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ORIGINAL ARTICLE

Syntenin-mediated regulation of Sox4 proteasomal degradation modulates transcriptional output

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The transcription factor Sox4 is aberrantly expressed in many human tumors and can modulate tumorigenesis and metastases of murine tumors *in vivo*. However, mechanisms that control Sox4 function remain poorly defined. It has recently been observed that DNA damage increases Sox4 protein expression independently of Sox4 mRNA levels, suggesting an as yet undefined post-transcriptional mechanism regulating Sox4 expression and functionality. Here, we show that Sox4 protein is rapidly degraded by the proteasome as indicated by pharmacological inhibition with Mg132 and epoxymycin. Sox4 half-life was found to be less than 1 h as evident by inhibition of protein synthesis using cycloheximide. Ectopic expression of Sox4 deletion mutants revealed that the C-terminal 33 residues of Sox4 were critical in modulating its degradation in a polyubiquitin-independent manner. Syntenin, a Sox4 binding partner, associates with this domain and was found to stabilize Sox4 expression. Syntenin-induced stabilization of Sox4 correlated with Sox4-syntenin relocalization to the nucleus, where both proteins accumulate. Syntenin overexpression or knockdown in human tumor cell lines was found to reciprocally modulate Sox4 protein expression and transcriptional activity implicating its role as a regulator of Sox4. Taken together, our data demonstrate that the Sox4 C-terminal domain regulates polyubiquitin-independent proteasomal degradation of Sox4 that can be modulated by interaction with syntenin. As aberrant Sox4 expression has been found associated with many human cancers, modulation of Sox4 proteasomal degradation may impact oncogenesis and metastatic properties of tumors.

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Introduction

Sox4 is a member of the highly conserved group C Sox transcription factors, which consists of two additional members; Sox11 and Sox12 (Bowles *et al*., 2000). The SoxC family members are grouped on the basis of the high degree of sequence similarity in their high mobility group box DNA-binding domain (DBD) that has specificity for A/TA/TCAAAG, and are further distinguished from other Sox transcription factors by their conserved C-terminal transactivation domain (van de Wetering *et al*., 1993; Dy *et al*., 2008). Sox4 is important in a variety of developmental processes including cardiogenesis, neurogenesis, T- and B-cell differentiation, osteoblast development and development of the endocrine pancreas (Schilham *et al*., 1997; Hunt and Clarke, 1999; Wilson *et al*., 2005; Bergsland *et al*., 2006; Nissen-Meyer *et al*., 2007; Dy *et al*., 2008). Sox4-deficient mice die at day E14 due to a circulatory failure caused by malformation of the heart (Schilham *et al*., 1996). In addition, Sox4 appears to have a dominant role in conferring oncogenic and metastatic transformation (Rhodes *et al*., 2004). High levels of Sox4 mRNA expression have been observed in a large number of human tumors including breast, lung, brain, prostate and ovarian cancer, and the Sox4 gene locus has been repeatedly found to be targeted by tumor-promoting retroviral integration in mice (Lund *et al*., 2002; Suzuki *et al*., 2002; Shin *et al*., 2004). Together, these studies support the inclusion of *Sox4* as one of the 64 genes that constitute a general cancer signature (Rhodes *et al*., 2004). Sox4 may thus be considered as a master regulator of cell fate and differentiation in numerous cell types and appears to have a dominant role in the development of neoplasia.

Post-transcriptional mechanisms regulating Sox4 protein expression and transcriptional activity have not yet been described, but may very well be important in tumorigenesis. miRNA-mediated reduction in Sox4 expression has been shown to correlate with reduced breast cancer metastases *in vivo*, demonstrating a relation between Sox4 expression levels and cancer progression (Tavazoie *et al*., 2008). In addition, a recent study described somatic stop mutants of Sox4 in a primary lung tumor, and showed that Sox4 stop mutants associated with transforming ability *in vitro*

and enhanced Sox4 protein levels (Medina *et al*., 2009). Induction of DNA damage has also been found to control Sox4 protein expression independent of its mRNA transcript levels, suggesting post-transcriptional regulation of Sox4 in response to DNA damage (Pan *et al*., 2009). It therefore appears that regulation of Sox4 protein expression levels may be important in the development of cellular transformation and metastases, and has an important role in regulating transcriptional pathways controlling tumorigenesis.

Here, we provide evidence that Sox4 protein has a remarkably short half-life, and that protein expression and transcriptional activity can be regulated through a syntenin-dependent mechanism. We have identified a novel degradation motif in the Sox4 C-terminus that targets the protein for proteasomal degradation in a polyubiquitin-independent fashion. We demonstrate that the Sox4-binding partner syntenin/melanoma differentiation associated gene-9 (*MDA-9*) associates with the Sox4 C-terminal domain and relocalizes to the nucleus, where it stabilizes Sox4 expression and transcriptional output. These data suggest that modulation of Sox4 degradation may contribute to human tumorigenesis, and that therapeutic intervention designed to regulate proteasomal degradation of Sox4 may impact cancer development.

Results

Sox4 expression is regulated by proteasomal degradation

To evaluate whether Sox4 protein expression may be post-transcriptionally regulated, we measured Sox4 mRNA transcript and protein levels in several human tumor cell lines. Sox4 protein was found differentially expressed in HepG2 cells (hepatocellular carcinoma), MCF7 (breast carcinoma), A549 cells (lung basal cell carcinoma) and U2OS cells (osteosarcoma; Figure 1a). In contrast, Sox4 mRNA levels were similar between HepG2, MCF7 and A549 cells, indicating that Sox4 protein expression may be regulated through cell type-specific post-transcriptional mechanisms (Figure 1b). Sox4 mRNA levels in U2OS cells were lower as compared with other cells tested, but were still detected at significant levels (Supplementary Figure S1). To investigate regulation of Sox4 expression under steady state conditions, we incubated HepG2, U2OS and MCF7 cells with cycloheximide (CHX) to inhibit protein synthesis and observed the Sox4 protein levels rapidly decreased, indicating the Sox4 half-life is less than 1 h (Figure 1c). Sox4 protein expression was found dramatically increased upon incubation of all cell lines tested with the peptide aldehyde Mg132 that inhibits the

