Differential regulation of myc homologs by Wnt/β-Catenin signaling in the early metazoan Hydra

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Keywords
cnidarian; development; gene regulation; oncogene; signal transduction

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(Received 29 October 2018, revised 8 January 2019, accepted 12 March 2019)
doi:10.1111/febs.14812

Introduction

The c-Myc protein is a transcription factor with oncogenic potential controlling fundamental cellular processes. Homologs of the human c-myc proto-oncogene have been identified in the early diploblastic cnidarian Hydra (myc1, myc2). The ancestral Myc1 and Myc2 proteins display the principal design and biochemical properties of their vertebrate derivatives, suggesting that important Myc functions arose very early in metazoan evolution. c-Myc is part of a transcription factor network regulated by several upstream pathways implicated in oncogenesis and development. One of these signaling cascades is the Wnt/β-Catenin pathway driving cell differentiation and developmental patterning, but also tumorigenic processes including aberrant transcriptional activation of c-myc in several human cancers. Here, we show that genetic or pharmacological stimulation of Wnt/β-Catenin signaling in Hydra is accompanied by specific downregulation of myc1 at mRNA and protein levels. The myc1 and myc2 promoter regions contain consensus binding sites for the transcription factor Tcf, and Hydra Tcf binds to the regulatory regions of both promoters. The myc1 promoter is also specifically repressed in the presence of ectopic Hydra β-Catenin/Tcf in avian cell culture. We propose that Hydra myc1 is a negative Wnt signaling target, in contrast to vertebrate c-myc, which is one of the best studied genes activated by this pathway. On the contrary, myc2 is not suppressed by ectopic β-Catenin in Hydra and presumably represents the structural and functional c-myc ortholog. Our data implicate that the connection between β-Catenin-mediated signaling and myc1 and myc2 gene regulation is an ancestral metazoan feature. Its impact on decision making in Hydra interstitial stem cells is discussed.

Abbreviations
Alp, alsterpaullone; bHLHZip, basic region/helix-loop-helix/leucine zipper; CEF, chicken embryo fibroblasts; ChIP, chromatin immunoprecipitation; ESI-MS, electrospray ionization mass spectrometry; GFP, green fluorescent protein; GSK, glycogen synthase kinase; HA, hemagglutinin; myc, myelocytomatosis oncogene; RACE, rapid amplification of cDNA ends; Tcf/LEF, T-cell factor/lymphoid enhancer-binding factor; Wnt, wingless/int.
gene expression and regulating fundamental cellular processes like growth, proliferation, differentiation, metabolism, and apoptosis [2,3,5–8]. c-Myc is a bHLHZip protein encompassing protein dimerization domains (helix-loop-helix, leucine zipper) and a DNA contact surface (basic region) that forms heterodimers with the Max (MAX) protein and binds typically to specific DNA sequence elements termed E-boxes (5'-CACGTG-3') [4,5]. Upstream acting signaling pathways regulate the c-Myc transcription factor network like those triggered by mitogenic receptor tyrosine kinases, or by wingless/int (Wnt) [9–12]. Human T-cell factor 4 (Tcf4), the effector of Wnt/β-Catenin signaling has been identified as an oncogenic regulator of the c-myc and cyclin D1 genes in colon cancer [13–15]. The Wnt signaling pathway is highly conserved throughout animal evolution representing one of the key cascades to regulate development and stemness [14,16,17].

c-Myc and Max homologs with conserved basic functions have been found in early diverging metazoans [18] and even in premetazoans [19], suggesting that principal functions of the c-Myc master regulator arose very early in the evolution of multicellular animals. In the diploblastic cnidarian Hydra, two c-myc homologs (myc1 and myc2) have been identified, which are transcriptionally activated in the interstitial stem cell system [18,20,21]. Both Hydra Myc proteins show the same degree of overall sequence identity (32%) compared to human c-Myc, but Myc2 is structurally more related to its human ortholog concerning protein size and the degree of Myc box conservation (MBI, MBIII) in the transactivation domain [18,22,23]. Myc proteins show the same transactivation domain [18]. Paradoxically, downregulation of myc1 by short interfering RNA or chemical inhibition promoted stem cell proliferation [22], suggesting a divergent role of Myc1 in the homeostasis of the interstitial stem cell lineage. In contrast to myc1, expression of the myc2 gene is not restricted to the interstitial stem cell system but also occurs in proliferating epithelial stem cells throughout the gastric region. Furthermore, myc2 is specifically activated in cycling precursor cells during early oogenesis and spermatogenesis, suggesting that the Myc2 protein has a possible nonredundant function in cell cycle progression [20].

