RESEARCH ARTICLE

Regulation of Cop9 signalosome activity by the EF-hand Ca^{2+}-binding protein tescalcin

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ABSTRACT

The Ca^{2+}-binding protein tescalcin is known to be involved in hematopoietic cell differentiation; however, this mechanism is poorly understood. Here, we identify CSN4 (subunit 4 of the COP9 signalosome) as a novel binding partner of tescalcin. The COP9 signalosome (CSN) is a multiprotein complex that is essential for development in all eukaryotes. This interaction is selective, Ca^{2+}-dependent and involves the PCI domain of CSN4 subunit. We then investigated tescalcin and CSN activity in human erythroleukemia HEL and promyelocytic leukemia K562 cells and find that phorbol 12-myristate 13-acetate (PMA)-induced differentiation, resulting in the upregulation of tescalcin, coincides with reduced deneddylation of cullin-1 (Cul1) and stabilization of p27^{Kip1} – molecular events that are associated with CSN activity. The knockdown of tescalcin led to an increase in Cul1 deneddylation, expression of F-box protein Skp2 and the transcription factor c-Jun, whereas the levels of cell cycle regulators p27^{Kip1} and p53 decreased. These effects are consistent with the hypothesis that tescalcin might play a role as a negative regulator of CSN activity towards Cul1 in the process of induced cell differentiation.

KEY WORDS: COP9 signalosome, Tescalcin, Cell cycle, Cullin, p27

INTRODUCTION

Tescalcin was first identified as a putative EF-hand Ca^{2+}-binding protein that is preferentially expressed in testis during the early stages of mouse gonadal development (Perera et al., 2001). Subsequent biochemical studies have shown that tescalcin is N-myristoylated, contains a single active EF-hand domain and binds Ca^{2+} with a low micromolar pK, which allows it to serve as a Ca^{2+} sensor and regulator (Gutierrez-Ford et al., 2003). The subcellular localization of tescalcin is predominantly cytoplasmic and perinuclear, but it has also been detected in the nucleus (Gutierrez-Ford et al., 2003; Levay and Slepak, 2007). Similar to its structural homologs calcineurin homologous proteins 1 and 2 (CHP1 and CHP2, respectively), tescalcin was reported to associate with sodium-hydrogen exchanger 1 (NHE-1) (Li et al., 2003; Lin and Barber, 1996; Mailänder et al., 2001; Pang et al., 2002; Zaun et al., 2008; Zaun et al., 2012).

Tescalcin has a tissue-specific distribution and is most abundant in the heart, stomach, brain, salivary and adrenal glands, bone marrow, and pancreas (Gutierrez-Ford et al., 2003). Tescalcin is also found in primary human granulocytes, megakaryocytes, platelets and some hematopoietic cell lines. Its expression is highly regulated upon induced cell differentiation and is dependent on sustained signaling through the mitogen-activated extracellular-signal-regulated kinase (MAP–ERK) pathway (Levay and Slepak, 2007; Levay and Slepak, 2010). Overexpression of tescalcin initiates spontaneous differentiation events, such as growth arrest and the appearance of lineage-specific markers in hematopoietic precursor cells. Conversely, short hairpin RNA (shRNA)-mediated knockdown inhibited signal-induced lineage commitment and differentiation in human primary CD34+ precursor cells, as well as in cell lines (Levay and Slepak, 2007; Levay and Slepak, 2010). We have also shown that tescalcin is required for the gene expression of several transcription factors of the Ets family. These transcriptional regulators are broadly involved in not only hematopoiesis but also in the development of the cardiovascular and nervous systems. Recent human genetics studies have supported this notion – genome-wide single nucleotide polymorphism and structural magnetic resonance imaging data analyses have identified a strong association between the levels of tescalcin gene expression and the volume of the hippocampus (Stein et al., 2012).

To gain insight into the mechanism of tescalcin activity, we performed a yeast two-hybrid screen and identified CSN4, a component of the COP9 signalosome (CSN), as a putative interacting partner. CSN is an evolutionarily conserved multiprotein complex that is composed of eight subunits CSN1–8, including two different isoforms of CSN7, CSN7a and CSN7b, and exhibits considerable similarity to the 26S proteasome regulatory particle (Chamovitz et al., 1996; Deng et al., 2000; Glickman et al., 1998; Seeger et al., 1998; Wei et al., 1994). Each of the CSN subunits contains unique domains. Subunits CSN1, CSN2, CSN3, CSN4, CSN7 and CSN8 comprise a proteasome-COP9-eIF3 (PCI) domain, whereas CSN5 and CSN6 comprise a Mpr1-Pad1-N-terminal (MPN) domain (Hofmann and Bucher, 1998). Studies from lower organisms and mammalian cells highlight that the CSN modulates a broad spectrum of processes – embryogenesis, the cell cycle, checkpoint controls, DNA repair, MAP kinase and steroid hormone signaling, axonal guidance, and differentiation. In regulating these diverse cellular processes, CSN impacts transcription, protein phosphorylation, protein stability and subcellular distribution (Kato and Yoneda-Kato, 2009; Wei and Deng, 2003).

One of the most studied functions associated with the CSN is the regulation of protein degradation. The CSN can affect the activity of cullin–RING E3 ubiquitin ligases by removing the ubiquitin-like modification Nedd8 from the cullin protein (Cope et al., 2002; Groisman et al., 2003; Lyapina et al., 2001; Pintard et al., 2003; Schwechheimer et al., 2001). The CSN5 subunit, which possesses metalloproteinase activity, plays a central role in the deneddylation of cullins (Cope et al., 2002). The CSN has also

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been shown to phosphorylate a number of protein substrates through CSN-associated kinases, such as casein kinase 2, protein kinase D and 1,3,4-triphosphate 5/6 kinase (Uhle et al., 2003; Wilson et al., 2001). These kinases can phosphorylate c-Jun, p53, ATF-2, IκBα and other important signaling molecules, affecting their stability and activity (Bech-Otschir et al., 2001; Filhol et al., 1992; Lin et al., 1992; Naumann et al., 1999; Seeger et al., 1998).

Over fifty diverse proteins have been shown to interact with CSN subunits. The majority of these partners are substrates rather than regulators of CSN activity, and more than forty interact with CSN5 (Kato and Yoneda-Kato, 2009). There is only one recent report describing a binding partner of CSN4 – TorsinA, which is involved in the regulation of synaptic activity (Granata et al., 2011). In this paper we provide evidence that CSN activity can be regulated by tescalcin, an interacting partner of CSN4.

RESULTS
COP9 signalosome subunit 4 interacts with tescalcin
We used the full-length sequence of tescalcin as bait in a yeast two-hybrid screen of a human bone marrow cDNA library. The screen of $\sim 1{\times}10^6$ cDNA clones identified a total of 32 positives. The development of an intense blue color in the presence of chromogenic substrate X-α-Gal indicated that 15 of these clones expressed strongly interacting partners. Of these, seven contained inserts ranging from 1.2 to 1.7 kb that represented partial polyadenylated cDNAs encoding CSN4. The direct protein–protein interaction of tescalcin with one of the identified fragments of CSN4 (amino acids 195–406) was confirmed by two independent assays that utilized purified recombinant proteins – far-western blots and a glutathione S-transferase (GST) pull down (Fig. 1A,B). Tescalcin specifically bound to CSN4 under our experimental conditions, whereas no binding between tescalcin and GST or control GST-fusion proteins was detected.

