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Et al.
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ABSTRACT
Snail-type transcription factors (TFs) are found in numerous metazoan organisms and function in a plethora of cellular and developmental processes including mesoderm and neuronal development, apoptosis and cancer. So far, Snail-type TFs are exclusively known as transcriptional repressors. They repress gene expression by recruiting transcriptional co-repressors and/or by preventing DNA binding of activators from the basic helix-loop-helix (bHLH) family of TFs to CAGGTG E-box sequences. Here we report that the Caenorhabditis elegans Snail-type TF CES-1 can activate transcription in vivo. Moreover, we provide results that suggest that CES-1 can share its binding site with bHLH TFs, in different tissues, rather than only occluding bHLH DNA binding. Together, our data indicate that there are at least two types of CES-1 target genes and, therefore, that the molecular function of Snail-type TFs is more plastic than previously appreciated.

INTRODUCTION
Snail-type TFs have been identified in numerous metazoan organisms, first in the fruit fly Drosophila melanogaster (1), and subsequently in others, including the nematode Caenorhabditis elegans (2), and human (3,4). Snail-type TFs are involved in many biological processes such as development, mesodermal and neuronal differentiation, apoptosis and cancer (5–7). The Snail-type family of TFs includes members that most resemble the firstly identified Snail, and proteins that resemble the homologous proteins Scratch and Slug. They possess three to five C2H2 zinc fingers with which they bind DNA in a sequence-specific manner. The DNA-binding specificity of several Snail-type TFs has been characterized to a limited extent. In vitro gel shift assays (2,8–11) and recently, in vivo (12) studies found that the Snail-type TFs bind the CAGGTG E-box and often more specifically the ACAGGTG sequence (referred to here as ‘Snail-box’).

So far, Snail-type TFs have been exclusively reported to function as repressors in vivo [see e.g. (3,4,13–15)]. The repressor activity of vertebrate Snail-type TFs depends on the SNAG repressor domain, which mediates repression by recruiting histone deacetylases (6,16). However, except for Scratch, C. elegans and Drosophila Snail-type TFs do not contain a SNAG domain. Other mechanisms of repression by Snail-type TFs include interactions with the co-repressors dCtBP (17) and Ebi (18). In addition, Snail-like TFs can repress gene expression by preventing the binding of members of the basic region helix-loop-helix (bHLH) family of TFs to DNA, thereby antagonizing the function of these transcriptional activators (3,10,13,15,19).

The C. elegans genome encodes three Snail-type C2H2 TFs: CES-1, K02D7.2 and SCRT-1 (6) (Figure 1a). A gain-of-function (gf) in ces-1 (cell death specification-1)
was first discovered in a genetic screen to identify genes involved in programmed cell death (20). The ces-1 gene product was found to be most homologous to the Scratch members of the Snail superfamily (2). Like other Snail-type TFs, CES-1 has been reported to function as a transcriptional repressor. Specifically, in ces-1(gf) animals, CES-1 is overexpressed and represses its only known target gene, egl-1, by preventing binding of activating HLH-2/HLH-3 heterodimers to the multiple E/Snail-boxes that are located in or around this target gene (15). So far no transcriptional effect has been reported in ces-1 loss-of-function (lf) animals. Surprisingly, we find that CES-1 can function as a transcriptional activator in vivo, by binding to a single Snail-box in the promoter of B0507.1. We find that CES-1 binds an extended Snail-box that is comprised of nine nucleotides and which we refer to as a ‘CES-box’. Finally, our findings strongly suggest that CES-1 shares rather than antagonizes the target B0507.1 promoter with HLH-6, another bHLH family member, although each TF utilizes the site in different tissues. Thus, there are at least two types of CES-1 target genes: those that are repressed by CES-1 through a bHLH occlusion mechanism and those that are activated by both types of TFs, but in different tissues. Taken together, the transcriptional function of Snail-type TFs as well as their functional interactions with other TFs may be more flexible than previously thought.

MATERIALS AND METHODS

Yeast one-hybrid assays

Yeast one-hybrid (Y1H) assays were performed as described previously (21–23).