Crucial components of the Wnt signaling pathway regulating c-myc in vertebrates are conserved in Hydra such as Wnt3a, Frizzled, Dsh (disheveled), GSK (glycogen synthase kinase)-3β, β-Catenin, or Tcf (T-cell factor)/Lef (lymphoid enhancer-binding factor) [23–26]. Further studies have revealed a surprising complexity of cnidarian Wnt proteins that are implicated in fundamental morphogenetic processes emphasizing the important role of Wnt signaling in organismal patterning throughout the animal kingdom [16,27–31]. Here, we report that myc1 is downregulated in the gastric region upon ectopic activation of β-Catenin signaling, whereas under the same conditions myc2 expression levels remain constant. Our results therefore imply that the Hydra myc1 gene represents a potential negative target of the Wnt/β-Catenin/Tcf signaling pathway and that myc2 presumably represents the functional ortholog of human c-myc.

**Results**

**Activation of β-Catenin signaling in Hydra leads to specific repression of myc1**

Possible effects of nuclear β-Catenin signaling on the expression of the myc1 and myc2 genes were analyzed by *in situ* hybridization. Polyps from transgenic Hydra (β-cat-Tg) express high levels of a β-Catenin-GFP fusion protein triggered by an actin promoter throughout their entire body columns. As a result of the enhanced nuclear signaling activity of β-Catenin in all cells, β-cat-Tg animals form multiple ectopic head and foot structures [31,32]. These animals were compared with wild-type polyps by testing for myc1 and myc2 expression. The *in situ* hybridization patterns showed that myc1 was significantly downregulated throughout the body column of β-cat-Tg animals, whereas myc2 expression remained unchanged or even appeared to be slightly upregulated in distinct cells (Fig. 1A). Expression of myc1 and myc2 was also analyzed after Alsterpaullone (Alp) treatment, and compared with nontreated wild-type control animals. This compound specifically blocks the activity of GSK-3β that normally contributes to β-Catenin degradation by phosphorylation [28]. Consequently, β-Catenin levels in cell nuclei are elevated due to protein stabilization, and ectopic tentacles and heads form along the entire body column [28,30]. Equivalent to the results obtained in β-cat-Tg animals, expression of myc1 was downregulated after 48 h in Alp-treated animals, whereas myc2 expression showed the same expression pattern as observed for the β-cat-Tg animals (Fig. 1B).

To confirm the specific downregulation of myc1, poly(A)+-selected RNAs isolated from whole β-cat-Tg animals and from whole wild-type controls were analyzed by Northern hybridization using DNA probes derived from the coding regions of Hydra myc1, myc2, and tcf. As a control for detection of the transgenic AN-ctnnb-GFP mRNA, a probe derived from the GFP-encoding portion was used. The Northern analysis confirmed that, compared to normal animals, the transgenic polyps contain distinctly lower amounts of myc1 mRNA, in contrast to myc2, or tcf whose overall
expression levels were almost not affected (Fig. 1C). To test myc1 and myc2 expression also at the protein levels, immunoblot analysis was performed using cell extracts from whole animals and immunoglobulin G-purified antisera directed against Myc1, or Myc2. Previous immunoprecipitation analyses using extracts from 35S-methionine pulse-labeled Hydra and a Myc1-specific antibody have detected the endogenous Myc1 protein with an apparent molecular mass of 36 kDa and a minor 32-kDa protein, whereas in vitro translated full-length Myc1 had a size of 39 kDa [18,20]. The 36-kDa Myc1 protein presumably results from the usage of an alternative translation start site [18]. On the other hand, endogenous Myc2 was expressed as a 41-kDa protein having the same size as the in vitro translated full-length product [20]. Here, immunoblot analysis using equal amounts of nonlabeled extracts from wild-type and transgenic Hydra detected Myc1 and Myc2 proteins with similar apparent molecular masses as the previously detected proteins from pulse-labeled cells, but in case of Myc1 the 32-kDa protein band was the dominant one and the 36-kDa isoform only weakly expressed (Fig. 2). Myc2, expressed at low levels as reported previously [20], displayed an apparent molecular mass of 41 kDa as expected. The 32-kDa Myc1 isoform, which was downregulated in transgenic polyps, could result from proteolytic processing of the 36-kDa protein. Although the nature of the smaller Myc1 protein needs yet to be determined, the results are in line with those obtained by in situ and Northern expression analyses.