Interaction of tescalcin with other CSN subunits
An analysis of the CSN subunits CSN1–CSN8 in a mammalian two-hybrid assay in HEK293 cells indicated that tescalcin can also bind to CSN5. As shown in Fig. 1C, co-expression of tescalcin with CSN5 resulted in a similar activation of the luciferase reporter as that observed upon co-expression of CSN4 (an average of 15-fold and 26-fold compared with control, respectively). However, the expression of CSN4 was 3–5-fold lower than CSN5, suggesting that the interaction of tescalcin with CSN4 is stronger than with CSN5. Our experiments also revealed a small increase in the luciferase activity in the presence of the CSN subunits 6 and 8 (Fig. 1C). Considering the low expression level of CSN4 and the strength of the luciferase reporter activity, our results suggest that the CSN4 subunit has the strongest interaction with tescalcin.
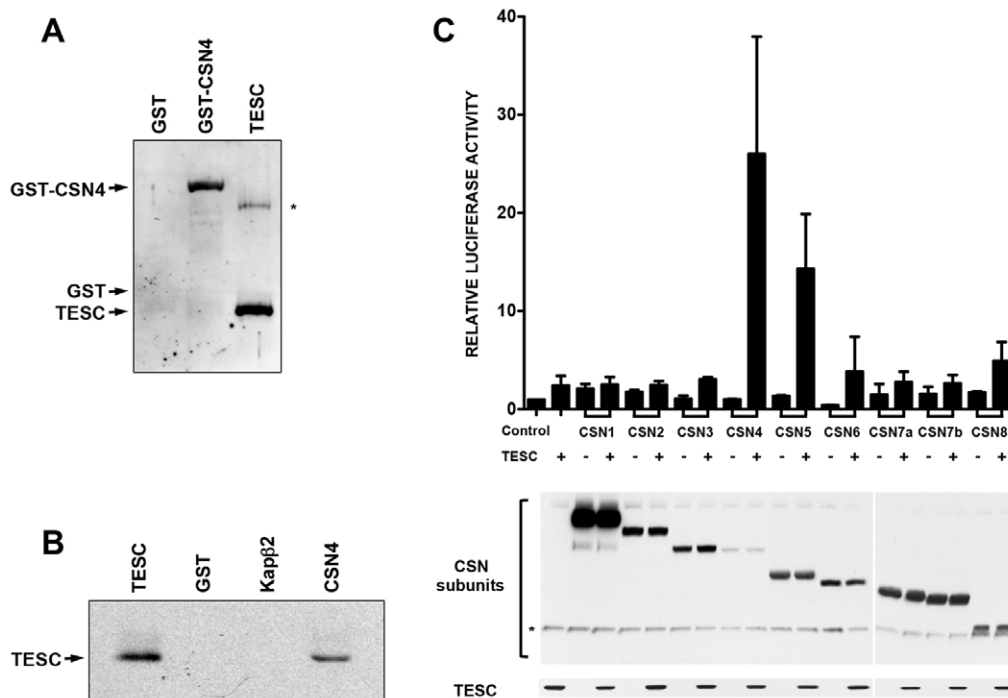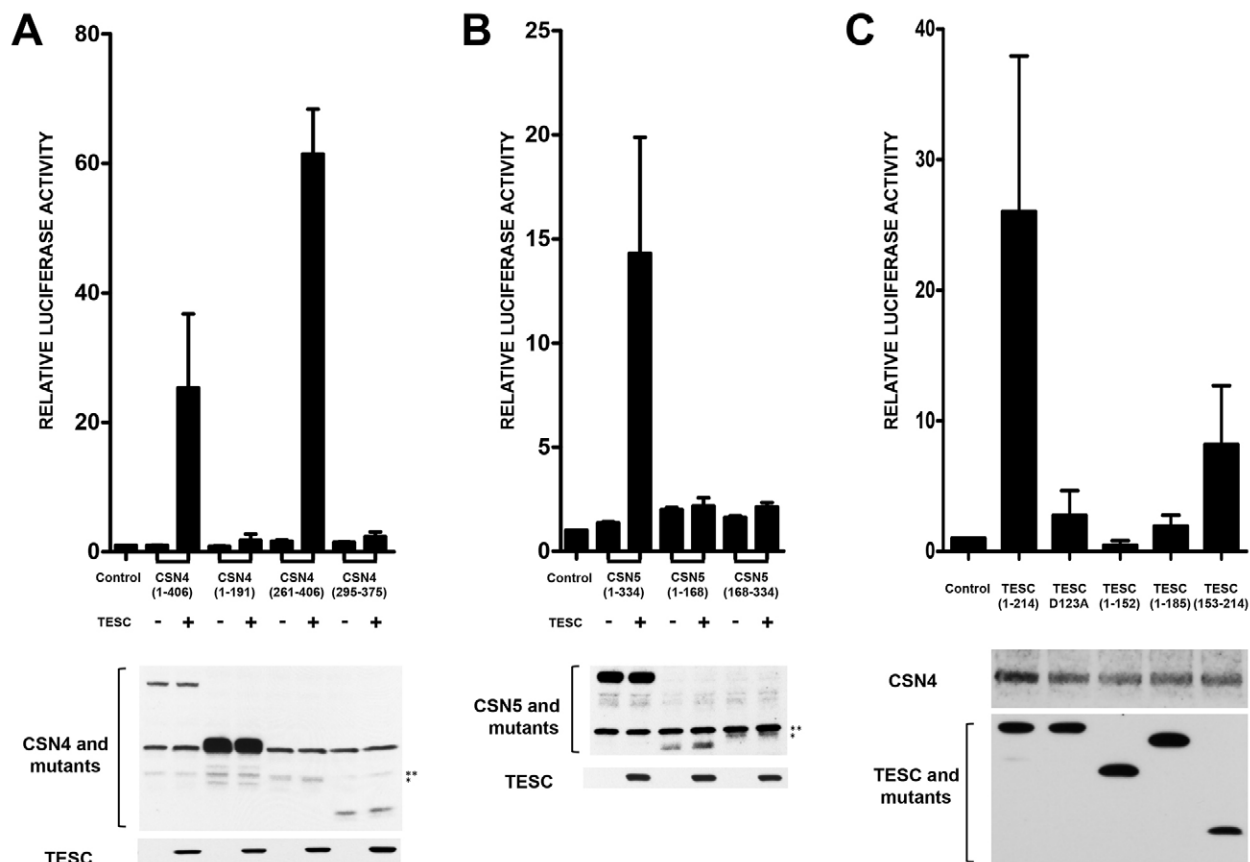

Fig. 1. Interaction of tescalcin with subunits of the CSN complex. (A) Far-western blots using recombinant CSN4. The membrane with immobilized CSN4 was incubated with recombinant tescalcin and the protein complex was detected by using an antibody against tescalcin (TESC). The asterisk indicates the position of a non-specific band detected by an antibody against tescalcin. (B) Pull downs with GST alone, GST–CSN4 (CSN4) and GST–Kapβ2 (karyopherin β2, a GST fusion protein control) bound to glutathione–Sepharose beads. Beads loaded with the GST proteins were incubated with recombinant His₆-tagged tescalcin. After washing, the bound proteins were eluted with SDS sample buffer, analyzed by electrophoresis and detected by western blotting for tescalcin. (C) CSN subunits (CSN1–8) were expressed in HEK293 cells as fusion proteins with the herpes simplex virus VP16 transcription activation domain. Tescalcin was expressed as a fusion protein with the GAL4 DNA-binding domain. Firefly luciferase under the control of GAL4 promoter was used as a reporter. The chart represents the strength of the interaction in folds (relative to the control). The means±s.d. are shown. At least five independent experiments for each construct were performed. The lower panel shows the expression levels of the CSN subunits in a typical two-hybrid experiment by western blotting for the VP16 tag. The asterisk indicates the position of a non-specific band detected by an antibody against the VP16 tag. The expression level of tescalcin was investigated by using an antibody against the GAL4 tag. The lanes of the western blot correspond to the aligned bar on the chart. No lane on the blot is shown for the negative control transfection (Control).

Structure–function analysis of the tescalcin–CSN interaction

To determine the regions of the CSN that are involved in the interaction with tescalcin, we tested deletion mutants of CSN4 and CSN5. Our experiments revealed that tescalcin strongly interacted with the C-terminal portion of CSN4 (amino acids 261–406) but does not interact with the N-terminus (amino acids 1–191) (Fig. 2A). The C-terminal fragment of CSN4 contains the PCI domain (amino acids 264–362), and its integrity is crucial given that the truncated PCI domain (amino acids 295–375) did not bind tescalcin. Interestingly, the luciferase reporter activity was much stronger in the presence of the PCI domain (the C-terminal fragment, amino acids 261–406) as compared with full-length CSN4, even though the expression level of the former was substantially lower. This observation indicates that the N-terminal portion of CSN4 attenuates the strength of the interaction with tescalcin. By contrast, only the full-length CSN5 protein, as opposed to its N-terminal or C-terminal truncated mutants, interacted with tescalcin (Fig. 2B). Based on these analyses, we conclude that the main binding site for tescalcin, within the CSN, is the PCI-domain-containing portion of CSN4. It is also possible that, in the HEK293 cells two-hybrid assay, recombinant CSN5,

CSN6 or CSN8 are able to activate the reporter because they partially integrate into the endogenous CSN complex, which in turn interacts with tescalcin through endogenous CSN4.

