HHS Public Access

Author manuscript

Nat Cell Biol. Author manuscript; available in PMC 2010 February 22.

Published in final edited form as:

Nat Cell Biol. 2009 June ; 11(6): 777–784. doi:10.1038/ncb1885.

TGF-β signalling is regulated by Schnurri-2 dependent nuclear translocation of CLIC4 and consequent stabilization of phospho Smad2–3

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Abstract

CLIC4, a multifunctional protein that traffics between the cytoplasm and nucleus, interacts with Schnurri-2, a transcription factor in the BMP pathway. TGF-β enhances the expression of both CLIC4 and Schnurri-2 and promotes their association in the cytoplasm and their translocation to the nucleus. In the absence of CLIC4 or Schnurri-2, TGF-β signalling is abrogated. Direct nuclear targeting of CLIC4 enhances TGF-β signalling and removes the requirement for Schnurri-2. Nuclear CLIC4 associates with phospho-Smad2 and 3 and protects them from dephosphorylation by nuclear phosphatases. An intact TGF-β signalling pathway is essential for CLIC4 to mediate growth arrest. These results reveal Schnurri-2 and CLIC4 as previously unidentified modifiers of TGF-β signalling through stabilizing phospho-Smad2 and phospho-Smad3 in the nucleus.

CLIC4 (Chloride intracellular channel 4) is a member of a family of intracellular chloride channel proteins that are ubiquitously expressed in multiple tissue types1. In addition to chloride channel activity in artificial and biological membranes, CLIC family members participate in cell cycle control, cytoskeletal function, mitosis and differentiation1, but the pathway(s) through which they function are undefined. Analysis of the molecular structure of CLIC1 and CLIC4 reveals dimorphic proteins that exist in both soluble and membrane-bound configurations at least in part regulated by redox potential2–4. CLIC4 is essential for apoptosis mediated by p53 and c-Myc, and the CLIC4 promoter is a direct downstream target of these transcription factors 5,6. Cytoplasmic CLIC4 translocates to the nucleus under conditions of metabolic stress, growth arrest, apoptosis and DNA damage mediated by a functional nuclear localization signal (NLS) on the carboxy terminus of the protein7. Nuclear CLIC4 residence is an essential component of its pro-apoptotic and growth arrest activity in keratinocytes 8. In contrast, CLIC4 is excluded from the nucleus in epithelial

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AUTHOR CONTRIBUTIONS

A.S. designed and carried out studies, analyzed data and wrote the manuscript, C.C. performed the analysis of *in vivo* samples and contributed to writing the manuscript, Y.H. and T.F. carried out studies, M.M. designed the yeast two-hybrid study, K.S.H. provided essential reagents, S.H.Y. designed experiments, analyzed and organized the data and wrote the manuscript. The manuscript was read by all authors.

cancer cells but upregulated in tumour stromal cells associated with myofibroblast conversion9. CLIC4 was previously linked to myofibroblast conversion in TGF-β treated mammary fibroblasts10. Thus, while CLIC4 participates in growth control and tissue remodelling, the signalling pathway through which CLIC4 participates is not known.

Yeast two-hybrid assays were carried out using six CLIC4 sequences spanning the entire protein as baits. The CLIC4 bait sequence with amino acids 120–254 interacted with several potential binding proteins, one of which was amino acids 814–1167 of Schnurri-2, a zinc finger protein known to function in the TGF-β superfamily signalling pathway11–13. The interaction of CLIC4 and Schnurri-2 was validated using co-immunoprecipitation assays in primary cultures of mouse keratinocytes. HA-tagged CLIC4 or empty vector was expressed and Schnurri-2 interacting proteins were co-immunoprecipitated using anti-Schnurri-2 antibody. Both endogenous and exogenous CLIC4 were revealed as Schnurri-2 interacting proteins upon immunoblotting of SDS-PAGE separated immunoprecipitates (Fig. 1a, Supplementary Information, Fig. S1a), confirming the yeast two-hybrid results. CLIC4 and Schnurri-2 do not associate outside of cell context, since *in vitro* transcribed and translated full length Schnurri-2 or its interacting domain or the interacting domains of both proteins failed to coimmunoprecipitate with recombinant full-length CLIC4 (data not shown).

Deletion constructs of C-terminal V5-His tagged CLIC4 (Supplementary Information Fig. S1b) in adenoviral vectors were expressed in primary keratinocytes Supplementary Information Fig. S1c indicates that Schnurri-2 antibody co-precipitates full length CLIC4 (aa 1–253) and the region of CLIC4 minus the NLS (1–197) but not the N-terminal CLIC4 half (1–120) or 1–60 that contains the transmembrane domain. Interaction with the 1–120 or 1–60 domains could not be detected even at the highest exposures. Thus, Schnurri-2 interacts between amino acid 121–197 of CLIC4 consistent with bait region attracting Schnurri-2 in the yeast two-hybrid assay.

Since CLIC4 causes cell cycle arrest and Schnurri-2 participates in TGF-β superfamily (Dpp/BMP) signalling, we asked if TGF-β could influence the interaction of CLIC4 and Schnurri-2. Whole cell protein lysates from keratinocytes that were treated with TGF-β1 for different periods of time show a persistent increase in CLIC4 and Schnurri-2 levels up to 24h of TGF-β1 treatment (Fig. 1b). The increase in CLIC4 and Schnurri-2 by TGF-β is transcriptional and is sustained (Supplementary Information, Fig. S2a). Furthermore, skin biopsies obtained from single and double transgenic mice overexpressing active TGF-β1 in the epidermis in response to doxycycline stimulation 14 (Supplementary Information Fig. S2b), show elevated transcripts for both Schnurri-2 and CLIC4 by 4–5 days after doxycline. At this time point, transcripts for the TGF-β response gene Smad7 are also increased as previously reported in this model. Thus, CLIC4 and Schnurri-2 respond to TGF- β stimulation similarly *in vitro* and *in vivo*. TGF- β also stimulates the interaction of CLIC4 and Schnurri-2.. Supplementary Information, Fig. S3 shows by co-immunoprecipitation that TGF-β1 treatment of keratinocytes increases the interaction of both endogenous and exogenous CLIC4 with Schnurri-2 within 30 minutes. The interaction in subcellular fractions appears to occur only in the cytoplasmic compartment of TGF-β1-treated cells and persists for at least 6 hours (Fig. 1c). Interaction of these proteins in the nucleus could not be detected. The TGF-β stimulated interaction of CLIC4 and Schnurri-2 is not Smad dependent

since siRNA mediated knockdown of Smad2, Smad3 or Smad4 did not interrupt this interaction (Supplementary Information, Fig. S4).