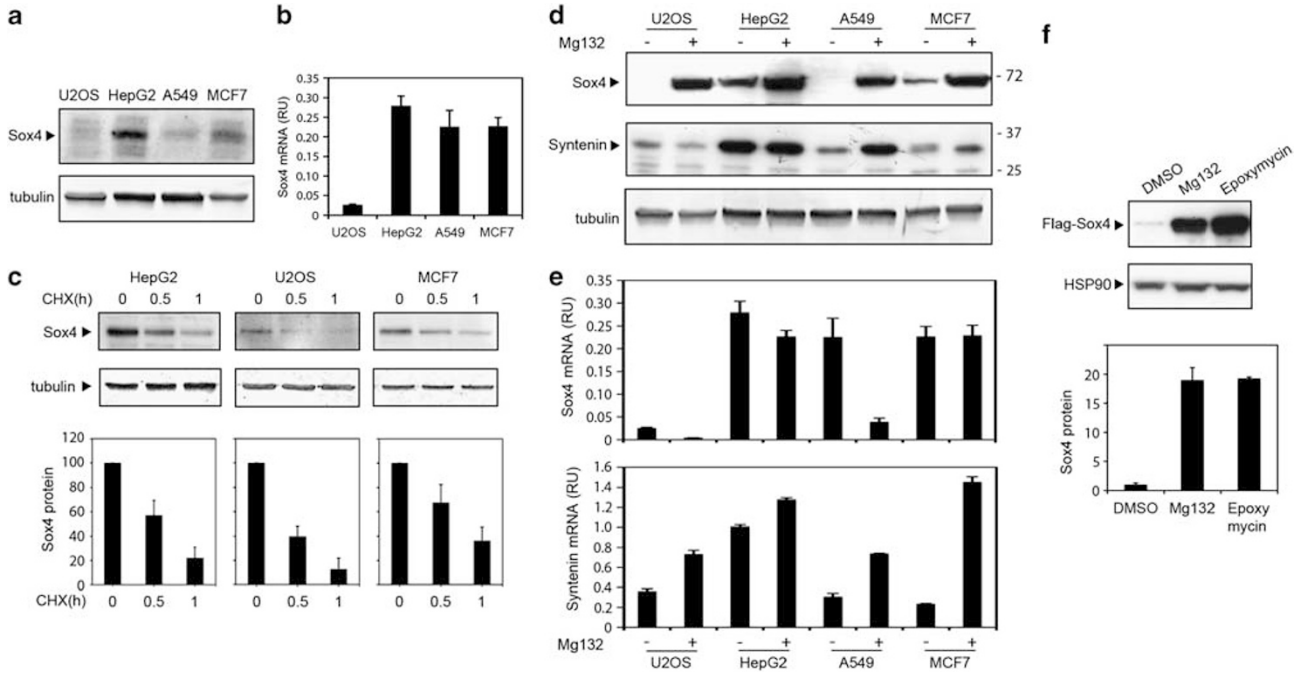


Figure 1 Sox4 protein expression is regulated post-transcritionally. (**a**) Western blots containing 50 μg protein of whole cell lysates (WCL) of U2OS, HepG2, A549 or MCF7 cells were incubated with Sox4 antibody (upper panel) and subsequently incubated with anti-tubulin antibody to demonstrate identical loading. A representative blot of three independent experiments is shown. (**b**) Quantification of Sox4 mRNA levels in multiple cell lines using qRT–PCR. Sox4 levels were normalized to β2m levels per cell line. Data (average ± s.d.) from three independent experiments are indicated. (**c**) HepG2 cells were incubated with cycloheximide (CHX, 0.15 mg/ml) for indicated timepoints and Sox4 protein expression analyzed by western blot (left panel). A representative experiment is shown and quantification of three independent experiments is indicated in the right panel (average ± s.d.). (**d**) Mg132 (16 h, 2 μM) increases Sox4 protein levels at the post-transcriptional level. WCL and RNA were prepared of cells incubated overnight with Mg132. Western blots were analyzed for Sox4 and syntenin expression. (**e**) Sox4 and syntenin mRNA levels were measured by qRT–PCR and expressed relative to β2m. Data from three independent experiments are indicated (average ± s.d.). (**f**) Upper panel, Flag-Sox4 was ectopically expressed in HEK in the presence or absence of Mg132 or epoxymycin (100 nM) and Sox4 protein levels were assessed by western blotting (one representative out of three independent experiments is shown). Lower panel, quantification of Mg132 or epoxymycin-induced Sox4 protein expression in HEK293 cells of three independent experiments (average ± s.d.).

proteasome, whereas mRNA levels remained unchanged or lowered (Figures 1d and e, and Supplementary Figure S1). Mg132-mediated upregulation of Sox4 was also observed in primary mammary epithelial cells and HEK293 cells (Supplementary Figure S1). Overnight incubation of HEK cells ectopically expressing Sox4 with epoxymycin further confirmed proteasomal degradation of Sox4 (Figure 1f). Taken together, these data demonstrate that Sox4 protein expression is regulated at the post-transcriptional level through proteasome-mediated degradation.

The Sox4 C-terminus contains a novel degradation motif

To define the region of Sox4 responsible for proteasomal targeting, we generated Sox4 deletion mutants on the basis of the original domain architecture defined by van de Wetering *et al*. (1993); Figure 2a). Sox4 mutants containing the C-terminal transactivation domain encoded by residues 299–440 were consistently expressed at low levels, but their expression was strongly increased upon Mg132 treatment (Figure 2b). These differences could not be explained by transfection efficiency (Supplementary Figures S2). Expression of the Sox4 299–440 was undetectable when cells were not pre-incubated with Mg132, but proteasomal inhibition resulted in dramatically increased expression. Taken together, these data suggest that Sox4 299–440 contains a functional degradation motif.

To determine whether Mg132-sensitive Sox4 protein expression correlated with subcellular localization, we studied the localization of all Sox4 mutants indicated in Figures 2a. Sox4 is primarily localized in the nucleus,

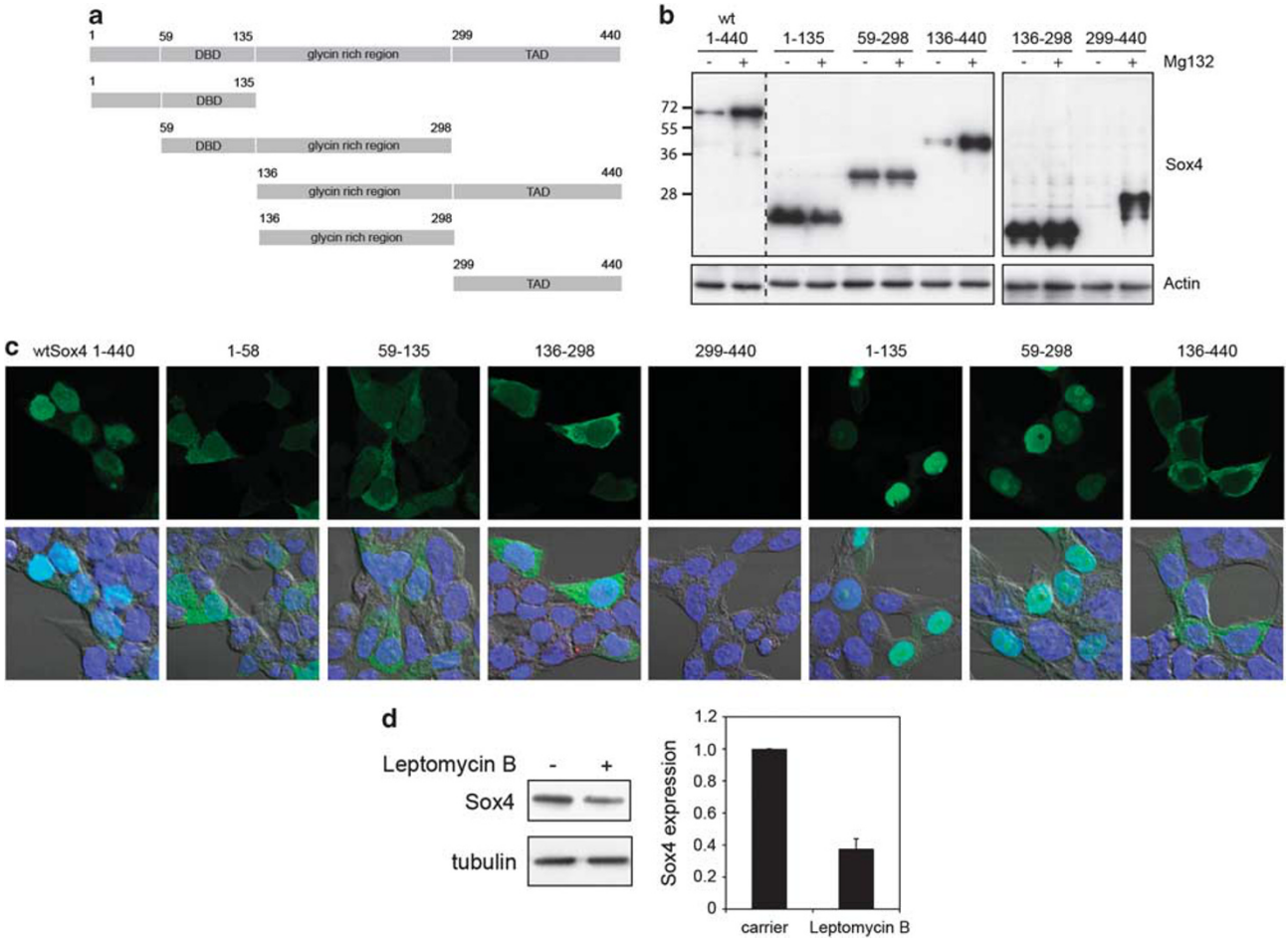


Figure 2 The Sox4 C-terminal domain is rapidly degraded by a proteasomal-dependent mechanism. (**a**) Domain architecture as indicated by van de Wetering *et al*. (1993). The predicted DNA-binding domain (DBD; residues 59–135) is separated by a glycine-rich region from the transcriptional activation domain (residues 299–440). (**b**) Mg132-sensitivity of (mutant) Sox4 protein upon ectopic expression in HEK293 cells and overnight incubation with Mg132. Western blots were prepared and incubated with Flag antibody to determine Sox4 protein expression levels (upper panel), and were reprobed with actin antibody to validate equal loading (lower panel). Samples from the left panel were present on the same membrane. Sox4 136–298 and 299–440 were analyzed on a different membrane. A representative blot out of three independent experiments is shown. (**c**) Subcellular distribution of Sox4 (mutants) in HEK293 cells as indicated by Flag-antibody conjugated to fluorescein isothiocyanate (upper panel). The lower panel shows differential interference contrast images overlaid with confocal images of fluorescein isothiocyanate to indicate Sox4, and 4′,6-diamidino-2-phenylindole to indicate the nucleus. A representative example from three independent experiments is shown. (**d**) HEK293 cells expressing Flag-Sox4 were overnight incubated with leptomycin B that inhibits nuclear export, and western blots were prepared and analyzed for Sox4 and tubulin as loading control. A representative example from two independent experiments is shown. The lower graph shows the quantification of Sox4 levels upon leptomycin B treatment (16 h, 2 ng/ml) of two independent experiments (average ± s.d.).

