 expression or could not be interpreted due to early lethality induced by RNAi (Table S6). However, since two of the LIN-53-associated complexes (NURD and Sin3a) each contain at least two histone deacetylase (HDAC) components and since the reprogramming role of *lin-53* may involve several complexes, we sought to broadly inhibit HDAC function by using two distinct chemical inhibitors, valproic acid and trichostatin A (24). At sub-lethal doses, animals treated with either drug survived and permitted heat-shock induced *che-1* to induce ASE fate in the germ line (and no other cell type) even in the presence of functional *lin-53* (Fig.6). Even though these drug effects may be unrelated to normal *lin-53* function, these results nevertheless provide a strong indication that histone modifications are key players in restricting the ability of a transcription factor to reprogram cellular identity.

Our finding that the removal of a single chromatin factor, together with the induction of single transcription factors, can produce distinct and specific neuron types in a heterologous cellular context is a testament to the simplicity of programs that control neuronal differentiation. The main role of complex, multi-stage neuronal developmental programs may be little more than to activate a terminal regulatory routine, that is, a single or a combination of terminal selectors which then induce a specific differentiated state. This notion may apply to more complex systems as well, since recent work has shown that it takes as little as two transcription factors to drive a differentiated fibroblast toward a specific neuronal fate (25). Our findings are also a testament to the pluripotency of germ cells (26). This totipotency is normally kept in check by a variety of transcriptional and post-transcriptional mechanisms (26), but is unleashed either spontaneously in pathological situations (germ cell tumors)(27) or upon culturing germ stem cells under specific conditions, which transform these cells into cells indistinguishable from pluripotent embryonic stem cells (28). We have shown here that the ability of germ cells to be directly reprogrammed into neurons can be unleashed, even in the adult animal, through removal of

a single gene that we speculate to be involved in rendering neuronal differentiation genes inaccessible to transcriptional induction by, for example, contributing to the formation of facultative, i.e. conditional and developmentally regulated heterochromatin.

Seen in a broader context, our results indicate that the reprogramming of cellular identity may critically depend not just on providing the correct transcription factor(s) that induce a specific fate, but also on the removal of inhibitory mechanisms that restrict transcription factor activity. We anticipate that the disablement of such inhibitory mechanisms may provide an efficient strategy to ectopically generate neuron types in other organisms or perhaps even in cell culture using isolated germ cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by the NIH (R01NS039996-05; R01NS050266-03). O.H. is an Investigator of the HHMI and B. T. is a Francis Golet Research Scientist. We thank T. Schedl, M. Krause, J. McGhee, P. Sengupta, Y. B. Qi, Y. Jin, M. Nonet, S. Strome and members of the Hobert lab for reagents, I. Greenwald and members of the Hobert lab for comments on the manuscript and Q. Chen for expert assistance in strain generation.

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Figure 1. Context-dependency of CHE-1 induction of target genes

Ectopic expression of CHE-1 can induce an artificial reporter of CHE-1 activity (a multimerized binding site, called the "8× ASE motif") throughout the animal (upper two panels; observed in 50/50 heat-shocked animals), but can induce ASE cell fate markers, such as the *gcy-5* chemoreceptor (exclusively expressed in ASER) or the *ceh-36* homeobox gene (expressed in ASEL/R and AWCL/R) only in 1–3 types of other sensory neurons (blue arrows), consistent with previous reports (6, 7). Heat-shock induction of CHE-1 was done at late larval stages. **Table S1** contains details on transgenic reporters and **Table S2** shows data quantification.

Figure 2. Removal of *lin-53* permits *che-1* to induce ASE fate markers in the germ line
(A) ASE cell fate markers (*gcy-5* and *ceH-36*), a transformation of nuclear morphology (from germ line with large nucleolus to neuronal with characteristic intranuclear speckles) and outgrowth of cellular extensions (blue arrows; also see panel B) are induced in the gonad of *lin-53*($-$) animals upon ubiquitous induction of *che-1* expression. Non-induced, *lin-53*($+$) controls are shown in Fig.1. This phenotype is observed in the germline of up to 52% of heat-shocked animals (see **Table S4**, Table S5 and **Fig.S3** for quantification) and in six out of six transgenic *che-1^{heat-shock}* lines; the line shown here is *otIs284*. Heat-shock of *lin-53*($-$) animals that do not carry the *che-1^{heat-shock}* array do not show any ectopic marker induction.