Expression of exogenous CLIC4 in mouse keratinocytes modestly enhanced the activity of the TGF-β dependent reporter plasmid p3TP-lux (Fig. 2a) in response to low doses (50 pg/ml) of TGF-β1. CLIC4 increased the reporter activity in a TGF-β1 dose dependent manner (Supplementary Information, Fig. S5a). This enhancement required the presence of Schnurri-2 as Schnurri-2 siRNA prevented CLIC4 from increasing TGF-β reporter activity (Fig. 2a, Supplementary Information, Fig. S5a). Growth inhibition is a major physiological response of keratinocytes to TGF-β. Fig. 2b shows that exogenous CLIC4 alone inhibited DNA synthesis and together with TGF-β 1 inhibited keratinocyte DNA synthesis to a greater extent than TGF-β 1 alone, and this required the presence of Schnurri-2. Keratinocyte growth inhibition by CLIC4 and TGF-β also followed a TGF-β 1 dose dependent course (Supplementary Information, Fig. S5b). Schnurri-2 is a Smad binding partner in Dpp and BMP signalling in Drosophila and mice respectively and alters downstream signalling12,13. We confirmed that Schnurri-2 also modifies TGF-β 1 signalling in a mammalian system by introducing a Schnurri-2 expression plasmid pAct-FLAG-hShn2 into HEK293, a readily transfectable epithelial cell line. By itself Schnurri-2 caused CLIC4 to translocate to the nucleus and in conjunction activated Smad2 and suppressed c-Myc (Supplementary Information, Fig. S6). With TGF-β1, Schnurri-2 increased TGF-β dependent signal transduction, transcriptional activity and growth inhibition (Fig. 2c, d, Supplementary Information, Fig. S7a). These results indicate that Schnurri-2 is involved in TGF-β signalling in addition to its known function in BMP signalling.

We next examined the biological significance of CLIC4 in TGF-β responses using RNA interference to knockdown CLIC4 expression in keratinocytes. TGF-β1-induced p3TP-lux activity and inhibition of thymidine incorporation by TGF-β1 in keratinocytes were reduced by expression of specific CLIC4shRNA further confirming participation of CLIC4 in TGF-β signalling (Fig. 2e, f). Expressing exogenous CLIC4 in keratinocytes also enhanced the TGF-β1 dependent downregulation of c-Myc and upregulation of p21 even in the absence of TGF-β1 (Fig. 2g). These changes are known to participate in TGF-β mediated growth inhibition15,16. Furthermore, suppression of CLIC4 by shRNA enhanced c-Myc and reduced p21 expression in the absence of TGF-β1 and reduced c-Myc inhibition and p21 induction in the presence of TGF-β1 (Fig. 2h).

TGF-β1 increases total CLIC4 and causes nuclear trafficking of CLIC4 in keratinocytes within 30 minutes (Fig. 3a). Nuclear CLIC4 can be detected by confocal microscopy following exposure to TGF-β1 as early as 1 hour (data not shown) and is sustained for at least 24 hours (Fig. 3b). Similarly, exogenous Schnurri-2 (pAct-FLAG-hShn2) translocates to the nucleus of HEK293 cells exposed to TGF-β1 in a time course virtually identical to that observed for endogenous CLIC4 in HEK293 cells (Fig. 3c). Confocal microscopy for FLAG staining confirmed nuclear trafficking of Schnurri-2 (Fig. 3d). Together these results support a mechanism whereby TGF-β1 enhances its activity through stimulating an interaction of CLIC4 and Schnurri-2 and promoting their nuclear translocation.

To confirm that nuclear translocation of CLIC4 by TGF-β1 has functional significance, we introduced a nuclear targeted CLIC4 expression adenovirus7 into keratinocytes and monitored the TGF-β responses. Both CLIC4 and nuclear CLIC4 enhanced TGF-β1 dependent reporter activity, but nuclear CLIC4 was 10 fold more potent than wild-type CLIC4 overexpression (Fig. 3e). TGF-β dependent inhibition of DNA synthesis in keratinocytes is enhanced by both wild-type and nuclear targeted CLIC4 but much more so by the latter. Furthermore, nuclear CLIC4 caused marked inhibition of DNA synthesis in the absence of exogenous TGF-β (Fig. 3f). In order to examine whether growth inhibition by nuclear CLIC4 is TGF-β dependent, we expressed exogenous wild-type CLIC4 and nuclear targeted CLIC4 in primary keratinocytes from Smad3 knockout mice and wild-type littermates and treated with TGF-β1 for 16h. Fig. 3g indicates that the lack of Smad3 increased thymidine incorporation in keratinocytes consistent with a growth inhibitory function of the TGF-β pathway for this cell type. CLIC4 and especially nuclear CLIC4 enhanced TGF-β mediated growth inhibition in wild-type keratinocytes but were much less effective in Smad3 knockout keratinocytes. While DNA synthesis in the wild-type keratinocytes was inhibited 83 fold by overexpression of nuclear CLIC4, the Smad3 knockout cells were only inhibited by 9 fold, thus confirming that a completely intact TGF-β signalling pathway is essential for CLIC4 to inhibit keratinocyte growth.