Fig. 1. Specific repression of myc1 mRNA expression upon β-Catenin activation. (A) Expression patterns of Hydra myc1 and myc2 in β-Catenin transgenic animals (β-cat-Tg) compared to wild-type polyps. Whole wild-type and transgenic polyps have myc1 and myc2 expressed throughout their body columns, but not in head and foot structures. myc1 is downregulated in the transgenic polyps, whereas myc2 levels are not significantly changed. (B) Differential expression of Hydra myc1 and myc2 48 h after onset of treatment with the GSK-3β inhibitor Alp. Inhibition of GSK-3β results in activation of β-Catenin in the canonical Wnt signaling pathway, and as a result in a reduction of the myc1 expression level. Upper halves of polyps and magnified views from gastric regions are shown. Positions of residual ribosomal RNAs (28S, 18S) are given on the left site. Ethidium bromide-stained RNAs used for blot analysis are shown below. The blot, which was hybridized to a second probe after filter washing is indicated by a hash sign (#). For transgenic mRNA detection, a GFP-specific probe (ΔN-ctnmb-GFP) was applied (arbitrary expression level 1.0). Representative blots from three independent experiments are shown where myc1, myc2, and tcf levels from wild-type animals were arbitrarily set to 1.0. Standard deviations (SD, n = 3) are shown by vertical bars. Statistical significance was assessed by using a paired Student t-test (**P < 0.01).
The lower amounts of endogenous Myc2 versus Myc1 are probably due to the higher instability of the Myc2 protein as reported previously [20]. Titer and specificities of the applied polyclonal antibodies were controlled using the carboxyl-terminal Myc1 p16 and the full-length Myc2 p41 protein (Fig. 2A,B). As expected, expression of the DN-ctnnb-GFP protein was exclusively detected in transgenic Hydra, whereas Tcf or Max were expressed at almost equal levels both in wild-type and in transgenic animals (Fig. 2C).

Loss of myc1 expression could result from either transcriptional downregulation of this gene in interstitial cells including stem cells, which occur as single cells (1s) and cell pairs (2s), or from potential β-Catenin-induced disappearance of myc1-expressing cells. We studied putative changes in the numbers of myc-expressing interstitial stem cells in the body columns of β-Catenin-activated versus wild-type polyps using the maceration technique (Fig. 3A). In a macerated cell suspension, cells maintain their original morphology, and thereby the different cell types can be clearly distinguished and precisely quantified (Fig. 3B) [33]. The results in Fig. 3C show that in both, β-cat-Tg and Alp-treated tissue, interstitial stem cells are embedded at higher densities in the ecto- and endodermal epithelial tissue layers. Thus, enhanced nuclear β-Catenin signaling seems to stimulate interstitial stem cell maintenance. Furthermore, our results clearly support the view that loss of myc1 expression is most likely caused by β-Catenin-regulated transcriptional repression in these cells.

**Hydra Tcf binds to the promoters of Hydra myc1 and myc2**

To investigate the mechanism of Hydra myc1 downregulation by Wnt/β-Catenin signaling, the promoter region of myc1 was defined by transcription start site mapping. cDNA prepared from whole Hydra was subjected to 5′rapid amplification of cDNA ends (5′RACE). Two closely spaced transcription start sites were detected in the myc1 promoter region, whereas transcription of myc2 starts at one defined site (Fig. 4A) as reported recently [20]. The sequence of the myc1 5′RACE product also allowed deduction of the relevant full-length mRNA sequence. Alignment of this sequence with the published Hydra genome [34] led to definition of the myc1 gene topography. In contrast to myc2, which consists of three exons similar to...
the vertebrate c-myc genes [20], Hydra myc1 has two exons only (Fig. 4B). Inspection of the 5′-untranscribed regions of myc1 and myc2 revealed that both promoters contain potential binding elements termed TBE [13] for the Tcf transcription factor, closely matching the consensus site 5′-CCTTTGWW-3′ [35].

(Fig. 4A). Whereas the myc2 promoter contains two TBE motifs located at positions −166 and −98 nucleotides (nt) upstream of the transcription start site, three canonical TBE sites are present in the myc1 promoter mapping to nt positions −128, −79, and +46 with reference to the proximal transcription start site (Fig. 4A). Interestingly, the myc2 promoter also contains a canonical Myc binding site (E-box) at nt position −60 (Fig. 4A). Inspection of the promoters for further transcription factor binding sites revealed the presence of Oct-1, C/EBPα, or NF-1 motifs in the myc1, and of hunchback, C/EBPα, or NFκB motifs in the myc2 promoter, respectively (Fig. 5).

To test if promoter regions of Hydra myc1 and myc2 are bound by Tcf in vivo, ChIP analysis was performed using cross-linked chromatin from whole wild-type, transgenic, and Alp-treated Hydra animals, and a Tcf-specific antibody followed by PCR amplification of the specific DNA regions (Fig. 4C). To generate the antiserum directed against Hydra Tcf, a partial recombinant Hydra Tcf protein was expressed in Escherichia coli, purified, and then used as an immunogen (Fig. 6). Bound Hydra Tcf was detectable on both promoter segments (Fig. 4C) suggesting that a β-Catenin/Tcf protein complex could indeed regulate the myc1 and myc2 genes. No significant changes in promoter occupancy were observed after β-Catenin activation either by overexpression (β-cat-Tg) or GSK-3β inhibition (Alp) (Fig. 4C). As a negative control for Tcf binding, a segment derived from the Hydra thrombospondin (TSP) promoter containing no Tcf binding site [36] was tested (Fig. 4D).
The myc1 promoter is selectively downregulated by ectopic β-Catenin/Tcf