To determine the region of tescalcin that is responsible for the interaction with the CSN, we analyzed several of its mutants (Fig. 2C). First, we established that the single point mutation D123A, which abrogates the Ca^{2+} and Mg^{2+} binding (Gutierrez-Ford et al., 2003), reduces the strength of the interaction of tescalcin with CSN4 (Fig. 2C) and CSN5 (data not shown) by ~10-fold. The truncated mutant of tescalcin, comprising the N-terminal portion (amino acids 1–152), did not bind to CSN4. Co-expression of a larger truncated mutant (amino acids 1–185) resulted in only a 2-fold elevation of reporter activity when compared with the basal level. However, when the C-terminal portion (amino acids 153–214) was assayed, it did interact with CSN4, although both the protein expression and the strength of the interaction were lower than that of the full-length tescalcin (Fig. 2C). The interaction of this C-terminal portion of tescalcin with CSN5, CSN6 or CSN8 was also detected (data not shown). Based on these results, we conclude that the intact EF-hand domain and the ability to bind Ca^{2+} are necessary, but not

Fig. 2. Structure function analysis of tescalcin interaction with CSN4 and CSN5 subunits. Different CSN and tescalcin constructs were tested using the mammalian two-hybrid system as described previously in the legend to Fig. 1C. (A) Luciferase activity obtained with full-length and deletion mutants of CSN4 with and without full-length tescalcin. * the position of CSN4 (261–406) fragment in corresponding lanes, ** the position of a non-specific band detected in HEK293 cell lysates by using the antibody against VP16. (B) Full-length and mutants of CSN5 (comprising the amino acid residues shown in the brackets) were tested in the assay shown in A. * the position of the CSN5 (168–334) fragment in the corresponding lanes; ** the position of a non-specific band detected in HEK293 cell lysates by using the antibody against VP16. (C) A Ca^{2+}-binding-deficient mutant (D123A), and three deletion mutants of tescalcin were tested for their interaction with full-length CSN4. Bar graphs represent the strength of interaction in folds (relative to control), means±s.d. are shown, $n \geq 5$. The expression levels of all the mutant constructs were verified by western blotting using antibodies against the VP16 tag to detect the CSN subunits (upper panels of blots) or against the GAL4 tag to detect tescalcin (bottom panel of blots). The lanes of the western blot correspond to the aligned bar on the chart. No lane on the blot is shown for the negative control transfection (Control).

sufficient, for the interaction with the CSN. In addition to the EF-hand domain, the sequence located within the C-terminal portion of tescalcin (amino acids 153–214) is required for the interaction with CSN4 (Fig. 2C).

CHP1 and CHP2 are homologs of tescalcin that do not interact with the CSN

The Ca^{2+}-binding proteins CHP1 and CHP2 are considered to be the closest relatives of tescalcin according to their amino acid sequence (Di Sole et al., 2012); all three proteins have been implicated in regulation of the Na^+/H^+ exchanger. We found that neither CHP1 nor CHP2 were able to bind to CSN4 or CSN5 (Fig. 3A). Alignment of all three protein sequences using Clustal Omega software (Sievers et al., 2011) revealed the presence of three additional unique eight- or nine-amino-acid blocks (I, II and III) in the tescalcin sequence (Fig. 3B). Two of these blocks (II and III) are located within the C-terminal portion of tescalcin (amino acids 167–214) and might play a role in its interaction with the CSN subunits. Taken together, our results indicate that tescalcin can selectively interact with CSN subunits in a Ca^{2+}-dependent manner.

## CSN activity is inhibited upon induced cell differentiation

The CSN was first discovered as a negative regulator of development in *Arabidopsis* (Chamovitz et al., 1996), and its role in development has been confirmed in a variety of eukaryotes, including mammals (Kato and Yoneda-Kato, 2009). Because tescalcin plays a role in the lineage commitment and differentiation of hematopoietic cells (Levay and Slepak, 2007; Levay and Slepak, 2010), we investigated whether there is a connection between the induction of cell differentiation and the activity of the CSN. First, we tested whether CSN activity changes upon the induced differentiation of HEL and K562 cells. For this purpose, we cultured these cells in the presence of 10 nM phorbol 12-myristate 13-acetate (PMA) to induce megakaryocytic differentiation. After 72 h, we analyzed the status of the known targets of CSN activity Cul1 and Skp2, which are components of the E3 ubiquitin ligase Skip–Cullin–F-box (SCF) complex. As expected, upon treatment with PMA, cells entered growth arrest, became polyploid (Fig. 4A) and developed markers that are characteristic of megakaryocytic lineage, such as increased expression of integrin αIIb and the transcription factors Fli-1 and Ets-1 (Fig. 4B). Analysis of the Cul1 neddylation status
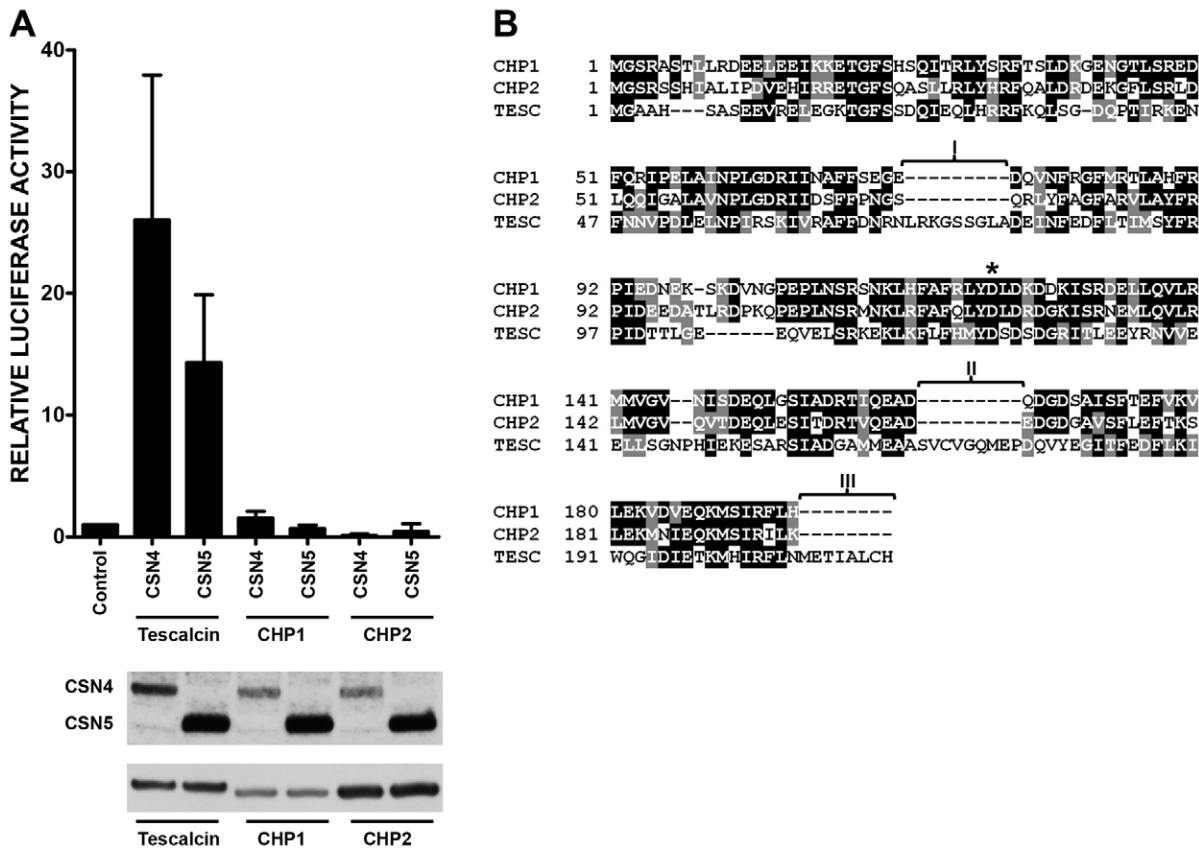