To understand the mechanism by which Schnurri-2 facilitates CLIC4 enhancement of TGF-β signalling, we analyzed the effect of Schnurri-2 siRNA on TGF-β1 dependent nuclear translocation of CLIC4. Knockdown of Schnurri-2 prevented CLIC4 from translocating to the nucleus in response to TGF-β1 treatment of keratinocytes (Fig. 4a, b) indicating that Schnurri-2 is important for CLIC4 nuclear translocation in response to TGF-β. Fig. 4c indicates that depletion of Schnurri-2 prevents wild-type CLIC4 from enhancing TGF-β1 transcriptional activation (also Fig. 2a) but does not prevent nuclear CLIC4 from enhancing TGF-β reporter activity. This result was confirmed using immortalized MEFs (mouse embryonal fibroblasts) from Schnurri-2 knockout mice. Supplementary Information, Fig. S7b shows that wild-type CLIC4 is unable to enhance TGF-β reporter activity in Schnurri-2 knockout MEFs. However, exogenous expression of Schnurri-2 restores the ability of wild-type CLIC4 to enhance TGF-β signalling in these cells. In contrast, nuclear targeted CLIC4 enhances reporter activity in the absence of Schnurri-2. Thus, the primary requirement for Schnurri-2 in this pathway is to facilitate CLIC4 nuclear translocation and is not required for CLIC4 nuclear action.

Fig. 5a shows that nuclear targeting of CLIC4 enhances nuclear phospho-Smad2 and phospho-Smad3 levels even in the absence of exogenous TGF-β stimulation. Expression of CLIC4shRNA decreased nuclear phospho-Smad2 and phospho-Smad3 in both untreated keratinocytes and keratinocytes treated with TGF-β1 for 1 hour (Supplementary Information, Fig. S8a). Targeting of a non-specific protein to the nucleus (GFP) by adenoviral vector7 did not enhance levels of phospho-Smad2-3 (data not shown) indicating that nuclear CLIC4 has a specific effect. Phospho- Smad2 and 3 levels are also increased when endogenous CLIC4 is targeted to the nucleus by TGF-β treatment, and the levels are sustained for extended periods when exogenous wild-type CLIC4 translocates to the nucleus by TGF-β treatment (Supplementary Information, Fig. S8b). Smad4 levels are not altered by nuclear CLIC4 (Fig. 5a). Phospho-Smad2 and 3 are predominantly nuclear proteins although

phosphorylation occurs in the cytoplasm. Nuclear CLIC4 primarily enhances nuclear phospho-Smad2 and 3 even in the absence of exogenous TGF-β (Fig. 5b). Nuclear CLIC4 does not enhance translocation of phospho-Smad2 or 3 from the cytoplasm to the nucleus in the absence or presence of exogenous TGF-β since cytoplasmic phospho-Smad2 or 3 are not reduced in its presence (Fig. 5b). The increase in nuclear phospho-Smads is not a consequence of enhanced total Smad protein levels since they remained unchanged (Fig. 5b).When keratinocytes were treated with TβRI inhibitor SB431542, the overall levels of phospho-Smad2 decreased due to blockade of basal Smad activation. Nevertheless phospho-Smad2 levels were partially sustained by nuclear CLIC4 (Supplementary Information, Fig. S9a). This result indicated that CLIC4 was not acting through receptor mediated phosphorylation and suggested that spontaneous dephosphorylation of phospho-Smads was delayed by nuclear CLIC4. Figure 5c indicates that CLIC4 directly interacts with phospho-Smad2-3 in keratinocytes. After TGF-β treatment for just a short time, endogenous CLIC4 coimmunoprecipitates from lysates of the nuclear fraction with endogenous phospho-Smad2 and 3 (Fig. 5c) although the abundance of phospho-Smads (and hence the level of immunoprecipitated phospho-Smads) diminishes over the 3 hour time course consistent with the reported degradation of nuclear phospho-Smads after TGF-β 17.Co-immunoprecipitation analysis demonstrates that CLIC4 also interacts with Smad4 (Supplementary Information, Fig. S9b).

Among the complexities of TGF-β signalling a dynamic interplay of Smad phosphorylation and dephosphorylation regulates nuclear responses. Ligand stimulation results in R-Smad phosphorylation and accumulation of Smad complexes in the nucleus. where they are dephosphorylated and exported from the nucleus thereby terminating the signal 18,19. In order to understand if physical association of CLIC4 with phospho-Smad2 and 3 could protect the two from dephosphorylation, we exposed nuclear lysates of keratinocytes transduced with either the control (Vector), wild-type or nuclear targeted CLIC4 adenovirus to lambda protein phosphatase, a serine threonine phosphatase. Fig. 5d shows that phospho-Smad2 and 3 in lysates containing nuclear CLIC4 are relatively more resistant to phosphatase treatment whether isolated from TGF-β treated or untreated keratinocytes. Relative to their respective untreated samples, 2 units of lambda phosphatase enzyme dephosphorylates p-Smad2 by ~75% and ~50%, in vector only and wild-type CLIC4 overexpressing samples respectively but only by 10% in nuclear targeted CLIC4 expressing samples. Similarly, p-Smad3 is dephosphorylated by ~80–95% in empty vector, ~70–80% in wild-type CLIC4 and only 50% in nuclear CLIC4 expressing samples. Thus in this assay nuclear CLIC4 and to a lesser extent wild-type CLIC4 protect phospho-Smad2–3 from the action of phosphatases. With the short TGF-β exposure (1 hour), only small amounts of wild-type CLIC4 are expected in the nucleus.

PPM1a has been identified as a Smad binding protein and a Smad phosphatase18. When nuclear CLIC4 is overexpressed in TGF-β treated keratinocytes, co-immunoprecipitation of phospho-Smad2 and 3 with PPM1a is inhibited (Fig. 5e) . Notably, wild-type CLIC4 also inhibits the interaction of phospho-Smad2-3 with PPM1a consistent with the earlier results that CLIC4 translocates to the nucleus after TGF-β treatment. RNAi mediated knockdown of PPM1a increased levels of phospho-Smad2 and 3 in lysates from keratinocytes transduced with empty vector or wild-type CLIC4 but did not further enhance the levels in samples with

nuclear targeted CLIC4 (Fig. 5f), indicating that at least part of the nuclear CLIC4 effect on p-Smad2/3 stabilization is due to its inhibition of PPM1a activity on phospho-Smads. That the levels of phospho-Smad2 and phospho-Smad3 in vector and wild-type CLIC4 groups did not rise to the same level as in nuclear targeted CLIC4 after PPM1a knockdown might indicate that other, yet to be described, Smad phosphatases are also inhibited by nuclear resident CLIC4 (or perhaps the knockdown of PPM1a is incomplete).