To test if the Hydra β-Catenin and Tcf proteins are involved in transcriptional regulation of the myc1 and myc2 genes, luciferase reporter assays were performed using the chemically transformed quail cell line QT6 as a test system. Cells were transiently co-transfected with reporter plasmids containing the Hydra myc1 and myc2 promoters inserted into luciferase reporter vectors (pGL3-hymyc1, pGL3-hymyc2), and expression vectors (pRc) encoding Hydra Tcf or β-Catenin proteins fused to a carboxyl-terminal hemagglutinin (HA) tag (Fig. 7A). Ectopic protein expression was verified by using the chemically transformed quail cell line QT6 as a test system. Cells were transiently co-transfected with reporter plasmids containing the Hydra myc1 and myc2 promoters inserted into luciferase reporter vectors (pGL3-hymyc1, pGL3-hymyc2), and expression vectors (pRc) encoding Hydra Tcf or β-Catenin proteins fused to a carboxyl-terminal hemagglutinin (HA) tag (Fig. 7A). Ectopic protein expression was verified...
by immunoblotting. Overexpression of Tcf-HA, β-Catenin-HA, or Tcf-HA together with β-Catenin-HA led to partial repression of the myc1 promoter, whereas the myc2 promoter was not influenced. The results suggest that, in contrast to myc2, the myc1 promoter is repressed by β-Catenin/Tcf although both promoters are bound by Tcf in vivo (Fig. 4C). Therefore, these biochemical analyses confirm the specific downregulation of myc1 upon activation of the β-Catenin/Tcf signaling complex suggesting that myc2 but not myc1 represents the functional ortholog of vertebrate c-myc.

To test, if Hydra β-Catenin is capable at all to activate transcription in the applied cell system, a reporter construct containing the chicken c-myc promoter containing multiple Tcf binding sites was co-transfected with pRc-hyCtnnb-HA into QT6 cells. The result of this luciferase assay showed that, in contrast to Hydra myc1 or myc2 promoters, Hydra β-Catenin moderately activates this vertebrate c-myc promoter (Fig. 7B). No luciferase activities were scored upon transfection of either the empty reporter plasmid, or the empty expression vector (data not shown). To investigate if overexpressed Hydra β-Catenin also induces cell transformation in avian cells similar to its vertebrate counterparts [37, 38], the coding region of Hydra β-catenin was inserted into the replication-competent retroviral RCAS expression vector (Fig. 8A) and transfected into chicken embryo fibroblasts (CEF). As a positive control, a RCAS construct encoding a LEF1/β-Catenin fusion protein (ΔLEF1ΔCTNNB) [37, 38] was employed. As expected, overexpression of ΔLEF1ΔCTNNB lead to efficient cell transformation manifested by colony formation in soft agar [37, 38] (Fig. 8B). Remarkably, also the cells overexpressing full-length Hydra β-Catenin were able to form colonies indicating a transformed phenotype (Fig. 8B). The ectopic proteins were efficiently expressed from their retroviral vectors, which was monitored by immunoblot analysis (Fig. 8C). This result shows that the principal oncogenic potential of β-Catenin has been conserved through metazoan evolution.

**Discussion**

Although substantial progress has been made in understanding the pleiotropic functions of the human c-Myc protein in cellular proliferation, growth, energy metabolism, differentiation, and apoptosis [5, 6, 12, 39], many open questions remain regarding the underlying molecular mechanisms. The recent identification of c-Myc to act as a possible general amplifier of gene expression controlling multiple transcriptional programs [8, 40–43] even enhances the complexity of Myc biology. Furthermore, several upstream signaling pathways like the mitogenic Ras/Raf cascade, or the Wnt/β-Catenin/Tcf4 axis regulate c-Myc expression and activity in cell proliferation, but they are also relevant in malignant cell growth leading to aberrant c-myc activation [2, 4, 5, 12].