Fig. 3. Homologs of tescalcin (CHP1 and CHP2) do not interact with CSN subunits. (A) The Ca^{2+}-binding proteins CHP1 and CHP2 were cloned and tested for their interaction with CSN4 and CSN5 using the two-hybrid luciferase reporter assay. Bar graphs represent the strength of interaction in folds (relative to control). Means±s.d. are shown, $n \geq 5$. The expression levels of the proteins were analyzed by western blotting using specific antibodies against the VP16 tag for detection of CSN subunits (top panel of the blot) or GAL4 tag for detection of tescalcin, CHP1 and CHP2 (bottom panel of the blot). The lanes of the western blot correspond to the aligned bar on the chart. No lane on the blot is shown for the negative control transfection (Control). (B) Clustal Omega multiple sequence alignment of human homologs of tescalcin (Genebank accession # NP_067319.2), CHP1 (NP_062743.1), and CHP2 (NP_081639.1). The HHalign algorithm and default settings were used for the alignment (Söding, 2005). The BOXSHADE v. 3.21 program was used for graphic output. Identical amino acid residues are boxed on a black background. Similar residues are boxed on gray background. * conserved aspartic acid residue that is important for Ca^{2+}-binding by the EF-hand domain.
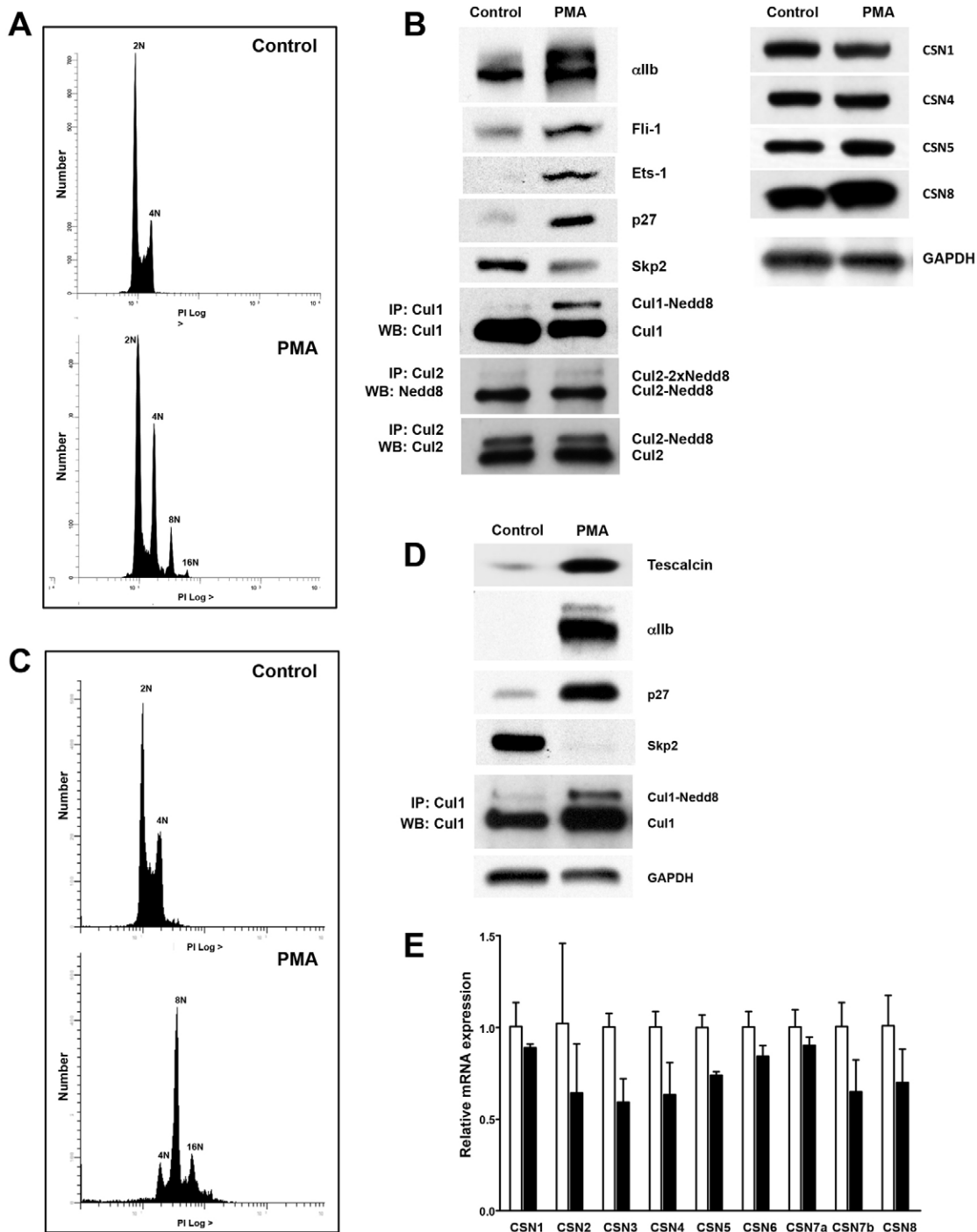
Fig. 4. Differentiation of HEL cells coincides with inhibition of CSN activity. (A) HEL cells were cultured in the absence (Control) or presence of PMA for 72 h, fixed and stained with propidium iodide, and their DNA content was analyzed by FACS. (B) HEL cells that had been cultured for 3 days in the presence of PMA were analyzed by western blotting for the expression of differentiation markers [αIIb, Ets-1, Fli-1] and the CSN subunits CSN1, CSN4, CSN5 and CSN8 (right panel). CSN activity was assessed by analyzing the neddylation (Nedd8) of Cul1and Cul2, and the stability of Skp2 and p27^{kip1}. Cul1 and Cul2 were subjected to immunoprecipitation (IP) before immunoblotting (WB). GAPDH was used as a loading control. Representative western blots from at least 4 independent cell differentiation experiments are shown. (C) K562 cells were cultured in the absence (Control) or presence of PMA for 72 h, then fixed and stained with propidium iodide, and their DNA content was analyzed by FACS. (D) Lysates of control and differentiated (PMA) K562 cells were analyzed by western blotting for the expression of tescalcin, αIIb, Cul1, Skp2 and p27^{kip1}. GAPDH was used as a loading control. Representative western blots from at least 3 independent experiments are shown. (E) Total RNA was isolated from control and differentiated K562 cells. SYBR green-based qPCR analysis using human GAPDH- and CSN-subunit-specific primers was performed. Data are from three independent experiments performed in triplicate and are expressed as the expression of the target mRNA in differentiated cells (black bars) relative to that of control cells (white bars). Means±s.d. are shown.
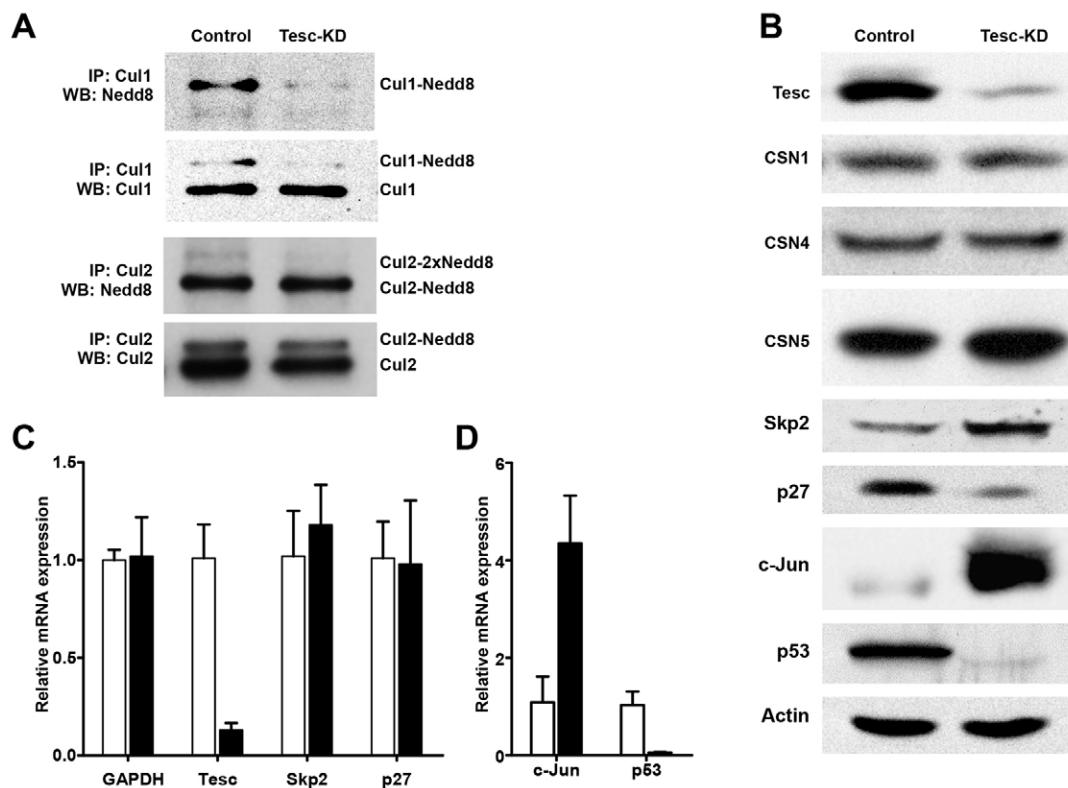
Fig. 5. Tescalcin knockdown effect on CSN activity. (A) The steady-state neddylation status of Cul1 and Cul2 in HEL cells expressing tescalcin-specific (Tesc-KD) and scrambled (control) target sequence shRNA was analyzed by immunoprecipitation (IP) and western blotting (WB). (B) Tescalcin knockdown did not affect the expression levels of CSN subunits. HEL cell lysates were resolved by 10-20%; gradient PAGE, using 25 mg of total protein per well. After transfer to nitrocellulose, blots were probed by using specific antibodies against tescalcin, CSN1, CSN4, CSN5, Skp2, p27, c-Jun, and p53. Probing for actin was used to demonstrate equal loading. (C,D) Total RNA was isolated from HEL cells expressing scrambled control and tescalcinspecific target shRNAs. SYBR green-based qPCR analysis using human GAPDH-, Tesc-, Skp2-, p27^{kip1-}, c-Jun-, and p53-specific primers was performed. Data are from four independent experiments that were performed in triplicate and are shown as the mRNA expression in tescalcin knockdown cells (black bars) relative to that of control cells (white bars). Means±s.d. are shown.