These results depict a novel mechanism of enhancement of TGF-β signalling. TGF-β stimulates the association of Schnurri-2 and CLIC4 in the cytoplasm leading to Schnurri-2 dependent nuclear translocation of the complex where it dissociates. Nuclear CLIC4 stabilizes nuclear phospho-Smad2 and phospho-Smad3 levels by associating with these proteins and protecting them from PPM1a dependent dephosphorylation and possibly from other Smad phosphatases, thus enhancing and sustaining the TGF-β nuclear signalling (Supplementary Information, Fig. S10). The protection of nuclear phospho-Smads by CLIC4 enhances both early (c-Myc downmodulation and p21 upregulation) and later (growth inhibition) consequences of TGF-β signalling also prolonging the response. A positive feedback loop may also be established since a consequence of TGF-β signalling is to increase the expression of both Schnurri-2 and CLIC4.

Previous data indicated that the CLIC4 NLS was required for nuclear translocation in response to cell stress7, suggesting that aside from TGF-β stimulation, there are additional mechanisms regulating CLIC4 nuclear translocation independent of Schnurri-2. Nevertheless, TGF-β signalling could be downstream from CLIC4 nuclear translocation in response to stress and cell cycle arrest as well as the participation of CLIC4 in myofibroblast conversion. CLIC4 loss in tumors could contribute to tumor resistance to TGF-β signalling. Schnurri-2 and CLIC4 now join a panoply of regulators of Smad signalling in the nucleus reflecting the need for precise control of TGF-β signalling. In addition to phospho-Smad dephosphorylation by PPM1a there is the Arkadia mediated degradation of the co-repressor SnoN 20, the direct action of Arkadia on both activation and degradation of phospho-Smads 17 and retention of Smad proteins in the nucleus by TAZ, a transcriptional co-regulator 21. Together, the combination of Schnurri-2 and CLIC4 present a unique duo of novel players in a critical pathway controlling diverse aspects of cell behaviour and may serve as targets for modifying TGF-β signalling under conditions where this pathway is involved in pathological changes.

METHODS

Cell culture, expression vectors and transfection

Primary keratinocytes from newborn Balb/c mice were prepared and cultured according to established methods22. Smad3 knockout mice were kindly provided by Dr. Lalage Wakefield (National Cancer Institute) and keratinocytes prepared and cultured similarly. The readily transfectable epithelial HEK293 cells were used in some experiments and cultured in DMEM supplemented with 10% newborn calf serum. Adenoviral Smad4 was provided by Dr. Adam Glick (Pennsylvania State University). Schnurri-2 expression plasmid pAct-FLAG-hShn2 was provided by Dr. Shunsuke Ishii (RIKEN Tsukuba Institute). Primary mouse embryonal fibroblasts from Schnurri-2 knockout mice (MEFs,

provided by Dr. Shunsuke Ishii)23 were immortalized by pRSV-T and cultured in DMEM with 10% newborn calf serum. Transfections were performed using Lipofectamine 2000 (Invitrogen). Generation of HA-CLIC4, nuclear targeted CLIC4, V5-His tagged full length CLIC4 and deletion constructs lacking the NLS and the C-terminal half has been described elsewhere7. The deletion mutant construct expressing 1–60 CLIC4 N-terminal amino acids was constructed similarly. Cells were transduced with CLIC4 expression vectors for 16h prior to TGF-β1 treatment.

Construction of adenovirus expressing CLIC4shRNA

CLIC4 sequence gggctgaaggaggaggacaaagag was sequentially cloned into pENTR/H1/TO and pLenti4/Block iT/Dest vectors (Invitrogen) to produce pLenti6/ Block iT-GW/U6 as the final vector. The inducible portion of this vector was subcloned into Ad5 RNAi adenovirus (ABM, Vancouver, BC) and amplified by the Adenovirus Core Facility (Frederick NCI, MD). The CLIC4 shRNA is constitutively active without requiring the TetR transactivator expression system and is homologous between human and mouse CLIC4 so that this shRNA can be used for both species. For nonspecific shRNA, 5'-gttctccgagagtgtcacgt -3' was used (Cellogenetics).

Schnurri-2, Smad2, Smad3, Smad4 and PPM1a siRNA

Schnurri-2 siRNA (uuaggauauaaagcccuuc), Smad2 siRNA (uaguaugcgauugaacacc), Smad3 siRNA (cgaguguaaauuauuucaa), Smad4 siRNA (agaugaauuggauucuuua), PPM1a siRNA (uuggaauucuuccuaaucg) and non-silencing siRNA (uucuccgaacgugucacgudtdt) were obtained from Qiagen and transfected at 20 nM concentration using HiPerfect (Qiagen). Cells were treated with TGF-β1 or infected with control, CLIC4 or nuclear CLIC4 adenovirus 24h post-transfection with Schnurri-2 siRNA and 48h post transfection with Smad2 and PPM1a siRNAs.

Yeast two-hybrid assay

The CLIC4 yeast two-hybrid screen was conducted by Myriad Genetics, based on original methodology24. CLIC4 cDNA fragments were cloned into pGBT.superB, a GAL4 DNA-binding domain (residues 1–441) plasmid, and used as bait to screen a mouse embryo cDNA library cloned into pGAD.PN2 plasmid containing a GAL4 activation domain (residues 2301–2643). Bait-transformed PYN200 cells were mated with prey-transformed BK100 cells and plated on selective media. Interactions were confirmed by transforming naïve yeast cells with purified bait and prey constructs and assaying for β-galactosidase using a chemiluminescent assay. While multiple bait constructs spanning the entire CLIC4 ORF were screened separately, only residues 120–254 successfully recruited prey.