but a significant cytosolic fraction was also observed (Figures 2c and 5d). We observed that the N-terminal region (residues 1–135 or 59–298 that share the DBD 59–135) regulated Sox4 nuclear localization. Mutants lacking the DBD such as Sox4 136–298 or 136–440 were found accumulated in the cytosol, similar to the individual domains of Sox4. Mg132 did not affect subcellular localization as demonstrated for full-length Sox4, or C-terminal domain containing Sox4 mutants (Supplementary Figure S3). This suggested that proteasomal degradation can occur both in the nucleus (wt Sox4) and the cytoplasm (Sox4 136–440). To further investigate whether Sox4 degradation can occur in the nuclear compartment, HEK293 cells expressing Sox4 were incubated with Leptomycin B to inhibit nuclear export and thereby facilitating nuclear accumulation of Sox4. Indeed, we observed reduced levels of Sox4 upon leptomycin B incubation, suggesting the Sox4 degradation occur predominantly in the nuclear compartment (Figure 2d). Together, these data demonstrate proteasomal degradation of Sox4 is dependent on its C-terminal transactivation domain (residues 299–440) and can occur both in the nucleus and cytoplasm.

The C-terminal 33 residues of Sox4 mediate proteasomal degradation

Proteasomal degradation of proteins is frequently regulated by polyubiquitination of lysine residues (Goldberg, 2003; Kerscher *et al*., 2006). However, the Sox4 299–440 deletion mutant contains no lysine residues, and we have not been able to demonstrate Sox4 polyubiquitination (Supplementary Figure S4). In addition, a Sox4 mutant in which all lysine residues were substituted for arginines (Sox4-K29R) was still degraded by the proteasome and was significantly degraded, albeit somewhat reduced compared with the wild-type Sox4, upon CHX treatment, supporting lysine-independent regulation as dominant mechanism for Sox4 proteasomal degradation (Supplementary Figure S2 and S4). Proline (P), glutamic acid (E), serine (S) and threonine (PEST) domains have been shown to control proteasomal degradation in a ubiquitin-independent manner (Rechsteiner and Rogers, 1996; Asher *et al*., 2006; Belizario *et al*., 2008). These domains are enriched for prolines (P), aspartic acid (D), glutamic acid (E), serines (S) and threonines (T) and act by destabilizing protein termini that are then directly recognized by the 20S proteasome and degraded. *In silica* PEST prediction analysis (PEST Find http://www.at.embnet.org/toolbox/pestfind) predicted multiple PEST-like domains in Sox4, including the C-terminal domain (residues 345–395), where a PEST domain was predicted with strong confidence in a region of Sox4 highly enriched for serines (Figure 3a). On the basis of these predictions, additional Sox4 mutants were generated that would disrupt potential PEST domains,

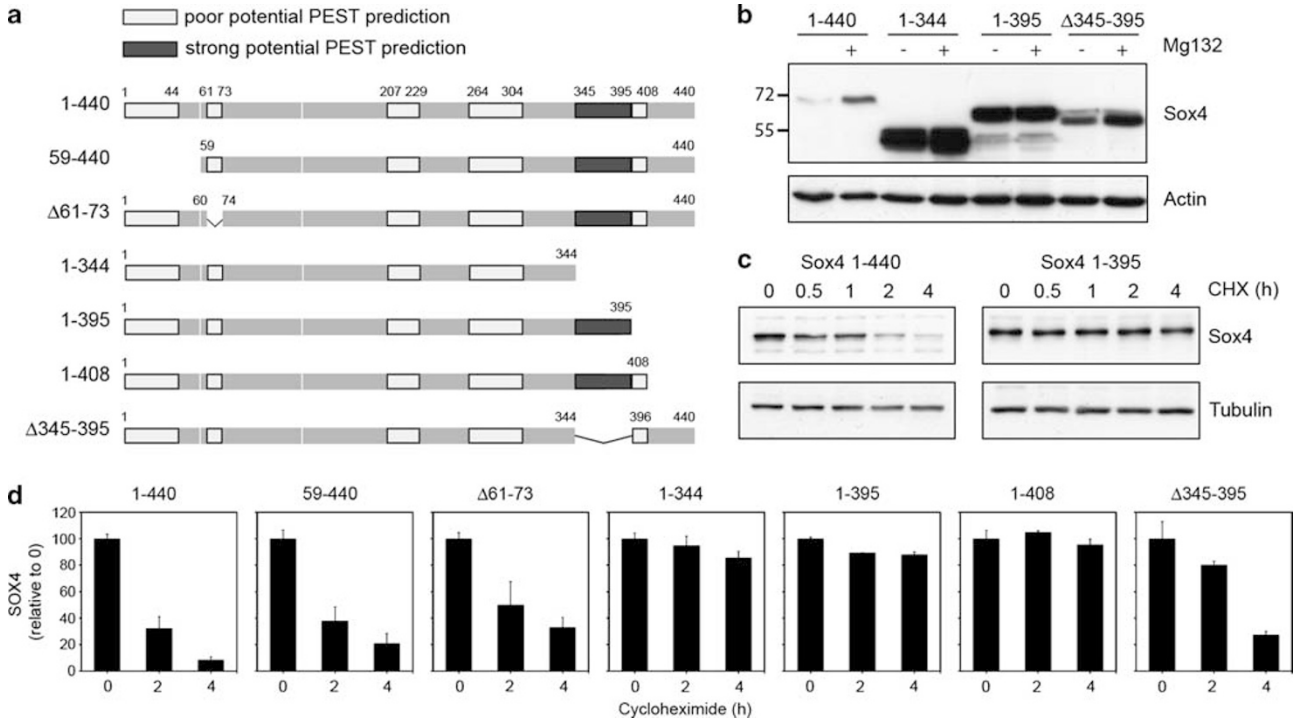


Figure 3 Ubiquitin-independent degradation of Sox4 is primarily modulated by the C-terminal 33 residues of Sox4. (**a**) Prediction of potential PEST sites using PESTfind (http://www.at.embnet.org/toolbox/pestfind). Light grey boxes indicate predictions with poor potential, whereas the dark grey box indicates a PEST site with high potential. (**b**) Sox4 C-terminal domain mutants were expressed in HEK293 cells and incubated overnight with Mg132. Western blots were prepared and probed for Sox4 and actin. A representative example of three independent experiments is shown. (**c**) Sox4 degradation is abrogated in C-terminal Sox4 deletion mutants. HEK293 cells were transfected with wild-type or Sox4 1–395 and incubated with cycloheximide (CHX, 0.15 mg/ml) to prevent *de novo* protein synthesis as indicated. Tubulin was used as loading control. A representative example from three independent experiments is shown. (**d**) Quantification of (mutant) Sox4 turnover upon incubation with CHX from three independent experiments (average ± s.d.).

and Mg132-sensitivity was assessed in HEK293 cells. We found that C-terminal Sox4 mutants lacking the last 46 or 95 amino acids were both highly expressed and insensitive to Mg132 (Figure 3b). Sox4 lacking the predicted C-terminal PEST domain (residues 345–395, Sox4Δ345–395) displayed an intermediate phenotype between wild-type Sox4 and C-terminal mutants. To directly demonstrate the relevance of these regions on Sox4 proteasomal degradation, we transfected cells with Sox4 deletion mutants lacking predicted PEST domains, as well as mutants lacking the N- and C-termini, and incubated cells with CHX to inhibit protein synthesis. Expression of Sox4 C-terminal deletion mutants were dramatically increased upon CHX treatment compared with wild-type Sox4, and the Sox4 degradation domain could be further localized to the last 33 amino acids (Figures 3c and d). Sox4 stability was unaffected by deletion of the N-terminus (1–58), whereas deletion of Sox4 345–395 (C-terminal PEST domain) or the N-terminal-predicted PEST domain (61–73) only modestly affected Sox4 stability (Figure 3d). Rapid degradation of the Sox4Δ345–395 mutant further validates the observation that it is the C-terminal 45 amino acids that are sufficient to drive degradation, as Sox4 1–344 expression is stable. These data demonstrated that the primary domain regulating rapid Sox4 proteasomal degradation resides within the last 33 C-terminal amino acids.