Mutant promoter and binding site constructs

Mutant promoter constructs were generated by PCR using plasmid templates and primers listed in Table S1. Mutant Promoter::GFP constructs were generated by mutating the promoter Entry vector construct and transferring the changed insert to pDD04 (24) by a Gateway LR recombination reaction (25). pENTRY::PB0507.1(ΔCES-box) was created using overlapping primers and the QuickChange Site Directed Mutagenesis Kit (Stratagene) that generates a mutant circular plasmid. All other mutant constructs were created using non-overlapping primers and PCR amplification with Platinum Pfx (Invitrogen) generating linear PCR products that require excision and extraction from agarose gel (Qiagen kit), treatment with PNK (New England Biolabs) and ligation using T4 Ligase (New England Biolabs). Binding site constructs were generated by cloning a DNA fragment containing a single copy of the putative binding site into BamHI/HindIII-digested Gateway-compatible Entry vector pMW#4 that we created for binding site cloning. These DNA fragments were created by annealing complementary primers (Table S1) with restriction enzyme-compatible overhangs. All constructs were confirmed by sequencing (Agencourt).

Caenorhabditis elegans transgenesis and mutant crosses

Transgenic animals were created by ballistic transformation in unc-119(ed3) animals as described (26,27). For each GFP construct we obtained multiple (up to eight) independent lines that all exhibited identical GFP expression patterns. Frozen stocks were generated for a maximum of three of these lines, and the best transmitting line was used for all experiments. For PB0507.1::GFP the strain VL456 was used because it contains the transcription regulatory network integrated in the genome. For PB0507.1(ΔCES-box)::GFP and PB0507.1(A+1C)::GFP, the non-integrated strains VL457 and VL708 were used respectively. Both VL457 and VL708 exhibited a >90% transmittal rate. The loss-of-function mutant strains ces-1(n703; n1434) (2), K02D7.2(bc366), scrt-1(tm509) and hhh-6(tm299) (28) were crossed into VL456.

Caenorhabditis elegans imaging and GFP expression scoring

Immobilized worms were viewed using a Zeiss Axioscope 2 and images were acquired using a Hamamatsu Orca-ER Digital Camera. GFP expression was scored for each genotype in least 100 worms (50 L1 to L3 larval stages, and 50 L4 to adult).

Bioinformatics

The sequence logo for the CES-1 binding site was created using http://weblogo.berkeley.edu/ (29). To search for promoters containing the CES-1-binding site we created a position weight matrix (PWM) using sequences from −5 to +7 (Figure 2a) from the five promoters initially found to bind CES-1 in our Y1H assays. We searched all C. elegans promoter sequences (24) (WS93) using Improbizer motif matcher (30) with this PWM. We considered promoters with an Improbizer score of 10.4 or higher as having a putative CES-1-binding site; this cut-off was defined by looking at the scores of the five Y1H positive promoters. For Y1H assays we selected 24 promoters for which a clone was available in the Promoterome (24).

RESULTS

CES-1 binds a single Snail-type DNA element

Interactions between TFs and their target genes can be modeled into transcription regulatory networks that provide insights into gene expression at a systems level (31). We have previously used Y1H assays to map transcription regulatory networks that pertain to the C. elegans digestive tract, nervous system and microRNA regulation (32–34). In each of these networks, we retrieved the Snail-type TF CES-1 with one or more promoters. These promoters include PB0507.1, Plin-32, Pmir-235 and Pmir-231. We recently also mapped a transcription regulatory network pertaining to fat biology and, in that study, have retrieved CES-1 with the promoter of C30F12.1 (H.E.A. and A.J.M.W., in preparation). We obtained clones encoding CES-1 and its homologs K02D7.2 and SCRT-1 from our TF array resource (23) to examine the
specificity of CES-1 DNA binding. We found that neither K02D7.2 nor SCRT-1 (Figure 1b) was capable of binding these promoters in Y1H assays, indicating that the interaction between CES-1 and the five promoters is specific.