A possibility to dissect the multiple Myc functions is the analysis of genetically defined invertebrate model organisms, like the simple eumetazoan Hydra, a classical diploblastic model system to study pattern formation, regeneration, and stem cell dynamics. About 600 million years ago, single-cell premetazoans and simple metazoans were predominant, but during the Cambrian period, a rapid diversification of multicellular lifeforms began. Hydra represents one of the earliest multicellular animals to evolve out of single-celled basal species, has a high regeneration potential, and is biologically immortal [21]. This ancient organism could represent a suitable model system to study basal

![Fig. 5. Promoter maps of Hydra myc1 and myc2. Bars represent the promoter regions. Positions of transcription factor binding sites were identified using the computer program AliBaba2 (gene-regulation.com) and are depicted on or above the bars (Tcf, T-cell-specific transcription factor; C/EBPα, CCAAT enhancer-binding protein alpha; NF-1, nuclear factor 1; Hb, hunchback; NFκB, nuclear factor kappa B; Myc, myelocytomatosis viral oncogene protein product).](image-url)
mechanisms in tumor biology because cancer is as old as multicellular life. Intriguingly, natural occurring tumors have been observed in *Hydra*, presumably resulting from differentiation-arrested female gametes [44], a cell type in which myc2 is expressed at high levels [20]. The occurrence of tumors in this early diverging organism is actually in line with the atavistic cancer model, in which the biological origin of malignant growth is traced back to the transition between unicellularity to multicellularity [45,46]. This theory was recently supported by molecular analyses of multiple sets of genes with common phylogenetic origin (phylostrata) in solid human tumors. The study showed that dormant genes conserved with unicellular organisms became strongly upregulated in tumors, whereas genes of metazoan origin were primarily inactivated [47].

*Hydra* is also one of the most basal metazoan organisms employed so far for analysis of the major cancer driver Myc and its signaling network [18,20] revealing that biochemical and oncogenic properties of c-Myc arose very early in metazoan evolution. In *Hydra* there are four myc-related genes (myc1–4),
which branched off from a basal position during cnidarian evolution [18,20,21]. Mycl and myc2 show the closest homology to vertebrate c-myc but significantly less homology to L-myc or N-myc [18], because the diversification of c-, L-, and N-myc subfamilies occurred later within the vertebrate lineage [18]. On the protein level, Hydra Mycl shares 32%, 22%, or 20% amino acid sequence identity with the human c-Myc, L-Myc, or N-Myc proteins, whereas Myc2 displays 32%, 22%, or 24% identity with c-Myc, L-Myc, or N-Myc, respectively. As reported previously, the structures of Hydra Mycl and Myc2 proteins display the same principal topography and similar evolutionary relationship with the human c-Myc protein with equal sequence identities (32%), but share only 24% overall identities among themselves [18]. Although Mycl or Myc2 cannot be assigned directly as ancestor of any specific vertebrate homolog, comparison of the sizes and conserved Myc boxes in the transactivation domain between Hydra Mycl or Myc2 and human c-Myc suggests that Myc2 is most likely the closest ortholog of human c-Myc [18]. Furthermore, the myc2 gene is composed of three exons like human c-myc, in contrast to mycl containing two exons only (Fig. 4B).

Concerning the differential regulation of the Hydra myc genes (Fig. 7), comparison of the promoter structures indicates, that besides Tcf/β-Catenin additional transcription factors may be involved in mediating transmission of upstream acting signaling pathways (Fig. 5). In addition, the presence of a Myc binding site in the myc2 promoter suggests that Hydra Myc proteins themselves may participate in myc2-specific gene regulation.

In mammalian Wnt/β-Catenin signal transduction, the transcription factor Tcf4 represents a crucial nuclear effector [9,14]. Comparison of the Hydra Tcf protein sequence with homologs from chicken or human reveals the highest degree of sequence identity with the vertebrate Tcf4 (Fig. 9), regulating vertebrate c-myc via TBE elements present in the relevant promoter regions [13]. In Hydra, wnt and tcf genes are transcriptionally activated in early bud formation and head regeneration namely in the putative Hydra head organizer, the upper part of the hypostome [23,28]. Furthermore, elevated levels of β-Catenin result in body columns that have high head organizer potency.
accounting for the formation of ectopic head structures in these animals [28] (Fig. 1A,B). Here, we present evidence that β-Catenin has an additional function in interstitial cells of the gastric region. Using pharmacologically treated or genetically modified Hydra, we have shown that activation of β-Catenin seems to enhance interstitial stem cell maintenance, and expression and promoter analyses demonstrate a specific repression of \( \text{myc1} \), but not of \( \text{myc2} \). According to our current working model (Fig. 10), this reduction in \( \text{myc1} \) expression by nuclear β-Catenin could in fact promote interstitial stem cell maintenance and self-renewal based on an earlier observation that \( \text{myc1} \) inhibition by \( \text{myc1} \)-specific antisense RNAs has the same effect [22]. The reason why in our biochemical analyses \( \text{myc2} \) is not significantly induced upon ectopic β-Catenin/Tcf activation is not yet clear. So far, our experiments were performed in intact polyps only. Therefore, it was not possible to resolve how \( \text{myc2} \) expression responds to changes in nuclear β-Catenin activity specifically in interstitial cells. A reason for the overall unchanged \( \text{myc2} \) expression could be that \( \text{myc2} \) is indeed activated in stem cells, but this is masked by a possible downregulation in epithelial cells. Since the two epithelial cell types form the major part of the Hydra body mass, a slight reduction in \( \text{myc2} \) expression upon β-Catenin activation could dilute a positive stimulatory effect in interstitial stem cells. Subtle shifts in Myc expression above or below distinct thresholds can trigger striking differences in biological output [48]. In addition, \( \text{myc2} \) expression may depend on possible Myc1-triggered transcriptional activation mediated by the E-box present in the \( \text{myc2} \) promoter (Fig. 4A). Consequently, a decrease in Myc1 levels due to Wnt signal-triggered \( \text{myc1} \) downregulation could mask a possible stimulatory effect of β-Catenin/Tcf on
the $\textit{myc2}$ promoter, a hypothesis, which has to be pursued by further investigations.