showed that there was a substantial increase in the level of the neddylated form (Fig. 4B). This change coincided with a decrease in Skp2 protein level. Accordingly, the cell cycle inhibitor protein p27^{kip1}, a substrate of the SCF-E3 ubiquitin ligase, was stabilized. It has been shown previously that downregulation of the activity of the CSN leads to similar changes in the stability of Skp2 and p27^{kip1}, and inhibits cell proliferation (Denti et al., 2006). Thus, our results indicate that, upon megakaryocytic differentiation of HEL cells, the CSN activity towards Cul1 is suppressed, which contributes to the stabilization of p27^{kip1} and leads to cell cycle arrest. There were no substantial changes in the expression of individual CSN subunits, as tested by western blotting (Fig. 4B). Similar results were obtained for K562 cells (Fig. 4C, D), and the quantitative real-time RT-PCR (qPCR) data from K562 samples revealed that the induction of cell differentiation did not bring about a reduction in expression of the CSN subunits (Fig. 4E). Thus, induction of HEL and K562 differentiation coincides with suppression of CSN activity towards Cul1.

We also tested whether inducing differentiation influences the activity of the CSN towards Cul2 and Cul3. Interestingly, there was no effect on the Cul2 neddylation status (Fig. 4B), suggesting that the deneddylation activity of the CSN might be selective. The analyses of Cul3 were inconclusive because the available antibodies were not effective in immunoprecipitation assays (data not shown).

Tescalcin knockdown increases CSN activity

To investigate whether tescalcin plays a role in the regulation of CSN function, we performed knockdown of the protein in HEL cells by using shRNA, as described previously (Levay and Slepak, 2007), and analyzed the neddylation status of Cul1, Cul2 and Cul3. We found a reduction in the steady-state level of neddylated Cul1 in tescalcin-depleted cells (Fig. 5A), whereas we did not detect changes in the protein levels of CSN subunits, as shown by western blotting (Fig. 5B). Therefore, reduced Cul1 neddylation must be the result of augmented CSN activity, rather than changes in the CSN expression level.

The rapid cycle of Cul1 deneddylation by the CSN, followed by its neddylation, is essential for maintenance of the SCF ubiquitin ligase activity (Liu et al., 2002). Therefore, we hypothesized that the reduced neddylation of Cul1 in tescalcin-depleted cells results in the increased stability and availability of the F-box protein Skp2. Indeed, we found that the steady-state level of Skp2 was higher in the tescalcin-knockdown cells (Fig. 5B). An increase in the Skp2 concentration is expected to cause an accelerated targeting, and successive proteasomal degradation, of SCF^{Skp2} substrates, such as p27^{Kip1} (Gstaiger et al., 2001). Consequently, we investigated the protein level of p27^{Kip1} by immunoblotting and found it to be significantly decreased after tescalcin knockdown (Fig. 5B). Because the mRNA levels of Skp2 and p27^{Kip1} did not vary between

the control cells and those with tescalcin knockdown (Fig. 5C), the differences in the Skp2 and p27^{Kip1} levels are attributed to protein stability, rather than gene transcription.

Similar to cell differentiation experiments, the depletion of tescalcin did not affect the neddylation status of any of the cullins tested. For example, we did not find any noticeable changes in the neddylation of Cul2 (Fig. 5A), and for Cul3 our results remain inconclusive (data not shown).

The tumor suppressor protein p53 and the transcription factor c-Jun are major players in cell cycle regulation and are also known to be substrates of CSN-associated kinase activity. Phosphorylation of p53 by this kinase activity signals for the protein to be degraded by the proteasome (Bech-Otschir et al., 2001). By contrast, the direct interaction of CSN5 with c-Jun, and its subsequent phosphorylation by the CSN-associated kinase, leads to c-Jun stabilization (Claret et al., 1996; Naumann et al., 1999). Our results show that the protein level of p53 in the tescalcin-knockdown cells was markedly lower than that in controls (Fig. 5B). These data might also indicate that depletion of tescalcin caused an increase in CSN activity – i.e. tescalcin might act as an inhibitor of CSN. Concomitantly, the level of c-Jun showed striking upregulation, which also supported our hypothesis. However, it appears that the mechanism associated with both p53 and c-Jun protein stability is not the only one regulating their levels upon tescalcin knockdown. mRNA analysis, by using qPCR, showed that the expression level of the p53 message decreased 6–10-fold, whereas the level of that for c-Jun was 3–4-fold higher (Fig. 5D). These results suggest that tescalcin might be involved in cell cycle control and cell differentiation by regulating p53 and c-Jun at both protein and mRNA levels.

Next, we suppressed CSN4 in tescalcin-depleted HEL cells using a lentivirus-mediated shRNA approach. Lentiviruses expressing control and CSN4-specific target sequences also encoded the extracellular region of the human low-affinity nerve growth factor receptor (LNGFR), which is not found in HEL cells. Expression of LNGFR was used to facilitate the isolation of transduced cells using immunomagnetic separation. By using qPCR analysis, the expression of CSN4 mRNA in transduced HEL cells was reduced more than 10-fold, whereas the expression of the other tested subunits was not affected (Fig. 6A). At the protein level, the knockdown of CSN4 also caused a reduction of CSN5, which is due, evidently, to the destabilization of CSN4-containing complexes (Fig. 6B). This was similar to the results of CSN4 knockdown in HEK293 cells (Denti et al., 2006). In tescalcin-depleted cells, the neddylation of Cul1 was higher in cells that had been transduced with a CSN4-specific lentivirus than in cells that had been transduced with control lentivirus (Fig. 6C). The overall loss of CSN4 in HEL cells induced a dramatic reduction in growth and survival and resulted in rapid apoptotic death, making further analysis difficult. Interestingly, the gene expression of the differentiation marker αIIb integrin, which was downregulated upon tescalcin knockdown (Levay and Slepak, 2007), was partially restored by the additional knockdown of CSN4 (Fig. 6D).

DISCUSSION
Cell differentiation involves numerous signal transduction pathways and mechanisms, such as gene expression and protein turnover. In our earlier studies we have explored and defined the role of the Ca^{2+}-binding protein tescalcin in the lineage commitment and terminal differentiation of human primary hematopoietic precursor cells and model leukemia cell lines. Modulation of tescalcin levels affected the appearance of cell-type-specific markers, as well as the
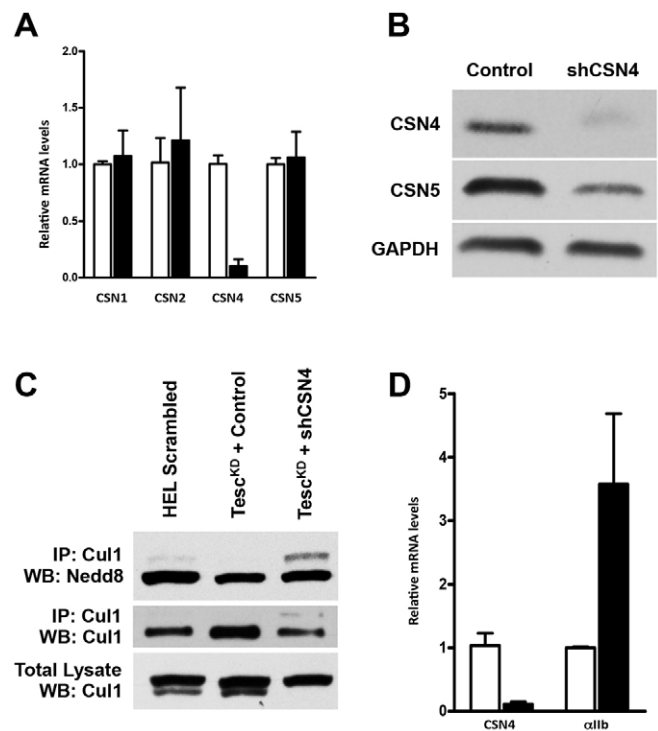
Fig. 6. The effect of CSN4 knockdown on CSN activity in tescalcin-depleted cells. HEL cells were transduced by using lentiviruses that expressed control and CSN4-specific shRNA with the LNGFR, and LNGFR-positive cells were purified by magnetic beads. (A) SYBR-green-based qPCR using CSN1-, CSN2-, CSN4- and CSN5-specific primers. Data are from two independent experiments run in triplicate, and the expression of the target mRNA in CSN4-knockdown cells (black bars) relative to that of controls (white bars), means±s.d. are shown. (B) Total lysates from HEL cells that had been transduced with control and shCSN4 lentiviruses were resolved by 10–20% gradient PAGE, using 25 μg of total protein per well. After transfer to nitrocellulose, blots were probed with specific antibodies against CSN4 and CSN5. An antibody against GAPDH was used to demonstrate equal loading. (C) HEL cells expressing control (Scrambled) or tescalcin-specific (Tesc^{KD}) shRNA were transduced by using lentiviruses expressing control or CSN4-specific shRNA (shCSN4). LNGFR-positive cells were isolated using magnetic beads. Total cell lysates (25 μg per well) and immunoprecipitated (IP) Cul1 were resolved by PAGE, transferred to nitrocellulose and probed with antibodies against Cul1 and Nedd8. (D) Tescalcin-depleted HEL cells were transduced with control and shCSN4 lentiviruses. Total RNA was isolated from LNGFR-positive cells and analyzed by using qPCR with CSN4- and αIIb-integrin-specific primers. Data are from two independent experiments performed in triplicate and are presented as the mRNA expression in CSN4-knockdown cells (black bars) relative to that of control cells (white bars). Means±s.d. are shown.