Like its evolutionary counterparts, CES-1 has been reported to interact with the E/Snail-box, (A)CAGGTG (2). We examined the sequence of each of the promoters that interact with CES-1 and found that four of these indeed contain a Snail-box (Figure 1c). Plin-32 does not

Figure 1. A single site is necessary and sufficient for CES-1 DNA binding. (a) The C. elegans Snail family contains three members: CES-1, K02D7.2 and SCRT-1. The C2H2 zinc fingers are indicated by purple boxes. Number in each of the boxes indicates percentage identity to the corresponding CES-1 zinc finger. (b) CES-1 binds PB0507.1, Pmir-235, Plin-32, PC30F12.1 and Pmir-231 in Y1H assays, whereas its closest homologs, K02D7.2 and SCRT-1 do not. P—permissive media; H—selective media lacking histidine, 20 mM 3-aminotriazole; Z—colorimetric assay for LacZ activity; AD—Gal4p activation domain. (c) Each of the five promoters that bind CES-1 possesses a Snail-box or Snail-box-like motif. Promoter length is indicated at the left; position, strand (+) or (−), and sequence are indicated at the right. Red—conventional Snail-box sequence (ACAGGTG), blue—Snail-box-like sequence (ACAGGGT). (d) Y1H assays showing that CES-1 cannot bind promoters in which the Snail-box or Snail-box-like motif is deleted. ZTF-2 binding to PB0507.1 and TBX-8 binding to Plin-32 are shown as positive controls for the functionality of mutant promoter constructs. The mutant yeast strains contain a wild-type promoter::LacZ construct to ensure functionality of AD-CES-1. (e) Y1H assays showing that a single copy of a CES-box is sufficient for CES-1 binding. Both the PB0507.1 and Plin-32 CES-box variants are shown.
possess a perfect Snail-box but does contain a sequence that differs from a Snail-box only in a single nucleotide (Snail-box-like sequence, ACAGGTT) (Figure 1c).

To test whether the Snail-box and Snail-box-like sequences function as CES-1-binding sites we deleted them from each promoter and examined CES-1 binding by Y1H assays. We deleted the putative CES-1-binding sites only in the context of the Promoter::HIS3 constructs and used wild-type Promoter::LacZ constructs. This enabled us to verify the functionality of CES-1 in yeast. As expected, we found that CES-1 failed to bind promoters in which the Snail-box was deleted, demonstrating that this sequence is required for CES-1 binding (Figure 1d, left panel, Figure S1 and data not shown).

When we deleted the CAGGTG Snail-box-like sequence from Plin-32, we found that CES-1 binding is similarly abolished (Figure 1d, right panel, see also below). Importantly, interactions with other TFs were not affected upon the deletion of the putative CES-1-binding sites, which demonstrates that each mutated promoter was functional in Y1H assays (Figure 1d and Figure S1).

Each of the CES-1-interacting promoters contains a single putative CES-1-binding site, suggesting that one site may be sufficient for CES-1 binding. To test this, we cloned each of the two different sites into our Y1H vectors and generated Y1H bait strains (21,22). We found that CES-1 is indeed capable of binding a single site, and can bind both Snail-box variants (Figure 1e). This demonstrates that a single site is sufficient for CES-1 binding. Additionally, the CES-binding site can occur on either strand (Figure 1c), which indicates that CES-1 binding is independent of binding site orientation.

**CES-1 binds an extended Snail-box**

Our finding that CES-1 can bind two different sites, CAG GTG and CAGGTG, suggests that CES-1 may have a broader DNA-binding specificity than previously reported. However, specificity of binding may also be influenced by sequences outside the core Snail-box, as CES-1 contains five C2H2 zinc fingers, each of which may contact up to three nucleotides upon binding of the protein to DNA (Figure 1a) (35). We used the CES-1-binding site sequences and flanking nucleotides from each of the five promoters that were bound by CES-1 to derive a CES-1-binding-site logo (Figure 2a). We identified two additional positions that were identical or highly similar in these promoters, all flanking the 5′ end of the Snail-box or Snail-like-box. The −3 position was a C in all cases and the −2 position was either a C or an A. We refer to this longer CES-1-binding site as the ‘CES-box’ (Figure 2a).

To test the contribution of each nucleotide to CES-1 binding, we created mutant CES-box constructs, all in the context of the B0507.1 promoter. We verified the integrity and functionality of each construct by sequencing, and by ensuring that the interaction with a non-related TF (ZTF-2) was unaffected (Figure 2b). We found that all positions in the CES-box are important for CES-1 binding, including positions −3 and −2. Surprisingly, changing the G in position +6 to a T also abolished CES-1 binding in the context of PB0507.1, even though that site now exactly matched that found in Plin-32 (Figure 2b). This suggests that promoter context may influence either the specificity or the affinity of CES-1 DNA binding. For example, nucleotides at the −4, +7 and +8 positions could influence the affinity of CES-1 for the Plin-32 site. Taken together, our DNA-binding specificity analysis revealed an extended CES-1-binding site that is composed of nine nucleotides and that contains a Snail box or Snail-box-like sequence.