Syntenin regulates Sox4 protein expression by association with the Sox4 C-terminal domain

We have previously reported that the adapter protein syntenin, also known as MDA-9, associates with Sox4 (Lin *et al*., 1998; Geijsen *et al*., 2001). Endogenous syntenin and Sox4 expression levels appear to correlate to some degree, particularly upon Mg132 treatment (Figure 1d). In contrast to Sox4, Mg132-increased syntenin protein levels were generally associated with increased mRNA levels (Figure 1e). To evaluate whether syntenin may regulate Sox4 protein expression, we ectopically co-expressed both proteins in HEK293 cells and observed increased levels of Sox4 (Figures 4a and c). Expression of Sox4 deletion mutants that lacked the C-terminal domain were not stabilized by syntenin, but addition of the last 45 amino acids to the Sox4 1–344 (Sox4Δ345–395) again rendered syntenin-dependent expression (Figures 4b and c). Expression of Sox4 299–440 was also enhanced by co-expression of syntenin. We have previously reported that the N-terminal domain of syntenin associates with Sox4 (Geijsen *et al*., 2001), and therefore investigated the syntenin-binding region within Sox4 by co-immunoprecipitation analysis. Sox4 and syntenin were found to co-immunoprecipitate, and both the Sox4 DBD (residues 59–135) and the C-terminal residues 299–440 were found to mediate association with syntenin (Figure 4d). We were unable to detect expression of Sox4 1–58 by western blot due to

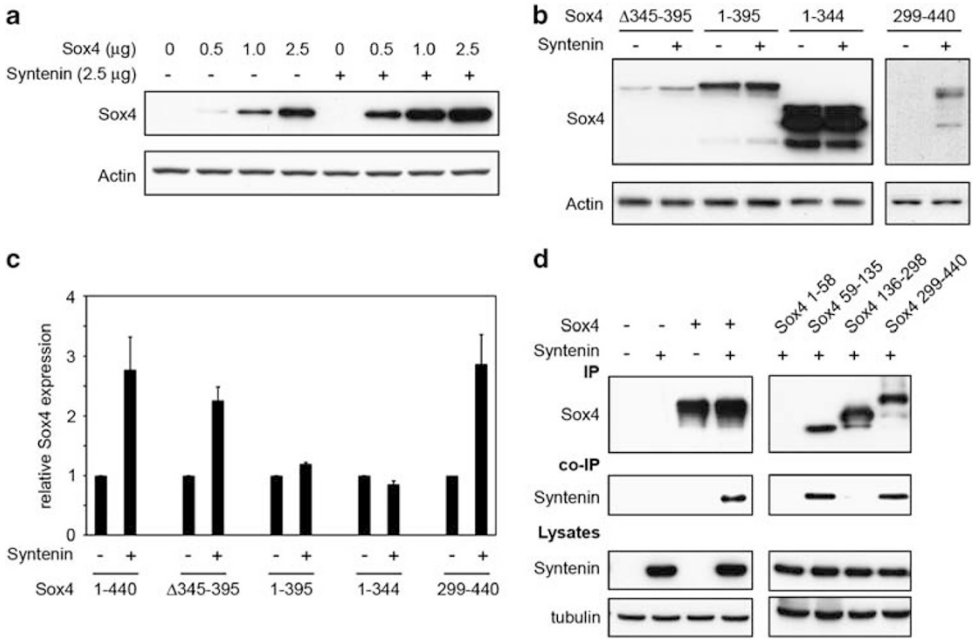


Figure 4 Syntenin increases Sox4 expression by binding to the Sox4 C-terminus. (**a**) Syntenin was co-expressed with increasing amounts of Sox4 in HEK293 cells and Sox4 levels analyzed by western blotting. Actin was used as loading control. A representative example from three independent experiments is shown. (**b**) Syntenin was co-expressed with Sox4 mutants for 48 h in HEK293 cells as indicated. Western blots were analyzed for Sox4 protein expression, and for actin to validate loading. A representative example from three independent experiments is shown. (**c**) Quantification of syntenin-mediated upregulation of Sox4 and deletion mutants from three independent experiments (average ± s.d.). (**d**) Co-immunoprecipitation of syntenin with Sox4 mutants. Flag-Sox4 was immunoprecipitated (IP) from lysates of HEK293 cells expressing (mutant) Sox4 and co-immunoprecipitation (co-IP) of HA-tagged syntenin was analyzed by western blot. Whole cell lysates (lysates) were analyzed for syntenin expression and tubulin was analyzed to validate equal loading. A representative example from three independent experiments is shown.

its small size, but observed the expression by immunofluorescence (Figure 2c). These data demonstrate that syntenin association with the Sox4 C-terminus is a critical determinant for its capacity to enhance Sox4 protein expression level.

Nuclear relocalization of syntenin by Sox4 associates with Sox4 accumulation

To better understand the mechanisms by which syntenin can stabilize Sox4 expression, we analyzed their subcellular distribution by confocal microscopy. Enhanced green fluorescent protein (EGFP)-tagged Sox4 was predominantly localized in the nucleus with a minor cytosolic component (Figure 5a). Syntenin fused to the red fluorescent protein mKATE2 was mostly localized in the cytosol. Upon co-expression of these proteins, both proteins accumulated in the nucleus as indicated by co-staining (Figure 5b). EGFP co-expressed with mKATE2-syntenin did not induce nuclear relocalization, indicating Sox4 as dominant factor in relocating mKATE2-syntenin (Figure 5c). These results were confirmed by analysis of Flag-tagged Sox4 and hemagglutinin (HA)-tagged syntenin expression (Figure 5d). Expression of Sox4 mutants not localizing to the nucleus failed to induce nuclear relocalization of syntenin, suggesting that Sox4 is the dominant factor in modulating subcellular localization (Figure 5e). These results were confirmed when nuclear and cytosolar fractions were analyzed upon co-expression of Sox4 and syntenin (Supplementary Figure S5) Together, these data suggest that interactions between Sox4 and syntenin result in nuclear relocalization of syntenin, which can subsequently prevent Sox4 degradation.