Our finding that $\textit{Hydra myc1}$ is a negatively regulated target of $\beta$-Catenin was unexpected, because $c$-$\textit{myc}$ is one of the best studied targets activated by mammalian $\beta$-Catenin. However, also in case of human $N$-$\textit{myc}$, a negative regulation of mRNA expression caused by activated Wnt signaling has been reported [49,50]. In murine embryonic stem cells, c-Myc controls the balance between stem cell self-renewal, pluripotency and differentiation [51,52] thereby driving a regulatory network to maintain this state.

Fig. 9. Amino acid sequence alignments of $\textit{Hydra magnipillata}$ (hy) Tcf with the $\textit{Homo sapiens}$ (h) and $\textit{Gallus gallus}$ (ck) homologs. (A) Alignment with human TCF4 (TCF7L2) and chicken Tcf4 (Tcf7l2). (B) Alignment with human LEF-1 and chicken Lef1. GenBank accession numbers are: hy Tcf, NP_001296662; h TCF-4, CAG38811; ck Tcf-4, NP_001193439; h LEF-1, Q9UJU2; ck Lef-1, NP990344. Identical residues are shaded in blue, and gaps are indicated by dashes. The $\beta$-Catenin (CTNNB) and DNA binding (HMG) domains are boxed in pink or yellow, respectively. Sequence identities between hy Tcf and h LEF1 or ck Lef1 are 29%, and between hy Tcf and h TCF4 or ck Tcf4 are 42%. The alignment was generated by using the computer program (omega) CLUSTALW with additional manual adjustments.
Furthermore, c-Myc potentiates the Wnt/b-Catenin signaling pathway by transcriptional repression of Wnt antagonists. This induces transcriptional activation of the endogenous myc family members, which in turn activate a Myc-driven self-reinforcing circuit [54]. The detailed actions of β-Catenin on Myc2 and the cross-talk between Myc1 and Myc2 are not yet known.

**Materials and methods**

**Animals**

*Hydra vulgaris* strains Basel, *Hydra magnipapillata* wild-type strain 105 and the *Hydra β-cat-Tg* strain containing a transgenic β-catenin gene fused in frame to the coding sequence of the enhanced GFP [31] were used in this study. Mass cultures were kept as described [57]. Experimental animals were collected 24 h after the last feeding. Treatment with the inhibitor Alp was done as described [28], and phenotypes of treated polyps were analyzed at 48 or 60 h after the onset of treatment.

**Maceration of *Hydra* tissue**

Maceration of excised tissue pieces from the gastric region was done as described using a maceration solution containing acetic acid (1 vol)/glycerol (1 vol)/water (7 vol) [33]. Fixed macerated cell suspensions were then spread onto microscope slides, and analyzed under phase contrast optics. For a single measurement, 10 tissue pieces were macerated, and about 500 epithelial cells and a corresponding number of 1s+2s interstitial stem cells were counted.

**Whole mount *in situ* hybridization**

*In situ* hybridization with digoxigenin-labeled RNA probes was done according to a protocol as described [58] using myc1- and myc2-specific cDNA probes [18,20].

**DNA cloning and nucleic acid analysis**

Molecular cloning, DNA sequencing, and Northern analysis have been described [18,59]. Total RNA isolation and poly(A)⁺-RNA selection from ~600 Hydra animals was performed as described [18]. The yield of total RNA per animal was ~2 μg. DNA fragments specific for *Hydra myc1*, and *myc2* have been described [18,20]. To detect the *Hydra tcf* mRNA, a DNA fragment encompassing the tcf
of pRc-hyTcf-HA and pRc-hyCtnnb-HA, the coding sequences were amplified by PCR from genomic DNA using the primer TGTTTTTTATGTAATG-3' and of Hydra tcf was excised from the HoTG plasmid [60]. To determine the myc1 transcription start site, 5' RACE was performed as described [61] using the primers 5'-GGATCCTAGTGGGAATGGCGAAG-3' and 5'-GTC TCGCAGAATCTGTTGAGGAAACACTTG-3' for first strand cDNA synthesis and subsequent PCR, respectively. Mapping of the myc2 transcription start site using the primers 5'-GGTGTTAGTTGGAAATGGCGAAG-3' and 5'-AATTATCCACGCTATTTGAC AATT-3' has been described previously [20].