**Predictive power of the CES-1-binding site**

To determine the predictive power of the different CES-1-binding sites, we searched all predicted *C. elegans* gene promoters (24) for the previously reported Snail-binding sites: the CAGGTG E-box and the ACAGGTT Snail-box, as well as the newly defined CES-box that is longer and that can tolerate a T at the +6 position (Figure 2a). Out of 20441 promoters examined, we found the CAGGTG E-box in 5250 (26%), the Snail-box in 1896 (9%) and the CES-box in 406 (2%) promoters (Table 1). Next, we examined the promoters that we had previously tested for binding to CES-1 in Y1H assays (32–34); H.E.A. and A.J.M.W., in preparation). We found that, of the Y1H positives that interact with CES-1, 80% contained an E-box, 80% contained a Snail-box and 100% contained a CES box sites, compared to 35%, 11% and 2% of the Y1H negatives (Table 1).

Conversely, we found that 4 out of 87 (5%) of the promoters harboring a CAGGTG E-box tested interacted with CES-1, compared to 4 out of 29 (14%) of the promoters with a Snail-box and 5 out of 10 (50%) of the promoters with a CES-box (Table 1). Using a Fisher exact test we determined that the association between the presence of a site and the binding of CES-1 in Y1H assays is more significant for the CES-box (*P* < 10⁻⁷) than either the E-box (*P* = 0.057) or Snail-box (*P* < 10⁻³) (Table 1). This demonstrates that the CES-box is a better predictor of CES-1 binding than either of the shorter sequences. As a control we used the reverse (but not complement) sequences that have similar nucleotide composition, and did not observe an increase in observed over expected occurrence in promoters that bind CES-1 (Table 1).

We have previously created a *C. elegans* ‘Promoterome’ resource that consists of ~6000 Gateway-cloned protein-coding gene promoters, complemented by ~75 microRNA promoters (24,36). We identified which of the cloned promoters contain CES-box, and selected 24 to test for CES-1 binding in Y1H assays. Of these 24, eight possess CAACA GGTG, five have CCACAGGTG, six have CCACAGGT T and five have CCACAGGTT (Figure 2c). We created Y1H DNA bait strains for all 24 promoters and transformed in AD-CES-1 plasmid and an AD-alone plasmid (negative control). Surprisingly, only six of these promoters were capable of interacting with CES-1 (Figure 2c, Figure S2). This suggests that additional nucleotides surrounding the extended CES-box may influence CES-1 binding. However, by examining the nucleotides flanking the 5′ and 3′ end of the CES-box, we could not find
obvious candidates. Alternatively, not all CES-boxes may be accessible due to nucleosome positioning. The latter may be more likely since Y1H baits are integrated into the yeast genome at a fixed location and copy number, and are therefore embedded into chromatin (21). Interestingly, CES-1 bound to 50% of the tested promoters with a $\text{PB0507.1}$-like CAACAGGTG motif. However, CES-1 bound only one of five promoters with a $\text{Plin-32}$-like CAACAGGTTG motif, one of six with a $\text{Pmir-235}$-like CCACAGGGTG motif, and none of five with the CCACAGGTT sequence that we did not observe in any of our original Y1H positive promoters (Figure 1c, Figure S2). This suggests that the latter may not constitute a functional CES-1-binding site and reflects the loss of information in binding site logos that compile multiple data points into a single model, but that do not reflect combinations of nucleotides that are avoided. Although the differences observed between the sites are based on small samples, these results indicate that the various CES-box variants may provide different degrees of predictive power.