Sox4 protein expression and transcriptional activity is modulated by syntenin

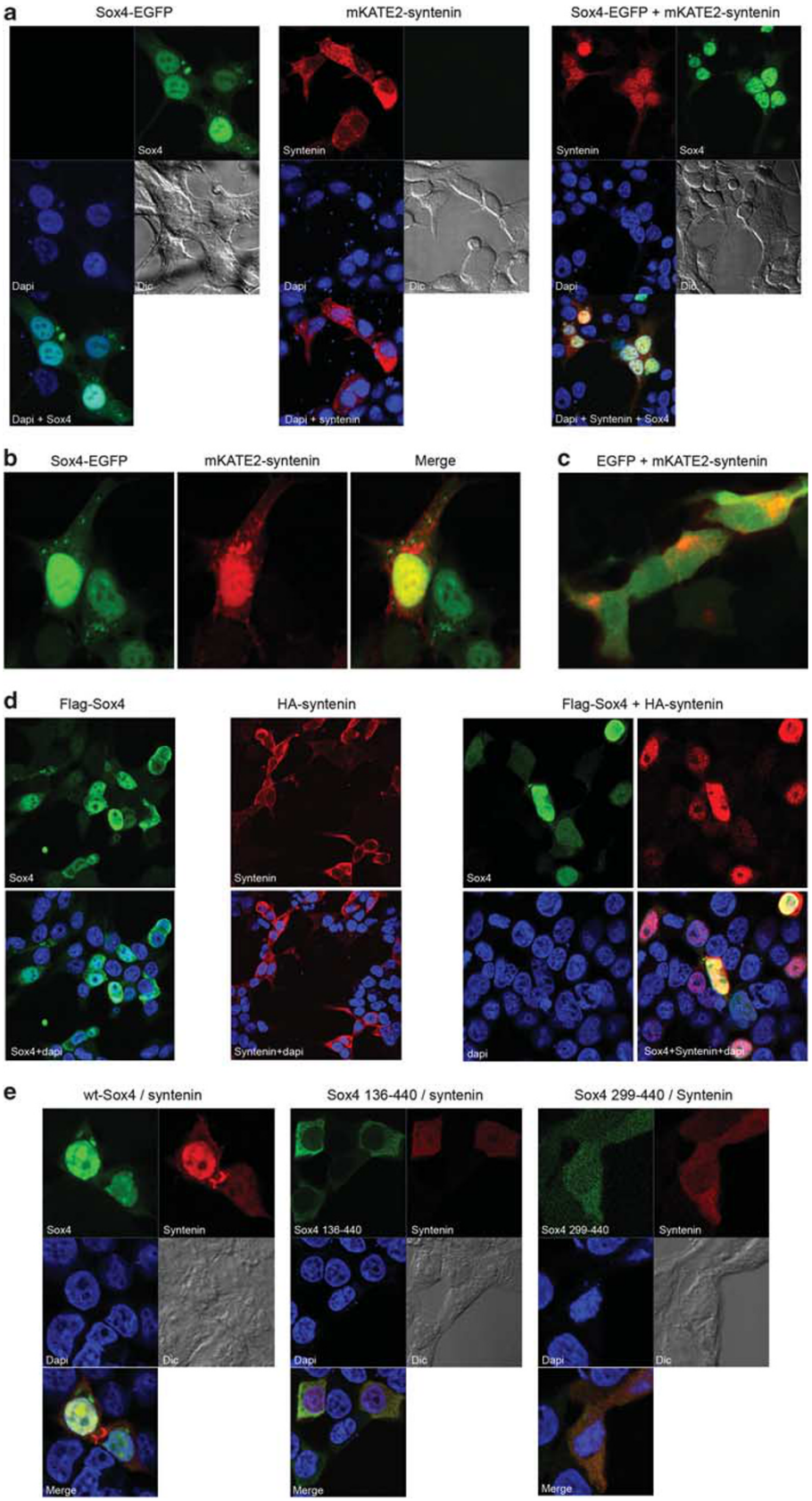
To further confirm that syntenin regulates endogenous Sox4 protein expression and function, we utilized two independent small inhibitory RNA to reduce syntenin expression level. As HepG2 cells have both high levels of syntenin and Sox4, we analyzed both Sox4 mRNA and protein levels upon syntenin knockdown in these cells. Reduced syntenin expression resulted in a 50% reduction in Sox4 protein expression, whereas mRNA levels for Sox4 remained unaffected (Figure 6a and Supplementary Figure S6). To study the capacity of syntenin to modulate endogenous Sox4 transcriptional activity, we utilized a luciferase reporter construct containing a Sox4 responsive promoter (Geijsen *et al*., 2001). We observed high promoter activity in HEK293 cells upon co-transfection of the reporter construct with wild-type Sox4, demonstrating Sox4 specificity (Figure 6b). Sox4 specificity was further confirmed by overexpression of mutant Sox4 1–135 or Sox4 59–298 that abrogate Sox4-induced reporter activity, indicating that both Sox4 1–135 and Sox4 59–298 can act as dominant-negative mutants. We next assessed the capacity of syntenin to enhance endogenous Sox4 transcriptional activity in cells with low Sox4 and syntenin levels (U2OS cells) or high Sox4 and syntenin-expressing cells (HepG2). In both cell types, we observed that ectopic syntenin expression increased Sox4 transcriptional activity as indicated by increased reporter activity that could be inhibited by overexpression of Sox4 59–298 (Figure 6c). Importantly, syntenin knockdown was found to reduce Sox4 transcriptional activity in both U2OS and HepG2 cells further establishing that syntenin can directly modulate Sox4 transcriptional activity. Taken together, these data demonstrate that syntenin can regulate Sox4 transcriptional output by stabilizing its expression through prevention of proteasomal degradation.

Discussion

The Sox4 transcription factor has an important role in regulating cellular differentiation, proliferation and oncogenesis; however, post-transcriptional mechanisms regulating the Sox4 protein functionality remain undefined. Here, we have established that a novel C-terminal degradation motif within Sox4 mediates rapid proteasomal degradation. Our data reveals that Sox4 protein half-life is <1 h, and that syntenin, a C-terminal interacting protein, can stabilize the Sox4 protein expression, thereby enhancing its transcriptional output. These data suggest that syntenin and Sox4, both independently implicated in modulating tumorigenesis, may cooperate in regulating cellular transformation and metastasis.

We found the C-terminal domain, which is devoid of lysine residues, can modulate the Sox4 protein expression by targeting the proteasome and were not able to demonstrate the Sox4 polyubiquitination. Sox4 proteasomal degradation appeared most prominent in the nucleus, but also cytosol-localized Sox4 mutants were sensitive to proteasomal degradation. Both nuclear and cytosolic proteasomal activity has been shown critical for regulation of multiple transcription factors by ubiquitin- and non-ubiquitin-dependent mechanisms (Kwak *et al*., 2010). Proteasome subunits diffuse throughout the cell during mitoses and the proportion of nuclear proteasomes are correlated to cellular proliferation rates, suggesting that Sox4 degradation and function may be regulated during various stages of the cell cycle (Reits *et al*., 1997; Kruger *et al*., 2001). In contrast, nuclear shuttling of Sox4, as well as nuclear import of the proteasomal subunits itself may regulate Sox4 degradation and function in a time and tissue-specific manner (Ziegler and Ghosh, 2005; Fietta and Delsante, 2010; Malki *et al*., 2010).

As the Sox4 C-terminus is highly conserved between Sox11 and Sox12, and fully conserved between human and mouse (Supplementary Figure S7), it is likely that other SoxC family members are regulated by mechanisms similar to those identified here for Sox4. It has been described that SoxC family members have overlapping expression patterns and molecular properties (Dy *et al*., 2008). Sox4 and Sox11 have both been implicated in cardiac and neuronal development, whereas Sox12 appears redundant (Bergsland *et al*., 2006; Hoser *et al*., 2008; Bhattaram *et al*., 2010; Potzner *et al*., 2010). As C-terminal domains of other Sox family members diverge from SoxC family members, protea-


a
Sox4-EGFP
mKATE2-syntenin
Sox4-EGFP + mKATE2-syntenin
Sox4
Syntenin
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Dapi + Syntenin + Sox4
b
Sox4-EGFP
mKATE2-syntenin
Merge
c
EGFP + mKATE2-syntenin
d
Flag-Sox4
HA-syntenin
Flag-Sox4 + HA-syntenin
Sox4+dapi
Syntenin+dapi
dapi
Sox4+Syntenin+dapi
e
wt-Sox4 / syntenin
Sox4 136-440 / syntenin
Sox4 299-440 / Syntenin
Sox4 136-440
Sox4 299-440
Merge

somal regulation of Sox proteins appears confined to the SoxC family. Thus far, however, evidence is lacking that either Sox11 or Sox12 is functionally regulated by the proteasome as we show here for Sox4.

Models have been proposed in which intrinsic unstructured features at protein termini can lead to protein degradation by the 20S proteasome in a polyubiquitin-independent fashion that can further be regulated by post-transcriptional mechanisms such as protein–protein interactions and phosphorylation (Prakash *et al*., 2004; Dyson and Wright, 2005; Asher *et al*., 2006). Early efforts to predict unstructured regions that are degraded by the proteasome from primary sequence information suggested PEST-rich regions to be important (Rechsteiner and Rogers, 1996; Asher *et al*., 2006), residues, also enriched within the Sox4 C-terminal domain. Secondary structure prediction indicated that apart from the DBD that contained helical structures, the remaining part of Sox4 is likely to be disordered (www.predictprotein.org). Short-lived proteins with recognized unstructured regions include p53, p73, p21, c-Fos and IκBα, and their turnover is significantly enhanced when compared with their turnover in multimerized protein complexes (Krappmann *et al*., 1996; Dyson and Wright, 2005; Asher and Shaul, 2006; Asher *et al*., 2006; Basbous *et al*., 2008). Our data are consistent with a model in which the Sox4 C-terminus is intrinsically unstructured and thereby targeted for proteasomal degradation. Upon multimerization of Sox4 into larger protein complexes through association with syntenin (or other binding partners), Sox4 degradation is abrogated by adopting a structurized conformation or masking its degradation motif, leading to enhanced Sox4 transcriptional activity.