Antibody generation, subcellular fractionation, co-immunoprecipitation and immunoblot

Monospecific polyclonal antibody generated against the N-terminal peptide of CLIC4 has been described elsewhere25. The polyclonal serum was purified through a protein A column (Pharmacia) and dialyzed in borate buffer. c-Myc and p-Smad2 antibodies were from Cell Signalling Technologies, p21 from BD Biosciences, Smad4, Schnurri-2 and Lamin A–C antibodies were from Santa Cruz Biotechnologies, PPM1a antibody from Abcam, while

alpha-tubulin antibody was from Bethyl Laboratories. p-Smad3 antibody was a kind gift from Dr. Edward Leof, Mayo Clinic. Keratinocytes or HEK293 cells were washed and scraped into lysis buffer (Cell Signalling Technologies). Nuclear and cytoplasmic extracts were made using NE-PER (Thermo Fischer Scientific). 25 μg of protein was subjected to immunoblotting and visualized using enhanced chemiluminescence (Thermo Fischer Scientific).

For immunoprecipitation, cells were washed with cold phosphate-buffered saline (PBS) and lysed in M-PER (Thermo Fischer Scientific) containing protease and phosphatase inhibitors. Lysates were precleared with protein A–G PLUS agarose beads and incubated overnight with the antibody of interest and protein A/G PLUS agarose beads at 4°C. Beads were washed and boiled prior to electrophoresis of the eluate. Co-immunoprecipitation from nuclear fractions was performed using the Nuclear Complex Co-IP kit (Active Motif).

Luciferase assay

Luciferase assay was carried out using TGF-β responsive p3TP luciferase reporter (p3TP-lux). Keratinocytes plated in 12-well culture plates were transfected with p3TP-lux (2.0 μg/well) and pRLTK (0.2 μg/well). Cells were transduced in quadruplicate with wild-type CLIC4, nuclear targeted CLIC4, Vector (empty), CLIC4shRNA or non-silencing shRNA expressing adenovirus and treated with TGF-β1 (50 pg/ml) 3h after infection for 14h. Luciferase activity was determined in cell extracts using the Dual Luciferase Reporter assay system (Promega) and normalized to renilla luciferase activity. For determining changes in TGF-β reporter luciferase activity by Schnurri-2, the same procedure as above was followed except that HEK293 cells were transfected with Schnurri-2 expression plasmid for 24 hours prior to exposure to TGF-β1.

^{3}H-Thymidine incorporation assay

Keratinocytes or HEK293 cells were plated in 24-well plates and transduced with control, wild-type CLIC4 or nuclear targeted CLIC4 viruses for 16h or transfected with Schnurri-2 expression plasmid for 24h. siRNAs were transfected 24h prior to viral transduction. TGF-β1 (50 pg/ml) was added to the wells 2h after transduction or 8h after transfection. ^{3}H-Thymidine (1 μCi / well) was added for 3 hrs before the end of the treatment. Cells were fixed using methanol and acetic acid (in a 3:1 ratio), solubilized in 5 N NaOH and incorporated counts measured using a scintillation counter.

Confocal microscopy

Cells were fixed in 2% paraformaldehyde/PBS (phosphate buffered saline) for 30 min, washed with PBS, permeabilized with cold methanol for 6 min and dried briefly. Samples were then re-hydrated and washed with PBS, treated with 100 mM glycine for 30 min, equilibrated with 0.2% Triton x-100 for 10 min, washed with PBS and then blocked for 1h in 0.5% bovine serum albumin/PBS. This was followed by incubation with primary antibody (anti-CLIC4 or anti-Schnurri-2) overnight at 4°C, wash with PBS and incubation in dark with fluorescence-labelled secondary antibody for 1h. The plates were washed again and Vectashield mounting medium with DAPI (Vector Laboratories) for nuclear stain was added, and cells were analyzed by confocal microscopy (Zeiss-NLO microscope).

Dephosphorylation of nuclear extracts using lambda protein phosphatase

Nuclear extracts of keratinocytes transduced with control, wild-type or nuclear targeted CLIC4 adenovirus ± treatment with TGF-β for 1h were dephosphorylated using lambda protein phosphatase (New England Biolabs) at 30°C for 30 min. Reactions were stopped by adding 2× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and heating for 5 min at 100°C.

RT-PCR

Total RNA from keratinocytes treated with TGF-β1 was isolated using Tri zol (Invitrogen), subjected to DNAse I (Ambion) treatment and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). PCR amplification of CLIC4 and Schnurri-2 was carried out using Platinum Supermix(Invitrogen). Sequences for Schnurri-2 primers were GGAAAGAGGGAAAGGAGAGATTCACGGAGAT and ATCTGAGTGTCATCACAAGAGTCACTGGGT. CLIC4 primers were from Superarray (catalog PPH08576A) and GAPDH primers were from Gene Link (catalog 40-1005-10). For in vivo analysis, cDNA (provided to us by Dr. Adam Glick, Pennsylvania State University) was reverse transcribed from RNA isolated from the skin of single transgenic control mice or double transgenic mice engineered to express active TGF-β1 from skin keratinocytes after induction by doxycycline 14. For real-time PCR analysis, predesigned Quantitect primers (Qiagen) and BioRad iQ iCycler and Gene Expression Macro were used.

Statistical analysis

All luciferase assays and ^{3}H-Thymidine incorporation assays were repeated at least three times in quadruplicate. Statistical analysis was performed using unpaired two-tailed t-test with confidence interval of 99%. The p values for significant differences are indicated in respective figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Dr. Lalage Wakefield for critically reading the manuscript, Mario Anzano for help with genotyping, Christina Cheng for help with immunoprecipitation assays and members of the Yuspa lab for providing constructive criticism throughout the study. This work was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