**CES-1 activates promoter activity in vivo**

To examine the effect of CES-1 on *C. elegans* promoter activity in vivo, we focused on the 300bp promoter of...
We used Gateway cloning (25,38) to create a transcriptional fusion with PB0507.1 upstream of an open reading frame (ORF) encoding the green fluorescent protein (GFP), and used the resulting construct to create transgenic C. elegans that carry the transgene integrated into the genome (27,36). Subsequently, we analyzed spatiotemporal promoter activity by examining where and when GFP is expressed in these animals. We found that PB0507.1 drives GFP expression in all developmental stages and in multiple tissues, including the anterior and posterior pharyngeal gland cells, pharyngeal-intestinal valve, rectal gland, spermatheca and distal tip cells (Figure 3a). This expression pattern was identical in multiple independent transgenic lines (data not shown). To test whether CES-1 and/or its closest homologs regulate the activity of PB0507.1 in vivo, we crossed the PB0507.1::GFP animals with animals carrying a deletion in ces-1, K02D7.2 or scrt-1. Surprisingly, we found that part of the expression pattern disappears upon removal of CES-1; while expression was maintained in the pharyngeal glands, expression in the pharyngeal-intestinal valve, spermatheca, distal tip cell and rectal gland was greatly reduced (Figure 3a, quantification in Figure 3d). This suggests that CES-1 functions as an activator of PB0507.1.

### Table 1. Occurrence of CES-1-binding sites

<table>
<thead>
<tr>
<th>Promoters with hits (20 441)</th>
<th>E-box</th>
<th>Snail-box</th>
<th>CES-box</th>
<th>Reverse E-box</th>
<th>Reverse Snail-box</th>
<th>Reverse CES-box</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1H neg promoters (238)</td>
<td>5250</td>
<td>1896</td>
<td>406</td>
<td>3543</td>
<td>1420</td>
<td>457</td>
</tr>
<tr>
<td>Y1H pos promoters (5)</td>
<td>83</td>
<td>25</td>
<td>5</td>
<td>53</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>P-value (Fisher exact test)</td>
<td>0.057</td>
<td>&lt;10⁻³</td>
<td>&lt;10⁻⁷</td>
<td>1</td>
<td>0.624</td>
<td>1</td>
</tr>
</tbody>
</table>

B0507.1. We used Gateway cloning (25,38) to create a transcriptional fusion with PB0507.1 upstream of an open reading frame (ORF) encoding the green fluorescent protein (GFP), and used the resulting construct to create transgenic C. elegans that carry the transgene integrated into the genome (27,36). Subsequently, we analyzed spatiotemporal promoter activity by examining where and when GFP is expressed in these animals. We found that PB0507.1 drives GFP expression in all developmental stages and in multiple tissues, including the anterior and posterior pharyngeal gland cells, pharyngeal-intestinal valve, rectal gland, spermatheca and distal tip cells (Figure 3a). This expression pattern was identical in multiple independent transgenic lines (data not shown). To test whether CES-1 and/or its closest homologs regulate the activity of PB0507.1 in vivo, we crossed the PB0507.1::GFP animals with animals carrying a deletion in ces-1, K02D7.2 or scrt-1. Surprisingly, we found that part of the expression pattern disappears upon removal of CES-1; while expression was maintained in the pharyngeal glands, expression in the pharyngeal-intestinal valve, spermatheca, distal tip cell and rectal gland was greatly reduced (Figure 3a, quantification in Figure 3d). This suggests that CES-1 functions as an activator of PB0507.1.
promoter activity. The activity of \textit{PB0507.1} was unchanged in \textit{K02D7.2(bc366)} (Figure 3b and d) and \textit{scrt-1(ok1228)} mutants (data not shown), which is in agreement with the observation that these CES-1 homologs cannot interact with this promoter in Y1H assays (Figure 1b).

We examined whether we could detect an overlap in \textit{Pees-1} and \textit{PB0507.1} activity but did not observe a clear overlap in later stages of development (38; data not shown). However, the promoter fragments used may be missing the regulatory elements required for such expression, or do not drive robust enough expression for the overlap to be observed. We did observed broad \textit{ces-1} promoter activity in embryos (38; data not shown). However, it is difficult to assess whether this activity overlaps with that of \textit{PB0507.1}. Thus, it is formally possible that CES-1 activates the \textit{B0507.1} promoter indirectly, for instance by repressing transcriptional cofactors that either activate or repress expression, or may function in a combinatorial manner with other TFs. Future studies are required to shed light on the mechanism of tissue-specific transcriptional activation and repression by CES-1; for instance, it may interact with tissue-specific transcriptional cofactors that either activate or repress expression, or may function in a combinatorial manner with other TFs.