Syntenin/MDA-9 is an adapter protein that consists of an N-terminal domain, followed by a tandem PDZ module and a small C-terminal domain (Beekman and Coffer, 2008; Sarkar *et al*., 2004, 2008). We have previously shown that interleukin-5 signaling activates Sox4 transcriptional activity by syntenin associating with the IL5Rα-chain, and further demonstrated syntenin to modulate interleukin-5-induced eosinophil differentiation of hematopoietic stem cells (Geijsen *et al*., 2001; Beekman *et al*., 2009). In the current study, we have shown that syntenin can directly stabilize Sox4 expression in a variety of (interleukin-5R negative) tumor cell lines. These data supports a much broader role for syntenin in regulating Sox-mediated transcriptional programs. Nuclear enrichment of syntenin, binding with its N-terminus to Sox4, would allow the selective recruitment of PDZ-domain interacting proteins that could modulate the transcriptional output of Sox4. Indeed, Sox4 but not Sox17 has been found to stimulate β-catenin/T cell factor (TCF) signaling, a transcriptional complex involved in colon cancer (Sinner *et al*., 2007). Other Sox4 C-terminal interacting proteins such as p53 may act in concert or compete with syntenin in regulating Sox4 protein stability and function (Pan *et al*., 2009). DNA damage has been described to stabilize Sox4 protein expression that is then critical for stabilization of the tumor suppressor p53 by a post-transcriptional mechanism. The molecular mechanisms by which Sox4 is stabilized by DNA damage are undefined, but may involve rescue of Sox4 from proteasomal degradation by syntenin or other binding proteins.

Syntenin was originally identified as *MDA-9* (Lin *et al*., 1998). Since then, it has been shown to regulate the metastatic phenotype of human melanoma cells through interactions with c-src, protein kinase C alpha and activation of nuclear factor-kB and p38 signaling pathways (Boukerche *et al*., 2008, 2010; Sarkar *et al*., 2008). Syntenin also bind and regulate syndecans as co-receptors for various growth factors and matrix components, thereby controlling cellular proliferation, differentiation, adhesion and migration (Grootjans *et al*., 1997; Lambaerts *et al*., 2009). The adapter molecule syntenin may provide crosstalk between these pathways and Sox4 that may be important for regulating the tumorigenic properties of transformed cells.

Sox4 gain-of-function or loss-of-function mutants may both contribute to oncogenesis, depending on tissue- and tumor-specific conditions (Penzo-Mendez, 2010). Recently, an acquired stop mutant in the C-terminal serine-rich region of Sox4 (S395X) was found to be associated with a primary lung tumor (Medina *et al*., 2009). Interestingly, this Sox4 mutant demonstrated enhanced protein expression and, although it was not transcriptionally active, its ectopic expression modulated *in vitro* transformation of NIH-3T3 cells expressing the weakly oncogenic RhoA-Q63L mutant. As this mutant lacks the C-terminal degradation domain identified in this study, the observed enhanced expression level of Sox4 S395X likely resulted from impaired proteasomal degradation. As demonstrated by our data, stop mutants of Sox4 may be expressed at dramatically increased levels compared with the wild-type Sox4 and may promote oncogenesis by dysregulation of transcriptional networks. Stop mutants of Sox4 may also sequester Sox4-interacting proteins such as syntenin or p53, thereby modulating their functions.

The remarkable short half-life of Sox4 implies its functional activity can be rapidly regulated by a post-transcriptional mechanism that stabilizes its expression. Our findings clearly show that profiling of Sox4

Figure 5 Nuclear accumulation of Sox4 and syntenin. (**a**) Sox4-EGFP tagged and mKATE2-syntenin were independently (left panel Sox4, middle panel syntenin) or co-expressed (right panel) in HEK293 cells, fixed and imaged by confocal microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue), and differential interference contrast images are indicated in the right bottom corner. Representative examples from three independent experiments are shown. (**b**) Magnification of Sox4-EGFP mKATE2-syntenin expressing Hek293 cells. (**c**) Expression of mKATE2-syntenin with EGFP tag alone ($n=3$). (**d**) Immunofluorescent staining of HA-syntenin and Flag-Sox4 upon expression in Hek293 cells. Three independent experiments were performed and one representative example is shown. (**e**) Co-expression of HA-syntenin with Flag-Sox4 mutants as indicated. Representative examples from three independent experiments are shown.

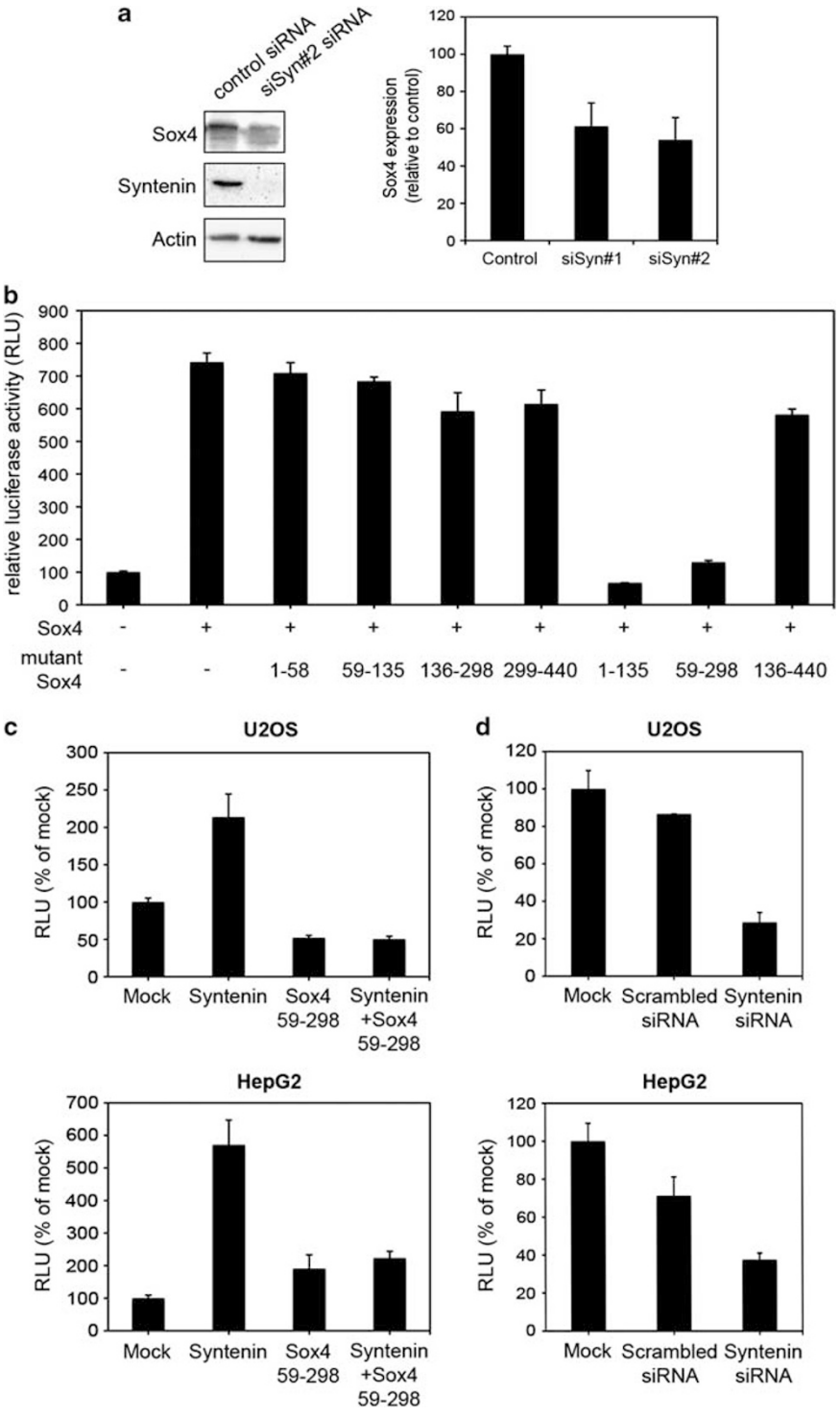

Figure 6 Regulation of endogenous Sox4 expression and transcriptional activity by syntenin. (**a**) HepG2 cells were transfected with control siRNA or a syntenin-targeting siRNA and western blots were prepared to assess Sox4 and syntenin levels (left panel). The right panel shows the quantification of Sox4 levels upon knockdown with two syntenin-targeting siRNA (siSyn#1 and siSyn#2) from three independent experiments (average ± s.d.). (**b**) HEK293 cells were transfected with limited amounts of wild-type Sox4 (0.1 μg) and 1 μg of mutant Sox4 construct. Sox4 transcriptional activity was assessed by a Sox4 luciferase reporter (Geijsen *et al*., 2001). A representative experiment (average ± s.d.) from three independent experiments is shown. (**c**) Sox4 transcriptional activity was assessed in U2OS cells (upper panel) and HepG2 cells (lower panel). Cells were transfected with syntenin with or without dominant-negative Sox4 (Sox4 59–298) and a Sox4 luciferase reporter construct. A representative example (average ± s.d.) from three independent experiments is shown. (**d**) Endogenous Sox4 transcriptional activity was assessed upon transfection of a Sox4 luciferase reporter plasmid in combination with syntenin or control knockdown in U2OS cells (upper panel) or HepG2 cells (lower panel). A representative experiment (average ± s.d.) from three independent experiments is presented.