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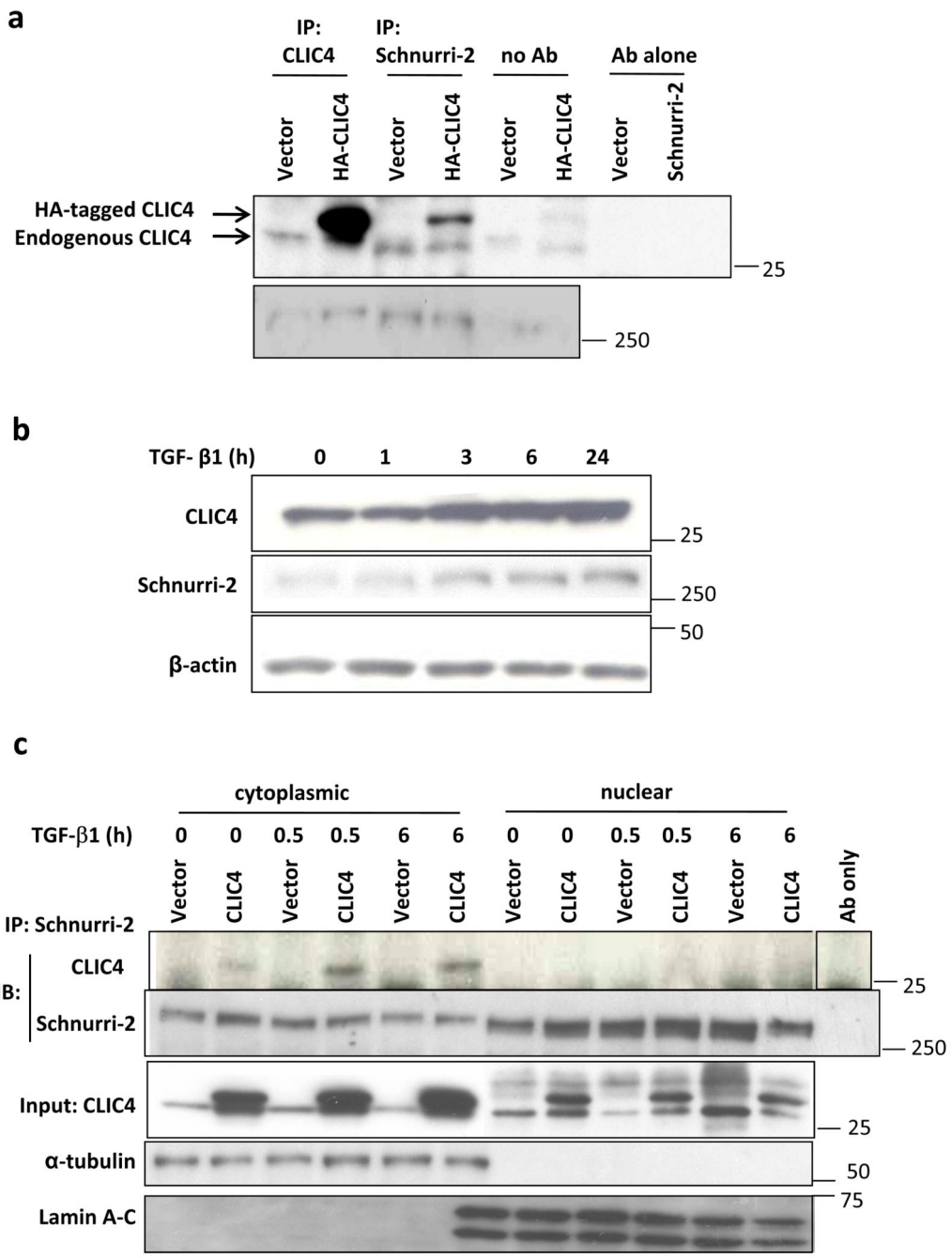

Figure 1. TGF-β enhances the expression and association of CLIC4 and Schnurri-2

(a) Empty vector or CLIC4 was expressed in primary Balb/c keratinocytes using an HA-tagged adenoviral construct. Whole cell lysates were immunoprecipitated using anti-CLIC4 or anti-Schnurri-2 antibodies and immunoblotted for CLIC4 and Schnurri-2. Arrows point to HA-CLIC4 and endogenous CLIC4 protein. Antibodies alone without cell lysates or cell lysates without antibodies were subjected to the immunoprecipitation procedure and used as controls. **(b)** Primary keratinocytes were treated with TGF-β1 (1 ng/ml) for indicated times. Whole cell lysates were immunoblotted for CLIC4 and Schnurri-2. β-actin was used as

loading control. **(c)** Adenoviral empty vector or HA-tagged CLIC4 were expressed in keratinocytes for 16h, and recipient cells were treated with TGF-β1 (1 ng/ml) for indicated times. Cytoplasmic and nuclear fractions were immunoprecipitated using anti-Schnurri-2 antibody and immunoblotted for CLIC4 and Schnurri-2. Because of co-migration of endogenous CLIC4 and IgG in the presence of cell fractionation buffers, only the exogenous CLIC4 can be monitored. Anti-Schnurri-2 antibody without cellular lysates was subjected to immunoprecipitation and immunoblotting and used as a control (Ab only). An aliquot of the cytoplasmic and nuclear fractions was used directly as input for immunoblotting for CLIC4, Lamin A–C and α-tubulin. The lanes separated by a line are from the same gel. Uncropped images of scans are shown in Supplementary Information, Fig. S11.