(B) Higher magnification image of axonal extensions induced by ectopic *che-1* expression in *lin-53(RNAi)* animals. The protruding vulva (Pvul) phenotype is a characteristic pleiotropy of loss of *lin-53*. Suppl. Movies 1, 2 and 3 show additional examples of axonal projections.

Figure 3. Reprogrammed germ cell express pan-neuronal markers and cluster presynaptic proteins

(A) Induction of pan-neuronal reporter genes in *che-1^{heat-shock}*; *lin-53(RNAi)* animals.

(B) Induction of the presynaptic SNB-1 protein, as assessed by antibody staining. An individual ASE-like neuron (labeled with *gcy-5::gfp*) is shown in higher magnification to show punctate staining of SNB-1 marker along the extension of the process (white arrowheads).

che-1 expression was induced in late (L3/L4) larval stages of animals exposed to *lin-53(dsRNA)* starting in the parental generation. Adult midbody regions are shown. White stippled lines indicate outline of the animal, yellow stippled line indicate outline of the gonad. The penetrance of induction of the markers shown here are the same as for the *gcy-5::gfp* marker, shown in Fig.2 and **Table S4**. See **Table S1** for information of transgenes and their wildtype expression pattern.

(A) ASE specific markers expressed in *che-1^{heat-shock}*; *lin-53(RNAi)* animals. These are not normally expressed in cells in the midbody region, therefore showing no signal in the left panel column.

(B) Other neuronal fates are not expressed in *che-1^{heat-shock}*; *lin-53(RNAi)* animals. Both panel columns show the same animals; the left panel column shows an overall view of the entire animal to illustrate the expression of non-ASE markers (all mCherry-based reporters) in other neuron types, the right panel shows a blow up (white box) of the gonad region, illustrating that these markers do not become induced in the *che-1* reprogrammed germ cells, labeled with *gcy-5::gfp*.

Figure 5. Reprogramming activity of other selector genes in *lin-53*(-) animals

(A) Heat-shock induction of the *unc-30* terminal selector induces GABAergic, D-type motor neuron fate (as assessed by neuronal morphology and *unc-47* vesicular GABA transporter expression) in the germline of *lin-53(RNAi)* animals. White asterisks indicate ventral cord neurons that normally express the marker and which are located in proximity to the gonad. 6/32 *unc-30^{heat-shock}; lin-53(RNAi)* animals (line #1, *otEx4442*), 4/21 animals (line #2, *otEx4443*) and 5/23 animals (line #3, *otEx4444*) show germline *unc-47::gfp* (*oxIs12*) expression. Without heat-shock and RNAi, 0/100 animals show the effect.

(B) Heat-shock induction of the *unc-3* terminal selector induces A/B-type motor neuron fate in the germline of *lin-53(RNAi)* animals. White stars indicate ventral cord neurons that

normally express the marker and which are located in proximity to the gonad. The white arrow indicates axonal-like extensions. 5/24 *unc-3^{heat-shock}*; *lin-53(RNAi)* animals show expression of a *gfp* reporter for the autoreceptor *acr-2* expression. 0/20 *unc-3^{heat-shock}* animals (no *lin-53(RNAi)*) show this effect and without heat-shock and RNAi, 0/>100 animals show the effect.

Figure 6. Inhibitors of HDACs also permit *che-1* to reprogram germ cells

Animals were grown on plates containing the respective drug, *che-1* was induced in late larval stages and one day later, adult P0 animals were scored for ectopic *gcy-5::gfp* expression in the gonad. Compared to *lin-53(RNAi)*, the drug affects appear to be weaker (5/40 animals affected show ASE-like neurons in valproic acid treated animals, 3/40 in TSA-treated; 0/50 mock-treated animals show an effect) and not as many germ cells appear to be transformed, possibly due to drug dosage issues (too high a dose results in no viable progeny, a phenotype similar to RNAi of at least one *C.elegans* HDAC; (29)). Heat shock in the absence *che-1* alone does not have an effect on drug-treated animals (40 animals scored).