mRNA expression levels is insufficient to draw any conclusions concerning the Sox4 protein expression levels and function. Here, we demonstrated that the Sox4 C-terminus mediates its rapid turnover, and that interacting proteins such as syntenin regulate Sox4 expression and function by binding this motif. These data may be exploited to design cancer therapeutics to manipulate Sox4 function by regulating its degradation by the proteasome.

Materials and methods

Antibodies, DNA constructs and reagents
The following antibodies were used: mouse anti-Flag (M2) (unconjugated, fluorescein isothiocyanate or horseradish peroxidase labeled), mouse anti-HA tag (12CA5), mouse anti β-tubulin, rabbit polyclonal serum against Sox4 were from Sigma Aldrich (Zwijndrecht, The Netherlands) and Diagenode (Liege, Belgium), rabbit anti-HA-TRITC, goat anti-actin and rabbit anti-syntenin were from Santa Cruz Biotechnology (Palo Alto, CA, USA). Blocking serum and conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA). Sox4 constructs were cloned and N-terminally tagged with Flag sequence by PCR using 5′-*Bam*HI and 3′-*Xho*I restriction sites, and ligated into pcDNA3. Deletion mutants were generated by standard quickchange PCR protocols. Sox4-EGFP-tagged fusion protein was constructed by ligating *Eco*RV-Sox4-*Cla*I and *BspE*I-EGFP-*Xba*I in pcDNA3 constructs described by Beekman *et al*. (2009). mKATE2 (kindly provided by Dr Chudakov, Shemiakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia) was ligated in *Not*I-*Cla*I restricted YFP2-syntenin (Beekman *et al*., 2009). All novel constructs were sequence verified. The optimal Sox4 luciferase reporter construct was kindly provided by Dr H Clevers (Hubrecht Institute, Utrecht, The Netherlands). Syntenin constructs have been described (Geijsen *et al*., 2001). Poly-L-lysine, Mg132, epoxymycin, CHX and leptomycin B were from Sigma Aldrich. Polyethylimine was purchased from Polysciences (Eppelheim). Syntenin-targeting small inhibitory RNA were previously described (Beekman *et al*., 2009).

Cell lines and transfection
HepG2, A549, MCF7 and Hek293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 8% heat-inactivated fetal calf serum, penicillin and streptomycin at 37 °C and 5% CO_2 as described (Beekman *et al*., 2009). Human mammary epithelial cells were cultured as described (Elenbaas *et al*., 2001). Cells were passed 1–7 surface area, and transfected the next day with a mixture of DNA/polyethylimine complexes as indicated, washed and analyzed 48 h post transfection. SiRNA (20 nM) was transfected using RNAi-max (Invitrogen) according to the manufacturer's instructions.

Western blots
Whole cell lysates were prepared and protein concentration was determined as previously described (Beekman *et al*., 2009). Briefly, samples were analyzed by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Milipore, Bedford, MA, USA). The membranes were blocked with 5% milk protein in Tris-buffered saline containing 0.3% Tween-20, and probed with antibodies as indicated. Blots of co-immunoprecipitation experiments were analyzed using HA and Flag antibodies conjugated directly to horseradish peroxidase. The membranes were washed, and developed by enhanced chemiluminescence (ECL) and exposure to Kodak XB films (Rochester, NY, USA). For quantification of protein levels, the integrated density values were determined and corrected for background using ImageJ software (http://rsb.info.nih.gov.ij/). Student's *t*-test was applied for statistics.

Quantitative reverse transcriptase PCR
RNA isolation, cDNA synthesis and quantitative PCR was performed as described (van Loosdregt *et al*., 2010). Forward Sox4 primer consisted of 5′-ggcctcgagctgggaatcgc-3′, reverse Sox4: 5′-gcccactcggggtcttgcac-3′; forward syntenin: 5′-gcggcga gcggttccttgt-3′, reverse syntenin: 5′-tgctggattggcagggtttgcag-3′, β-2-microglobulin primers have been described (van Loosdregt *et al*., 2010).

Immunofluorescence and confocal microscopy
HEK293 cells were cultured on poly-L-lysine-coated microscope glasses (Thermo Scientific, Waltham, MA, USA) and transfected with Sox4-EGFP, mKate2-syntenin or mKate2-syntenin together with Sox4-EGFP. After 48 h, slides were washed, cells were fixed in phosphate-buffered saline containing 3% paraformaldehyde, and mounted in Mowiol (Calbiochem, Darmstadt, Germany) containing 3% DABCO (1,4-diazabicyclo[2.2.2] octane) and 4′,6-diamidino-2-phenylindole Sanofi-Aventis, Paris, France). Immunofluorescent staining of HA and Flag was performed as described (Beekman *et al*., 2004). Briefly, cells were paraformaldehyde-fixed, permeabilized in phosphate-buffered saline containing 0.25% saponin and 5% mouse serum and goat serum, and stained by mouse anti-Flag-fluorescein isothiocyanate and rabbit anti-HA-TRITC, followed by goat anti rabbit-DyLight555-conjugated (serum and secondaries from Jackson Immunoresearch). Cells were examined with the Carl Zeiss confocal microscope LSM 710 (Oberkochen, Germany) with a 63× objective.

Co-immunoprecipitation
HEK293 cells were transfected by polyethylimine–DNA complexes in poly-L-lysine-coated 70-cm^2 culture dishes. Two days later, cells were resuspended in phosphate-buffered saline and lysed in 1-ml radioimmunoprecipitation assay buffer. The lysates were spun down and incubated with 10 µl 50% slurry Flag-antibody-coated agarose beads (Sigma Aldrich) for 2 h. The beads were washed three times for 3 min and boiled for 5 min in reducing sample buffer before western Blot analysis.

Luciferase assays
Hek293, HepG2 and U2OS cells were grown on six-well plates and transfected with Sox constructs together with an optimal luciferase reporter construct that contains seven Sox4-binding sites (AACAAAG). In each sample, pRLTK renilla (Promega, Leiden, The Netherlands) was co-transfected to normalize for transfection efficiency. Two days after transfection, cells were washed twice with phosphate-buffered saline and lysed in 50 µl passive lysis buffer (Promega) for 15 min according to the manufacturer's instructions. The insoluble cell debris was spun down and the supernatant fraction was assayed for luciferase activity, using a dual-luciferase reporter assay system (Promega), and for protein expression by western blot.

Conflict of interest

The authors declare no conflicts of interest.

References

Asher G, Reuven N, Shaul Y. (2006). 20S proteasomes and protein degradation "by default". *Bioessays* **28**: 844–849.

Asher G, Shaul Y. (2006). Ubiquitin-independent degradation: lessons from the p53 model. *Isr Med Assoc J* **8**: 229–232.

Basbous J, Jariel-Encontre I, Gomard T, Bossis G, Piechaczyk M. (2008). Ubiquitin-independent- versus ubiquitin-dependent proteasomal degradation of the c-Fos and Fra-1 transcription factors: is there a unique answer? *Biochimie* **90**: 296–305.

Beekman JM, Bakema JE, van de Winkel JGJ, Leusen JHW. (2004). Direct interaction between FcgammaRI (CD64) and periplakin controls receptor endocytosis and ligand binding capacity. *Proc Natl Acad Sci USA* **101**: 10392–10397.