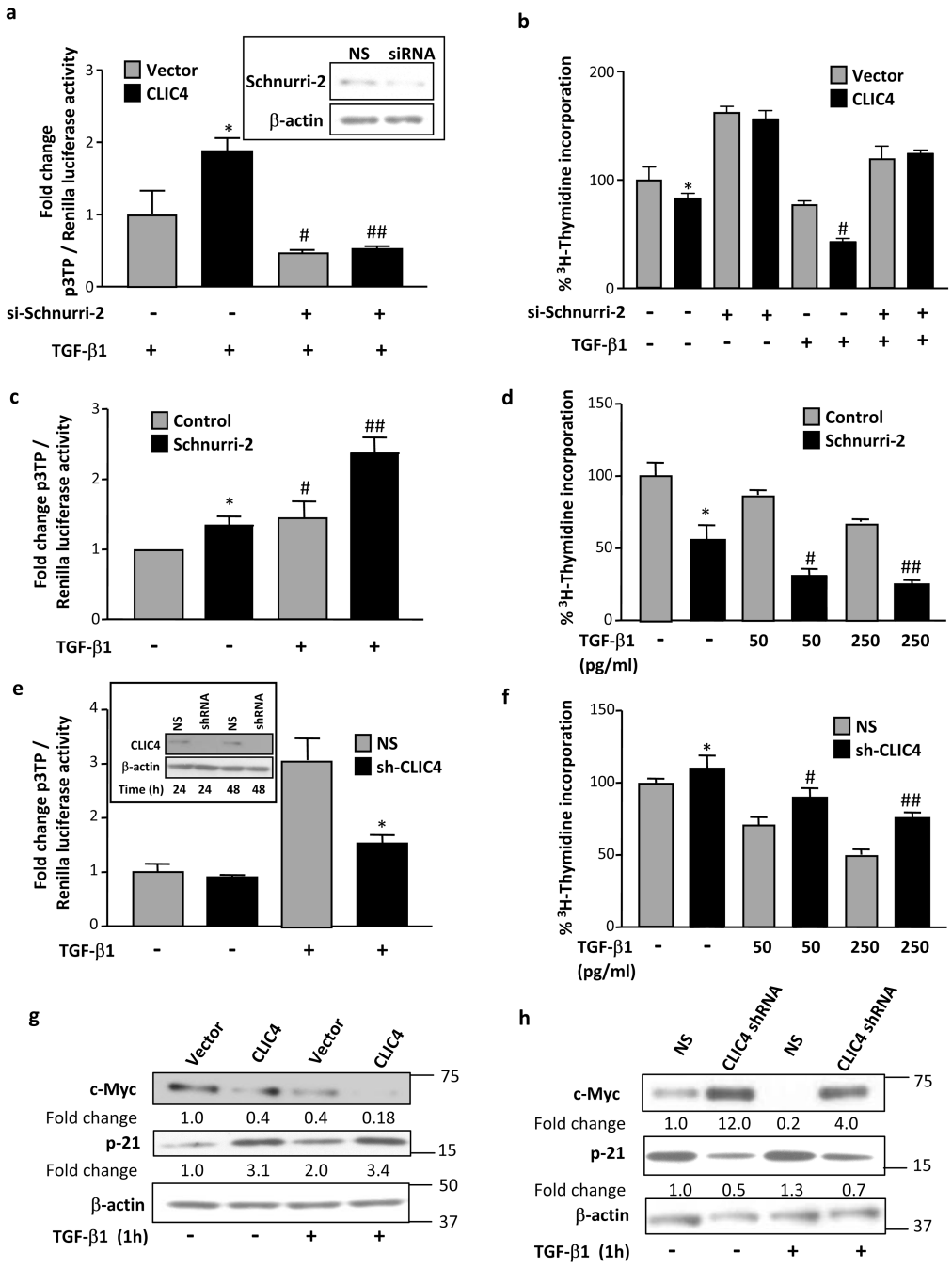

Figure 2. CLIC4 and Schnurri-2 enhance TGF-β signaling

Primary keratinocytes **(a,e)** or HEK 293 cells **(c)** were transfected with p3TP-lux and plasmid pRLTK. Keratinocytes **(a,b)** were transfected with non-silencing (−) or Schnurri-2 (+) siRNA (inset) for 24h **(a)**, before transduction with empty or CLIC4 adenoviruses and treated with TGF-β1 (50 pg/ml) for 14 **(a)** or 16h **(b)**. **(a)** Luciferase data is presented as fold change of non-silencing siRNA empty adenovirus sample ± SD *p=0.0125 and # p=0.0155 compared to non-silencing siRNA empty adenovirus sample, # # p=0.0092 compared to non-silencing siRNA CLIC4 adenovirus sample. For **(b)** ^{3}H-thymidine incorporation is a

percentage of the untreated non-silencing siRNA empty adenovirus ± SD. *p=0.0279 compared to untreated non-silencing siRNA empty adenovirus sample, #p<0.0001 compared to non-silencing siRNA transfected, TGF-β1 treated empty adenovirus sample. **(c,d)** HEK293 cells were transfected with pAct-FLAG-hShn2 or transfection reagent alone and treated with TGF-β1 (50 pg/ml) for 16 **(c)** or 24h **(d)**. Luciferase assays **(c)** *p=0.0253 and #p=0.0291 compared to untreated control, ##p=0.0357 compared to TGF-β1 treated sample. For ^{3}H-thymidine incorporation **(d)** *p=0.015 compared to untreated control, #p<0.0001 compared to 50 pg/ml TGF-β1 treated control, ##p=0.0001 compared to 250 pg/ml TGF-β1 treated control. For **a,b,c,d** each data point is an average of n=4. **(e)** Keratinocytes were transduced with non-silencing (NS) or CLIC4 specific shRNA (inset) adenovirus for 24h prior to TGF-β1 (50 pg/ml) treatment for 14h. *p=0.0102 compared to TGF-β1 treated non-silencing shRNA (NS) transduced sample. **(f)** Keratinocytes were transduced with sh-CLIC4 as in **(e)** and treated with TGF-β1 for 16h. ^{3}H-Thymidine incorporation is a percentage of the untreated non-silencing shRNA sample ± SD. *p=0.0177 compared to untreated non-silencing shRNA sample, #p=0.0005 compared to 50 pg/ml and ##p<0.0001 compared to 250 pg/ml TGF-β1 treated non-silencing shRNA sample. **(g,h)** Keratinocytes were transduced with empty or CLIC4 expressing adenovirus **(g)** or the CLIC4 knockdown vectors **(h)** for 16h and treated with TGF-β1 (1 ng/ml) for 1h. The ImageJ software quantified c-Myc and p21 bands are normalized to their respective β-actin and represented as fold change of the untreated empty vector samples. Uncropped images of scans are shown in Supplementary Information, Fig. S11.