kinetics and extent of differentiation into megakaryocytic and granulocytic lineage (Levay and Slepak, 2007; Levay and Slepak, 2010). A recent study describing the knockout of the tescalcin gene in mice appears to contradict such a role, because the number of megakaryocytes and platelets, in the tested adult mice remain unchanged (Ukarapong et al., 2012). However, the knockout of most genes does not result in an obvious phenotype in a resting state, and the difference between wild-type and knockout animals might only be revealed upon stress or otherwise, eliciting a functional response.

We initiated this study in order to find binding partners of tescalcin, and the unbiased yeast two-hybrid screen resulted in the identification of the CSN4 subunit. The CSN is essential for the regulation of development in all eukaryotes that have been tested

(Kato and Yoneda-Kato, 2009; von Arnim, 2003; Wei and Deng, 2003). Therefore, this interaction could potentially explain how changes in the expression level of tescalcin could influence cell differentiation (Levay and Slepak, 2007; Levay and Slepak, 2010) or human brain development (Stein et al., 2012). At the molecular level, CSN is known to have several activities that affect ubiquitination, neddylation and the turnover of several proteins involved in cell division and differentiation. However, mechanisms regulating the functions of the CSN complex itself are generally unknown. We found reports describing only three proteins that are able to interact with the CSN and influence its activity. All of them were shown to regulate the function of CSN5; macrophage migration inhibitory factor (MIF) negatively regulates CSN5 activity in fibroblasts (Kleemann et al., 2000). Another protein, RIG-G, was shown to interfere with CSN complex activity by sequestering CSN5 in the cytoplasm. This affected the gene expression of Cdk inhibitor p21, suppressed the SCF E3 ligase activity towards p27^{Kip1} and induced morphologic features of partial differentiation in the human leukemic cell line U937 (Xiao et al., 2006; Xu et al., 2013). Of particular interest, in relation to our study, is that a small EF-hand Ca^{2+}-binding protein psoriasin (also known as S100A7) has been shown to interact with CSN5 in breast cancer cells. Overexpression of psoriasin correlated with the destabilization of p27^{Kip1}, upregulation of AP-1 transcriptional activity, and tumor progression *in vitro* and *in vivo* (Emberley et al., 2004; Emberley et al., 2003). However, it was not shown whether the psoriasin–CSN5 interaction was regulated by Ca^{2+}. In the current report, we provide experimental evidence that the CSN can be regulated by the CSN4-interacting Ca^{2+}-binding protein tescalcin.

Data presented in Figs 1–3 demonstrate that tescalcin specifically binds to the PCI domain of CSN4 in a Ca^{2+}-dependent manner. So far, our attempts to co-immunoprecipitate tescalcin and CSN subunits from a native tissue or differentiated and non-differentiated cells have been unsuccessful. This can be interpreted by the highly dynamic nature of this interaction or attributed to the quality of available antibodies. The homologs of tescalcin (CHP1 and CHP2) did not bind to CSN4, and the structure–function analysis revealed that the unique C-terminal region of tescalcin is essential for the interaction (Fig. 2C and Fig. 3B). It is worth noting here that the tescalcin-knockout strategy described previously by Ukarapong and colleagues (Ukarapong et al., 2012) did not lead to complete elimination of the gene product but, rather, produced mRNA comprising the sequences of exons 1, 2, 7 and 8. Translation of this mRNA could potentially result in a protein containing the C-terminal portion of tescalcin (amino acids 175–214), which might be sufficient for the interaction with the CSN (as we show in Fig. 2C) and other binding partners.

We assayed the expression and status of CSN substrates before and after the induction of the differentiation of hematopoietic precursor cells (Fig. 4) and examined the effects of knockdown of tescalcin and CSN4 (Fig. 5, 6). Our results are consistent with the following hypothesis (Fig. 7) – during the differentiation of precursor cells into a certain lineage, the sustained MAP–ERK signaling induces a change in the tescalcin expression level (Levay and Slepak, 2007; Levay and Slepak, 2010). Tescalcin interacts with the PCI domain of CSN4. This interaction appears to be direct, Ca^{2+}-dependent and highly selective (Figs 1–3). The connection between tescalcin and CSN activity is also supported by the fact that the expression level of both proteins changes upon
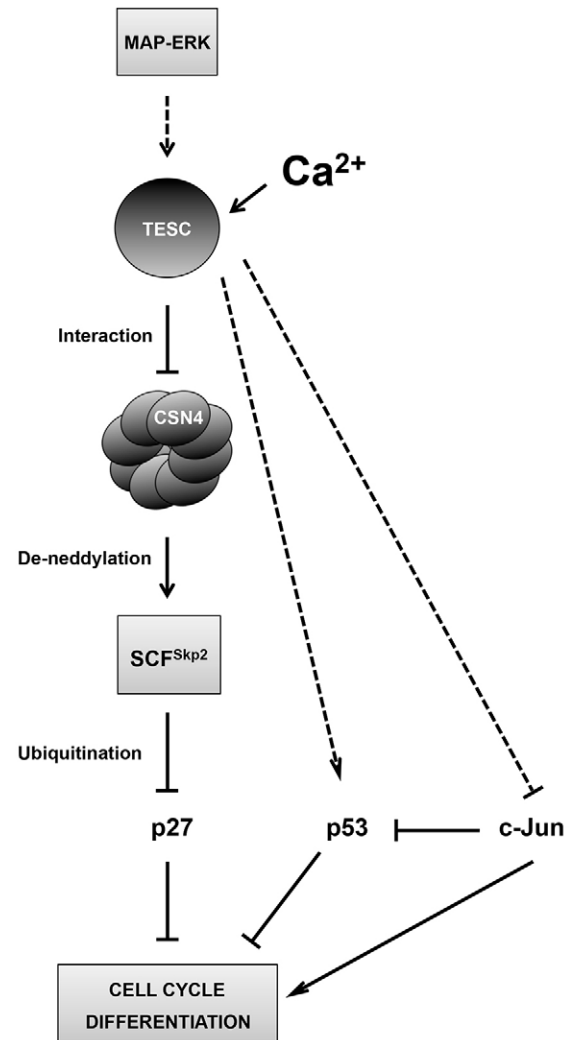
Fig. 7. Hypothetical tescalcin- and CSN-mediated signaling pathway. Representation of the molecular events that are associated with tescalcin and the CSN complex. Tescalcin is upregulated in response to MAP–ERK activation (Levay and Slepak, 2007). A direct, Ca^{2+}-mediated interaction with CSN4 inhibits the CSN-mediated regulation of the SCF E3 ubiquitin ligase. In the process of differentiation, an increase in Cul1 neddylation leads to stabilization of p27^{kip1} and other cell cycle regulators, and cell cycle arrest. Tescalcin can also regulate expression of p53, c-Jun and other transcription factors through regulation of their transcription and, possibly, other currently unknown mechanisms (see text for details).

differentiation (Fig. 4). The knockdown of tescalcin results in the apparent stimulation of CSN activity towards SCF^{Skp2}, evidenced by decreased levels of neddylated Cul1 and the accumulation of Skp2 (Fig. 5). Increased activity of the CSN leads to the destabilization of p27^{Kip1}, which occurs at the protein level rather than the mRNA level. Depletion of the p27^{Kip1} pool stimulates cell cycle progression. The suppression of CSN4 appears to partially restore the effects of tescalcin knockdown (Fig. 6C), which might be consistent with our hypothesis that they act in the same cellular pathway. Thus, one of the ways that tescalcin might participate in the regulation of cell differentiation is by influencing CSN-mediated protein degradation.