Beekman JM, Coffer PJ. (2008). The ins and outs of syntenin, a multifunctional intracellular adaptor protein. *J Cell Sci* **121**: 1349–1355.

Beekman JM, Verhagen LP, Geijsen N, Coffer PJ. (2009). Regulation of myelopoiesis through syntenin-mediated modulation of IL-5 receptor output. *Blood* **114**: 3917–3927.

Belizario JE, Alves J, Garay-Malpartida M, Occhiucci JM. (2008). Coupling caspase cleavage and proteasomal degradation of proteins carrying PEST motif. *Curr Protein Pept Sci* **9**: 210–220.

Bergsland M, Werme M, Malewicz M, Perlmann T, Muhr J. (2006). The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev* **20**: 3475–3486.

Bhattaram P, Penzo-Mendez A, Sock E, Colmenares C, Kaneko KJ, Vassilev A. (2010). Organogenesis relies on SoxC transcription factors for the survival of neural and mesenchymal progenitors. *Nat Commun* **1**: 9.

Boukerche H, Aissaoui H, Prévost C, Hirbec H, Das SK, Su ZZ. (2010). Mda-9/Syntenin promotes metastasis in human melanoma cells by activating c-Src. *Oncogene* **29**: 3054–3066.

Boukerche H, Su ZZ, Prévot C, Sarkar D, Fisher PB. (2008). Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-kappaB. *Proc Natl Acad Sci USA* **105**: 15914–15919.

Bowles J, Schepers G, Koopman P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* **227**: 239–255.

Dy P, Penzo-Mendez A, Wang H, Pedraza CE, Macklin WB, Lefebvre V. (2008). The three SoxC proteins—Sox4, Sox11 and Sox12—exhibit overlapping expression patterns and molecular properties. *Nucleic Acids Res* **36**: 3101–3117.

Dyson HJ, Wright PE. (2005). Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol* **6**: 197–208.

Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev* **15**: 50–65.

Fietta P, Delsante G. (2010). Proteasomes and immunoproteasomes. *Riv Biol* **103**: 29–50.

Geijsen N, Uings IJ, Pals C, Armstrong J, McKinnon M, Raaijmakers JA. (2001). Cytokine-specific transcriptional regulation through an IL-5Ralpha interacting protein. *Science* **293**: 1136–1138.

Goldberg AL. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* **426**: 895–899.

Grootjans JJ, Zimmermann P, Reekmans G, Smets A, Degeest G, Dürr J. (1997). Syntenin, a PDZ protein that binds syndecan cytoplasmic domains. *Proc Natl Acad Sci USA* **94**: 13683–13688.

Hoser M, Potzner MR, Koch JM, Bosl MR, Wegner M, Sock E. (2008). Sox12 deletion in the mouse reveals nonreciprocal redundancy with the related Sox4 and Sox11 transcription factors. *Mol Cell Biol* **28**: 4675–4687.

Hunt SM, Clarke CL. (1999). Expression and hormonal regulation of the Sox4 gene in mouse female reproductive tissues. *Biol Reprod* **61**: 476–481.

Kerscher O, Felberbaum R, Hochstrasser M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**: 159–180.

Krappmann D, Wulczyn FG, Scheidereit C. (1996). Different mechanisms control signal-induced degradation and basal turnover of the NF-kappaB inhibitor IkappaB alpha *in vivo*. *EMBO J* **15**: 6716–6726.

Kruger E, Kloetzel PM, Enenkel C. (2001). 20S proteasome biogenesis. *Biochimie* **83**: 289–293.

Kwak J, Workman JL, Lee D. (2010). The proteasome and its regulatory roles in gene expression. *Biochim Biophys Acta* **1809**: 88–96.

Lambaerts K, Wilcox-Adelman SA, Zimmermann P. (2009). The signaling mechanisms of syndecan heparan sulfate proteoglycans. *Curr Opin Cell Biol* **21**: 662–669.

Lin JJ, Jiang H, Fisher PB. (1998). Melanoma differentiation associated gene-9, mda-9, is a human gamma interferon responsive gene. *Gene* **207**: 105–110.

Lund AH, Turner G, Trubetskoy A, Verhoeven E, Wientjens E, Hulsman D. (2002). Genome-wide retroviral insertional tagging of genes involved in cancer in Cdkn2a-deficient mice. *Nat Genet* **32**: 160–165.

Malki S, Boizet-Bonhoure B, Poulat F. (2010). Shuttling of SOX proteins. *Int J Biochem Cell Biol* **42**: 411–416.

Medina PP, Castillo SD, Blanco S, Sanz-Garcia M, Largo C, Alvarez S. (2009). The SRY-HMG box gene, SOX4, is a target of gene amplification at chromosome 6p in lung cancer. *Hum Mol Genet* **18**: 1343–1352.

Nissen-Meyer LS, Jemtland R, Gautvik VT, Pedersen ME, Paro R, Fortunati D. (2007). Osteopenia, decreased bone formation and impaired osteoblast development in Sox4 heterozygous mice. *J Cell Sci* **120**: 2785–2795.

Pan X, Zhao J, Zhang WN, Li HY, Mu R, Zhou T. (2009). Induction of SOX4 by DNA damage is critical for p53 stabilization and function. *Proc Natl Acad Sci USA* **106**: 3788–3793.

Penzo-Mendez AI. (2010). Critical roles for SoxC transcription factors in development and cancer. *Int J Biochem Cell Biol* **42**: 425–428.

Potzner MR, Tsarovina K, Binder E, Penzo-Mendez A, Lefebvre V, Rohrer H. (2010). Sequential requirement of Sox4 and Sox11 during development of the sympathetic nervous system. *Development* **137**: 775–784.

Prakash S, Tian L, Ratliff KS, Lehotzky RE, Matouschek A. (2004). An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat Struct Mol Biol* **11**: 830–837.

Rechsteiner M, Rogers SW. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem Sci* **21**: 267–271.

Reits EA, Benham AM, Plougastel B, Neefjes J, Trowsdale J. (1997). Dynamics of proteasome distribution in living cells. *EMBO J* **16**: 6087–6094.

Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D. (2004). Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. *Proc Natl Acad Sci USA* **101**: 9309–9314.

Sarkar D, Boukerche H, Su ZZ, Fisher PB. (2004). Mda-9/syntenin: recent insights into a novel cell signaling and metastasis-associated gene. *Pharmacol Ther* **104**: 101–115.

Sarkar D, Boukerche H, Su ZZ, Fisher PB. (2008). Mda-9/Syntenin: more than just a simple adapter protein when it comes to cancer metastasis. *Cancer Res* **68**: 3087–3093.

Schilham MW, Moerer P, Cumano A, Clevers HC. (1997). Sox-4 facilitates thymocyte differentiation. *Eur J Immunol* **27**: 1292–1295.

Schilham MW, Oosterwegel MA, Moerer P, Ya J, de Boer PA, van De WM. (1996). Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. *Nature* **380**: 711–714.

Shin MS, Fredrickson TN, Hartley JW, Suzuki T, Akagi K, Morse III HC. (2004). High-throughput retroviral tagging for identification of genes involved in initiation and progression of mouse splenic marginal zone lymphomas. *Cancer Res* **64**: 4419–4427.

Sinner D, Kordich JJ, Spence JR, Opoka R, Rankin S, Lin SC. (2007). Sox17 and Sox4 differentially regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells. *Mol Cell Biol* **27**: 7802–7815.

Suzuki T, Shen H, Akagi K, Morse HC, Malley JD, Naiman DQ. (2002). New genes involved in cancer identified by retroviral tagging. *Nat Genet* **32**: 166–174.

Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451**: 147–152.

van de Wetering M, Oosterwegel M, van NK, Clevers H. (1993). Sox-4, an Sry-like HMG box protein, is a transcriptional activator in lymphocytes. *EMBO J* **12**: 3847–3854.

van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O. (2010). Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* **115**: 965–974.

Wilson ME, Yang KY, Kalousova A, Lau J, Kosaka Y, Lynn FC. (2005). The HMG box transcription factor Sox4 contributes to the development of the endocrine pancreas. *Diabetes* **54**: 3402–3409.

Ziegler EC, Ghosh S. (2005). Regulating inducible transcription through controlled localization. *Sci STKE* **2005**: 1–6.

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