**Promoter analysis**

ChIP analysis was carried out as described [20,59,61,62] using sheared extracts from ~ 300 Hydra animals treated with formaldehyde for 30 min. Immunoprecipitations were performed with specific antibodies followed by PCR amplification of 248- and 201-bp fragments from the Hydra myc1 or myc2 regulatory regions, respectively, using the specific primer pairs 5'-TCAGTACTAAAGTTGCT GTTCCT-3'/5'-GTCCTTGTGCGTTGAC-3' (myc1), and 5'-ACTTGGTTTTATGTAATG-3'/5'-GAAT GACTTTGATTTCCG-3' (myc2). In addition, the primer pair 5'-AGGTGTCGGTCTGCTACTTCCGTCGAGCAAACAAAGG-3' (myc1) was employed to amplify a 129-bp fragment from the myc1 promoter region. The primers used to amplify a 173-bp segment from the TSP promoter have been described [36].

Transcriptional transactivation analysis using the luciferase reporter system has been described [20,59,62]. To generate the reporter constructs pGL3-hymyc1 and pGL3-hymyc2, a 416-bp and a 319-bp segment encompassing nucleotides −369 to +47 or nucleotides −216 to +103 of the Hydra myc1 or myc2 promoter regions, respectively, were amplified by PCR from genomic DNA using the primer pairs 5'-GGATCCTAGTGGGAATGGCGAAG-3' and 5'-GTC TCGCAGAATCTGTTGAGGAAACACTTG-3' for first strand cDNA synthesis and subsequent PCR, respectively. Mapping of the myc2 transcription start site using the primers 5'-GGTGTTAGTTGGAAATGGCGAAG-3' and 5'-AATTATCCACGCTATTTGAC AATT-3' has been described previously [20].

**Cells and retroviruses**

Cultivation of CEF and of the methylcholanthrene-transformed cell line QT6 and cell transformation assays were performed as described [18,59,61]. The construct pRCAS-ΔLEF1ACTNNB (pRCAS-AN-bAN) encoding a truncated mouse LEF1/CTNNB fusion protein has been described [38]. To construct pRCAS-hyCtnnb-HA, the Hydra ctnnb insert from pRe-hyCtnnb-HA was released with XbaI (blunt ended) and inserted into the eukaryotic pRC/RSV expression vector (Thermo Fisher Scientific, Vienna, Austria) which had been opened with HindIII (blunt ended)/XbaI. Calcium phosphate-mediated DNA transfection, and luciferase assays were performed as described [20].

**Protein expression, purification, and analysis**

To construct pET11d-hyTcf(1-151) a DNA segment encoding amino acid residues 2-151 from Hydra Tcf was amplified by PCR using the primers 5'-CCCCCACTACC AACTTCA-3'/5'-AAGCTTGATCTGCTCATA TGCCAAGG-3' and the plasmid pGEX6p3-hyTcf [28] as a template. The PCR product was digested with BamHI and inserted into pET11d vector yielding the construct pET11d-hyTcf(1-151) with the initiating methionine codon derived from the vector. The encoded Hydra Tcf(1-151) protein (151 amino acids; M, = 17 146; pl = 4.13) represents a truncated version of the 418-amino acid full-length Hydra Tcf protein. DNA of the construct pET11d-hyTcf(1-151) was transformed into E. coli strain BL21 (DE3) CodonPlus-RIL (Stratagene/Agilent, Vienna, Austria). To express recombinant hyTcf(1-151) protein, bacteria from a single colony were grown overnight at 37 °C with shaking at 220 r.p.m. in 10 mL of LB medium containing 100 µg·mL⁻¹ ampicillin and 25 µg·mL⁻¹ chloramphenicol. The bacteria were transferred into 400 mL LB medium containing 100 µg·mL⁻¹ ampicillin, and grown at 37 °C with shaking at 220 r.p.m. to an optical density of 0.5 (600 nm). To induce recombinant protein expression, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 1 mM and bacteria were incubated as above for 3 h. The bacteria were pelleted and resuspended in 25 mL of buffer A (20 mM Tris HCl pH 7.5, 80 mM NaCl, 1 mM EDTA, 0.5 mM DTT). The recombinant hyTcf(1-151) protein was purified by Ni²⁺-NTA affinity chromatography, and its purity was verified by SDS-PAGE.