Tescalcin can also regulate differentiation through other mechanisms, such as gene transcription (Levay and Slepak, 2007). Similarly, expression of multiple transcription factors and

other genes early in development is affected by a null mutation in CSN4 in the *Drosophila* model (Oron et al., 2007). In our study, we found that tescalcin knockdown leads to changes in the expression levels of the transcription factors p53 and c-Jun (Fig. 5). This result is consistent with previous reports that have demonstrated that the CSN-associated kinases casein kinase 2 and protein kinase D phosphorylate p53 and c-Jun, resulting in the degradation of the former protein and stabilization of the latter (Bech-Otschir et al., 2001; Naumann et al., 1999; Uhle et al., 2003). It is worth noting that, although HEL cells express a mutant form of p53 that is ineffective in transcriptional activation (Jia et al., 1997), p53 itself still might be phosphorylated and targeted for ubiquitin-dependent proteasomal degradation. It is possible that, similar to p27^{Kip1}, the protein levels of p53 and c-Jun are regulated by the tescalcin–CSN signaling pathway. We also found that changes in p53 and c-Jun protein levels are accompanied by substantial changes in the amount of their mRNA (Fig. 5) and, thus, we cannot yet conclude whether this mechanism is CSN-dependent. Regardless of a specific mechanism, our study establishes a connection between tescalcin and the expression levels of p53 and c-Jun, which is similar to the previously reported link between tescalcin and the Ets family of transcription factors.

MATERIALS AND METHODS
Materials
cDNA of human tescalcin has been described previously (Levay and Slepak, 2007). cDNA of mouse CHP1 was a gift from Diane Barber (UCSF, San Francisco, CA). cDNA of mouse CHP2 was obtained by RT-PCR using total RNA isolated from small intestine. Full-length cDNAs of human CSN1 (GenBank accession number BC000155), CSN2 (GenBank accession number BC012629), CSN3 (GenBank accession number BC001891), CSN4 (GenBank accession number BC004302), CSN5 (GenBank accession number BC007272), CSN6 (GenBank accession number BC002520), CSN7a (GenBank accession number BC011789), CSN8 (GenBank accession number BC003090) and mouse CSN7b (GenBank accession number BC012659) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). The mouse monoclonal antibody against tescalcin has been described previously (Levay and Slepak, 2010). Rabbit polyclonal antibodies against CSN4 (386-400, Sigma-Aldrich, St Louis, MO), CSN1 (PW8285, Enzo Life Sciences, Farmingdale, NY), and Cullin-1 (RB-042, NeoMarkers, Fremont, CA) were used. Rabbit polyclonal antibodies against GAPDH (sc-25778) and p27^{Kip1} (sc-528) were purchased from Santa Cruz Biotechnology (Dallas, TX), and against c-Jun (#9165) and Nedd8 (#2745) were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies against Cullin-1 (sc-17775), VP-16 (sc-7546) and p53 (sc-126) were from Santa Cruz Biotechnology; against β-actin (MAB1501R) and integrin αIIb (MAB1990) were from Millipore (Billerica, MA); against CSN5 were from Transduction Laboratories (Lexington, KY). A goat polyclonal antibody against Skp2 (sc-1567) was from Santa Cruz Biotechnology. Mouse antibodies against Cul2 (SC-166506) and Cul3 (SC-166054), and a goat antibody against Cul3 (SC-8556) were from Santa Cruz Biotechnology. Secondary antibodies against mouse, rabbit and goat IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Glutathione Sepharose 4B was from GE Healthcare Biosciences (Pittsburgh, PA). Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) was supplemented in all cell lysates. Protein electrophoresis reagents and markers were from Bio-Rad (Hercules, CA). Cell culture plastic and media were purchased from BD Biosciences (San Jose, CA). Other chemicals were from Sigma-Aldrich.

Two-hybrid library screening and evaluation of protein–protein Interactions
The entire 645-bp tescalcin cDNA in the pGBKT7 bait vector was used to screen a human bone marrow cDNA library constructed in the pGADT7 pray vector. The yeast two-hybrid screen (Matchmaker Gold,

Clontech Laboratories, Mountain View, CA) was based on the overnight mating of two yeast strains – the MATα reporter strain AH109 transformed with pGBKT7-tescalcin bait and the MATα strain Y187 transformed with the human bone marrow cDNA library in the pGADT7 vector. The library screening and subsequent confirmation of interactions were performed under high-stringency selection, and positive interactions were initially identified by the growth of cells on medium lacking adenosine, leucine, tryptophan and histidine with 5 mM 3-amino-1,2,4-triazole (3-AT, an inhibitor of histidine synthase) at 30°C for 3–4 days. Positive colonies obtained from the screen were confirmed by β-galactosidase activity in a colony filter-lift assay. To minimize the number of false positives, and to increase the likelihood of obtaining pure clones, the initial isolates were grown in −His medium with 30 mM 3-AT. Plasmids from individual positive tescalcin-interacting clones were isolated, sorted by restriction analysis and propagated in the DH5α-strain of *Escherichia coli*. All inserts were GenBank accession numberd and identified using NCBI Blast. Bait and individual identified pray plasmids were co-transformed into AH109 yeast and the interaction was re-confirmed.

GST pulldown experiments
To produce a recombinant GST-tagged CSN4 subunit, the coding portion of the CSN4 cDNA was cloned into the pGEX-6P vector. The fusion protein was expressed in BL21-RIL cells and purified using standard protocols. Recombinant His$_6$-tagged tescalcin was produced as described previously (Gutierrez-Ford et al., 2003). To study the interaction between CSN4 and tescalcin, glutathione–Sepharose beads were preloaded with either GST–CSN4, GST alone or an unrelated GST–fusion protein for 1 h at 4°C. After washing with PBS, 20 μg of recombinant tescalcin was added to 20 μl of Sepharose beads with GST proteins and incubated for 1 h at room temperature. After the beads had been washed, the bound proteins were eluted with 2×SDS sample buffer and analyzed on SDS-PAGE, followed by transfer to nitrocellulose membrane and immunoblotting with antibody against tescalcin.

Far-western blots
Far-western experiments were performed, essentially, as described previously (Berse et al., 2004; Kapelari et al., 2000; Wu et al., 2007). Briefly, 0.5–1 μg of purified recombinant GST–CSN4 or GST alone were separated on a 12% SDS polyacrylamide gel and blotted onto nitrocellulose. The membrane was stained with Ponseau S to mark the position of proteins, washed three times (5 min each with in PBS) and incubated in blocking buffer (5% milk in PBS, 5% glycerol and 0.1% Tween-20) for 1 h at room temperature. The immobilized proteins were incubated for 2 h with 1 μg/ml of recombinant tescalcin in PBS-glycerol-Tween buffer. After washing, the blots were probed by using an antibody against tescalcin (Levay and Slepak, 2010) and developed by the enhanced chemiluminescence technique.

Cell culture, transient transfection and stable cell lines
HEK293 cells (a human embryonic kidney line) were maintained in Dulbecco's modified Eagle's medium (DMEM) that had been supplemented with 10% fetal bovine serum (FBS), and 100 μg/ml of penicillin and streptomycin. HEL cells (a human erythroleukemia line; TIB-180, ATCC) and K562 cells (a human chronic myelogenous leukemia line; CCL-243, ATCC) were maintained in RPMI-1640 medium, which had been supplemented with 10% heat-inactivated FBS and 100 μg/ml of penicillin and streptomycin, at a density of 1×10^5–1×10^6 viable cells/ml. Transfections for mammalian two-hybrid assays were performed using the TransIt-LT1 reagent according to the manufacturer's recommendations (Mirus Bio, Madison, WI). For the transfection of HEL cells, exponentially growing cells were washed twice with PBS and resuspended in serum-free RPMI-1640 at a density of 2×10^7 cells/ml. Electroporation was performed using 80 μg of plasmid DNA that had been mixed with 0.8 ml of cell suspension. The cells were subjected to a single electric pulse (960 μF, 250 V) in 0.4-cm gap electroporation cuvettes using a Bio-Rad Gene Pulser, followed by dilution to 5×10^5 cells/ml in RPMI-1640 cell culture medium. To

generate stable cell lines, neomycin-resistant cells were selected by using 1.5 mg/ml Geneticin (Invitrogen, Grand Island, NY) and screened for tescalcin expression by western blotting and qPCR analyses. Stable cells were routinely maintained in RPMI-1640 with 10% FBS and 1 mg/ml Geneticin.