\textbf{DISCUSSION}

In this study we report the first example of transcriptional activation by a Snail-type TF \textit{in vivo}, as well as the first example of target sharing between Snail-type and bHLH TFs, as opposed to the widely reported Snail-type occlusion of bHLH DNA binding. Our data suggest that there are at least two types of CES-1 target genes (Figure 4c).

First, there are the classical target genes that, within one cell or tissue, are either repressed by CES-1 or activated by bHLH-type TFs. In this case, these TFs compete for binding to their DNA recognition sequence. Second, there are targets that are activated by CES-1 and by bHLH-type TFs, but within different cells or tissues. In this case, these TFs do not compete for their DNA recognition sequence in the same tissue. To date, only two \textit{in vivo} targets of any \textit{C. elegans} Snail-type TF have been identified: \textit{egl-1} (15) and the promoter of \textit{B0507.1} (this study). CES-1 represses the first target, at least in \textit{ces-1(gf)} animals that overexpress CES-1, but activates the second. The regulation by CES-1 of both \textit{egl-1} and \textit{PB0507.1} is highly tissue-specific; \textit{egl-1} is repressed in the NSM sister cells in \textit{ces-1(gf)} animals and \textit{PB0507.1} is activated by CES-1 in the rectal gland, pharyngeal-intestinal valve, distal tip cells and spermatheca in wild-type animals.

Although CES-1 expression is tissue-restricted in larvae and adults, it is broadly expressed in embryos (38; data not shown), suggesting that the expression patterns may be established early in development. No phenotype is known for \textit{ces-1(gf)} mutants. Likewise, genome-scale RNAi studies have not yet revealed a biological function for either \textit{ces-1} or \textit{B0507.1} (WormBase). Thus, it is not yet conceivable to understand the transcriptional activation of \textit{PB0507.1} by CES-1 in a phenotypic, or biological, context. Future studies are required to shed light on the mechanism of tissue-specific transcription activation and repression by CES-1; for instance, it may interact with tissue-specific transcriptional cofactors that either activate or repress expression, or may function in a combinatorial manner with other TFs.

At least two additional TFs contribute to the complex tissue-specific expression pattern conferred by the 300 bp
promoter of B0507.1. First, HLH-6 activates PB0507.1 in the anterior pharyngeal gland cells. Although HLH-6 itself is expressed in both the anterior and posterior pharyngeal gland cells, it only regulates PB0507.1 in the anterior pharyngeal gland cells. However, upon loss of HLH-6, the activity of PB0507.1 was only partially reduced in this tissue. Both of these observations may result from hlh-6(tm299) not being a complete loss-of-function, or null allele. Expression in the anterior pharyngeal gland cells is reduced both in hlh-6 mutants and in PB0507.1 variants that carry mutations in the CES-box. In addition, it has previously been shown that HLH-6 regulates pharyngeal gland expression through an E-box sequence (28,41). These observations indicate that HLH-6 most likely activates PB0507.1 by binding to the CES-box. Second, at least one as yet unidentified TF activates PB0507.1 in the posterior pharyngeal gland cells. This TF has to act through a cis-regulatory element that is distinct from the CES-box because none of the PB0507.1 mutants that we tested exhibited changes in GFP expression in these cells. We have previously identified another pharyngeal TF, ZTF-2, binding to PB0507.1 (32). However, mutations in the ztf-2 gene do not affect PB0507.1 activity in vivo (data not shown). Thus, the remaining TF(s) that activate(s) PB0507.1 may either be one of the two additional factors we identified by Y1H assays (ETS-5 and LIN-48) (32). However, these TFs are expressed in different tissues (WormBase). More likely, it is a TF that we have not retrieved in Y1H assays, for instance because the clone was not available (23), or because the TF binds DNA as an obligate heterodimer (detection of which is not yet feasible in Y1H assays).

In sum, CES-1 can activate transcription in vivo and share a DNA target with bHLH proteins, albeit in different tissues. Future studies will determine whether these molecular functions are a common feature of Snail-type TFs and whether these TFs exhibit flexible functions in other systems as well. In addition, it will be interesting to see if binding site sharing in different tissues is a common feature of metazoan TFs.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement
None declared.

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