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1 mM DTT, 1 mM PMSF), and then lysed at 1300 psi using a French Press. DNase I was added to a final concentration of 5 μg·mL⁻¹, and the lysate was incubated at 4 °C for 30 min. The sample was centrifuged at 18 000 g for 20 min at 4 °C. Ammonium sulfate was added to the clarified supernatant at 40% (w/v) saturation, and the solution was stirred on ice for 30 min. The precipitated proteins were pelleted by centrifugation at 11 000 g for 30 min at 4 °C. The pellet was dissolved in 10 mL of buffer A and dialyzed for 36 h at 4 °C against 2.5 L of buffer A, centrifuged at 18 000 g for 20 min, and then loaded onto a Mono Q anion exchange column using an automated liquid chromatography system (ÄKTA purifier; GE Healthcare). Chromatography was carried out with a linear gradient from 0 to 1 M NaCl in buffer A at a flow rate of 1 mL·min⁻¹. Fractions containing hy Tcf(1-151) protein were combined and applied onto a Superdex-200 gel filtration column (GE Healthcare, Vienna, Austria) equilibrated with buffer A, and then eluted with the same buffer at a flow rate of 0.5 mL·min⁻¹. hy Tcf (1-151) containing fractions were pooled, dialyzed against PBS, and stored at −80 °C. The final yield of purified hy Tcf(1-151) from the 400-mL bacteria culture was approximately 500 μg. For electrospray ionization mass spectrometry (ESI-MS), hy Tcf(1-151) was desalted using Vivaspin 500 PES centrifugal concentrators (MWCO 5000) (Sartorius, Vienna, Austria). Centrifugal concentration and addition of 100 mM ammonium acetate in H₂O (18 MΩ, Vienna, Austria) to the supernatant was repeated six times, followed by six cycles of concentration and dilution with H₂O (18 MΩ). The final protein concentration of the ESI solution (H₂O : CH₃OH vol/vol) was 1 μM. For ESI-MS analysis in positive and negative ion mode, 1% vol/vol acetic acid and 0.1% 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) were added to aliquots of the ESI solution, respectively. SDS-PAGE and immunoblotting were done as described [18,59]. To prepare cell extracts from whole Hydra, ~10 animals were dissolved in 200 μL of RIPA buffer [59] and the extract clarified by centrifugation at 20 000 g for 1 h at 4 °C. The yield of total protein per animal was ~20 μg.

A polyclonal rabbit antiserum directed against the purified recombinant hyTcf(1-151) protein was generated as described previously using 3 x 100 μg recombinant protein as immunogen [18,20]. The antiserum directed against Hydra Max has been described [18]. The monoclonal mouse antibodies anti-α-tubulin, anti-GFP, and anti-HA were purchased from Sigma-Aldrich, Vienna, Austria (T5168), Roche, Vienna, Austria (11 814 460 001), and Covance, Vienna, Austria (MMS-101P), respectively. The polyclonal rabbit antibody directed against mouse anti-β-Catenin was purchased from Sigma-Aldrich, Vienna, Austria (C2206).

Hydra Myc1- and Myc2-specific antisera [18,20] were subjected to immunoglobulin G (IgG) purification. To each 20 mL antiserum, an equal volume of saturated ammonium sulfate solution was added dropwise resulting in a final concentration of 50% (w/v), and stirred on ice for 3 h. After centrifugation at 3000 g for 20 min (4 °C), the pellets were washed twice in 50% (w/v) ammonium sulfate (20 mL), and centrifuged as above. Pellets were dissolved in 4 mL of PBS, and dialyzed against 20 mM sodium phosphate pH 7.0 for 48 h. Prior to protein G affinity chromatography, samples were clarified by centrifugation at 13 000 g for 30 min (4 °C), and then loaded onto a 5-mL HiTrapG column (GE Healthcare, Vienna, Austria) equilibrated with 20 mM sodium phosphate pH 7.0. Bound IgG were eluted with 0.1 M glycine pH 2.7, and 1-mL fractions immediately neutralized with 200 μL of 1 M Tris-HCl pH 9.0. IgG fractions were pooled resulting in final volumes of 8 mL each, and dialyzed against 20 mM sodium phosphate pH 7.0 for 48 h. The IgG preparations were quantified (anti-hy Myc1 540 μg·mL⁻¹; anti-hy Myc2 1080 μg·mL⁻¹), and stored in aliquots at −80 °C. For immunoblotting, 1 : 50 dilutions of IgG-purified antisera were applied.

Acknowledgements

We thank Hans Bode for providing β-cat-Tg polyps, and Masahiro Aoki and Peter K. Vogt for providing the construct RCAS-ΔN-hΔN. This work was supported by Austrian Science Fund (FWF) grants P23652 (to KBi), P20734 (to BH), and P27347 (to KBr). SG was a recipient of fellowships from the Austrian Academy of Sciences and from the University of Innsbruck.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

MH, KBi, and BH planned experiments, MH, SGl, SGu, AR, KP, and KBr performed experiments, MH, KBr, KBi, and BH analyzed data, MH and BH wrote the paper, with contributions from KBi.

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