Mammalian two-hybrid assay

cDNAs encoding tescalcin, CHP1 and CHP2 were cloned into the pBIND vector to be expressed as fusion proteins with the GAL4 DNA-binding domain. cDNAs encoding CSN subunits and their mutants were cloned into the pACT vector to be expressed as fusion proteins with the VP16 activation domain. Plasmid pG5luc was used as a firefly luciferase reporter. Mammalian two-hybrid assays were performed in HEK293 cells according to manufacturer's recommendations with some modifications (CheckMate, Promega, Madison, WI). Briefly, 20 h before transfection, $7–8\times10^5$ cells were seeded onto 6-well tissue culture dishes. Cells were transfected with the pACT, pBIND and pG5luc plasmid DNAs, mixed in a ratio of 3:1:1 at 2.5 μg/well. At 24 h after transfection, the cells were lysed in a passive lysis buffer (PLB; Promega, Madison, WI) and the reporter activities, representing the strength of each interaction, were assayed using the Dual Luciferase Reporter Assay System (Promega) and Fluoroskan Ascent FL luminometer (Thermo Fisher Scientific, Waltham, MA). To correct for transfection variations, each sample was normalized for the internal control, the *Renilla* luciferase. Basal luciferase activity in cells that had been co-transfected with empty binding domain and activating domain plasmids was used as the negative control. All transfection experiments were performed in triplicate a minimum of five times. The expression of proteins was verified by immunoblotting the lysates of cells that had been transfected with similar efficiency by using antibodies against the VP16 and GAL4 tags.

Sample preparation and western blotting

Exponentially growing suspension cells were collected by centrifugation for 10 min at 300 *g* and 4℃, and the cell pellet was washed twice with ice-cold PBS. To obtain a total cell lysate, the pellet was gently resuspended in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_2P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and protease inhibitors) and incubated on ice for 30 min. Adherent cells were rinsed twice with PBS on ice to remove residual media, and monolayer cells were gently scraped into lysis buffer. Further homogenization was performed by using an ultrasonic cell disruptor (Misonix, Farmingdale, NY), and the lysate was centrifuged at 15,000 *g* for 15 min at 4℃. The protein concentration was determined using the Bio-Rad Bradford protein assay unless otherwise stated. To prepare samples for loading, the total cell lysate was mixed with 5×SDS sample buffer (10% SDS, 62.5 mM Tris-HCl pH 6.8, 25% glycerol and 125 mM dithiothreitol). The proteins were resolved on 12% or 10–20% gradient polyacrylamide gel and transferred onto nitrocellulose (Schleicher & Schuell BioScience, Keene, NH). Membranes were blocked in 1% w/v bovine serum albumin or 5% nonfat dry milk in TBST (20 mM Tris, 50 mM NaCl and 0.1% Tween-20, pH 7.4), probed with primary antibodies for either 1–2 h at room temperature or overnight at 4℃ and then incubated with the appropriate horseradish-peroxidase-conjugated secondary antibodies. Visualization of protein bands was performed by enhanced chemiluminescence (SuperSignal western blotting kit, Thermo Fisher Scientific) and exposure to Kodak X-Omat film. After developing, the films were scanned by using a high-resolution film scanner (Epson, Long Beach, CA), and the images were resized for presentation without any significant manipulation.

Analysis of Cul1 neddylation

Cultured cells were collected by centrifugation, washed twice with ice-cold PBS and resuspended in lysis buffer (50 mM Tris pH 7.5, 2% SDS, 10 mM iodoacetamide). After brief sonication, the lysates were diluted to 0.1% SDS in reconstitution buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5 mM 2-mercaptoethanol and protease inhibitors), incubated on ice with gentle rocking for 30 min and filtered through a 0.45-μm syringe filter. Total protein concentration was determined by using the BCA Protein Assay Kit (Thermo Fisher

Scientific). Cul1 was immunoprecipitated from 500 μg of total protein by using a mouse monoclonal antibody against Cul1 and 20 μl of protein A/G PLUS-agarose (Santa Cruz Biotech). The beads were washed four times, 5 min per wash, with ice-cold reconstitution buffer and then eluted with SDS-PAGE sample buffer. Samples were boiled for 5 min, resolved on 10–20% gradient gels (Novex, Invitrogen), transferred to nitrocellulose and probed with rabbit polyclonal antibodies against Cul1 and Nedd8.

Flow cytometry

To analyze the DNA content, cells were collected by centrifugation and fixed with ice-cold 70% ethanol overnight at 4℃. The DNA was stained with propidium iodide solution in the presence of RNase A (Sigma-Aldrich). All flow cytometry experiments were performed using a Becton Dickinson FACScan instrument with a minimum of 10,000 events acquired per sample. CellQuest (BD Biosciences) and WinList 3D (Verity, Topsham, ME) software packages were used for data analysis.

RNA isolation and quantification of gene expression

Total RNA was extracted from cells, by using Trizol reagent, and then treated with recombinant DNase (Ambion, Invitrogen), purified over an RNeasy column (Qiagen, Valencia, CA) and converted to cDNA using the High-Capacity Reverse Transcription kit (Applied Biosystems, Invitrogen). 100 ng of total RNA was used in a qPCR with Power SYBR Green PCR Master Mix (Applied Biosystems) and specific primers (see supplementary material Table S1). Target-specific primers for qPCR were designed and selected using the Primer-Blast tool (Ye et al., 2012) and validated as described previously (Kurrasch et al., 2004). All reactions were run in triplicate on the 7900HT Fast Real-Time PCR system (Applied Biosystems) and normalized to the endogenous control GAPDH RNA.

Lentivirus-mediated shCSN4 knockdown

The lentiviral small hairpin (sh)RNA plasmid pLKO.1 that targeted human CSN4 (Clone ID: TRCN0000118346 with the target GenBank accession number 5′-ATTCTTGAAGTTGATTTCCTC-3′) was purchased from Open Biosystems. The TRC eGFP shRNA plasmid was used as a control. A 835-bp DNA fragment that encodes the extracellular domain of human low-affinity nerve growth factor receptor (LNGFR) was amplified by using RT-PCR of total RNA isolated from HEK293T cells. The primers used to generate the LNGFR cDNA fragment were 5′-CAATGGATCCATGGGGGCAGGTGCCAC-3′ (forward) and 5′-CAATGGTACCTCAGGCTATGTAGGCCAC-3′ (reverse). The 679-bp puromycin resistance coding GenBank accession number in pLKO.1 shRNA vectors was substituted with LNGFR cDNA, by using the *Bam*HI and *Kpn*I sites, to generate the pLNGFR-shCSN4 and pLNGFR-shGFP transfer vectors.

To generate lentiviruses, 6×10^6 HEK293T cells were plated on 100-mm plates. The following day, 5 μg of shRNA vector pLNGFR (control or shCSN4), 4 μg of the packaging vector pCMVdR8.74 and 1 μg of the vesicular stomatitis virus-glycoprotein envelope vector pMD2.VSVG were co-transfected using the TransIt-LT1 reagent (Mirus Bio). The medium was changed the next day and replaced with DMEM that had been supplemented with 2% FBS and 10 mM sodium butyrate. Supernatants containing lentivirus were collected 48 h after transfection and centrifuged for 15 min at 4℃ and 1000 *g* to remove cell debris. Virus titer was determined by transduction of HEK293T cells, followed by staining with an antibody against LNGFR that had been conjugated to phycoerythrin (Miltenyi Biotec, Auburn, CA) and flow cytometry analysis. For transduction, HEL cells were seeded at 5×10^5 in 6-well plates in the Opti-MEM medium (Invitrogen) supplemented with 8 μg/ml of polybrene, transduced by spinoculation (60 min at 900 *g*) at MOI=2 and then incubated for 16 h. Next, cells were washed with Opti-MEM and resuspended in regular RPMI 1640 culture medium with 10% FBS. After 48 h, LNGFR positive cells were purified using the magnetic CD271 (LNGFR) MicroBead kit, MS columns and MiniMACS separator (Miltenyi Biotec) according to manufacturer's instructions.

Supplementary material
Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.139592/-/DC1

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