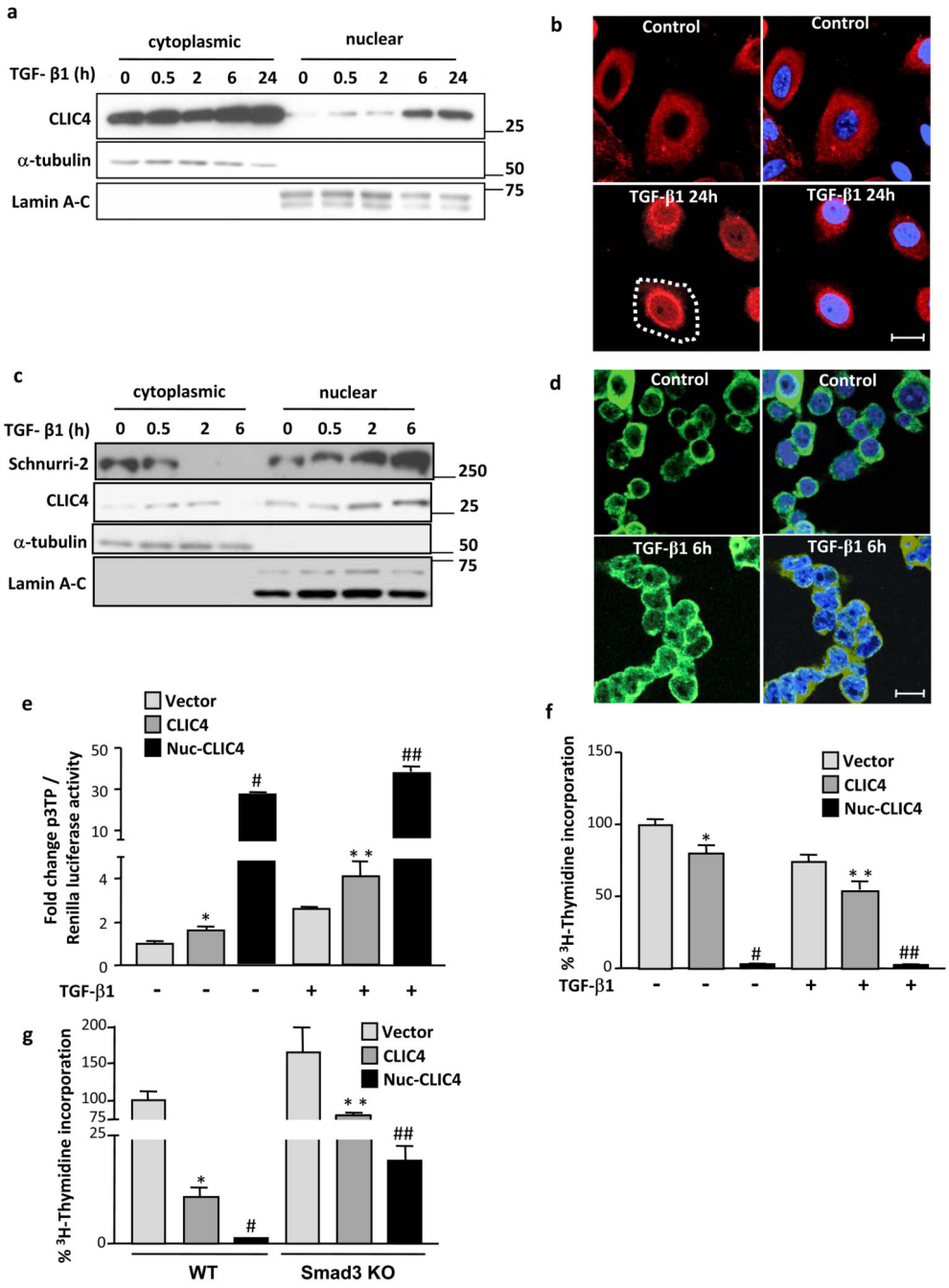

Figure 3. TGF-β induces nuclear translocation of CLIC4 to enhance TGF-β signalling

(a,b) Primary keratinocytes were treated with TGF-β1 (1 ng/ml) for the times indicated. **(a)** Cytoplasmic and nuclear fractions were immunoblotted or **(b)** immunostained for CLIC4. In **(a)** α-tubulin and Lamin A–C were used as controls for the cytoplasmic and nuclear fraction respectively. Uncropped image of scan is shown in Supplementary Information, Fig. S11. In **(b)** DAPI was used to visualize nuclei. The dotted line defines the cell periphery. **(c)** HEK293 cells were transfected with Schnurri-2 expression plasmid pAct-FLAG-hShn2 and treated with TGF-β1 for the times indicated. **(c)** Cytoplasmic and nuclear fractions were

immunoblotted for Schnurri-2 and CLIC4 or **(d)** immunostained with anti-FLAG primary and FITC labelled secondary antibody. In **(c)** α-tubulin and Lamin A–C were used as controls and in **(d)** DAPI was used to visualize nuclei. Scale bar, 20 μm. **(e)** Primary keratinocytes were transfected with p3TP-lux and control plasmid pRLTK. Cells were transduced with adenoviral control (vector), wild-type CLIC4 or nuclear targeted CLIC4 and treated with TGF-β1 (50 pg/ml) for 14h. Luciferase assays were performed and data presented as fold change of untreated empty vector sample ± standard deviation. Values significantly different from control, *p=0.0074 and #p<0.0001 compared to untreated vector sample, **p=0.0056 and ##p<0.0001 compared to TGF-β1 treated vector sample. **(f)** Keratinocytes were transduced as in **(e)** and treated with TGF-β1 (50 pg/ml) for 16h. ^{3}H-Thymidine incorporation is presented as a percentage of the untreated vector sample ± standard deviation. Values significantly different from control, *p=0.03 and #p<0.0001 compared to untreated vector sample, ** p <0.032 and ##p <0.0001 compared to TGF-β1 treated vector sample. **(g)** Keratinocytes from Smad3 knockout mice or wild-type littermates were transduced as in **(e)** and treated with TGF-β1 (50 pg/ml) for 16h. ^{3}H-Thymidine incorporation is presented as a percentage of the wild-type vector sample ± standard deviation. Values significantly different from control, * <0.0005 and #p<0.0001 compared to wild-type vector sample, ** p=0.0022 and ## p=0.0001 compared to Smad3 KO vector sample. For **a,b,c** each data point is an average of quadruplicates (n=4) from a representative experiment.

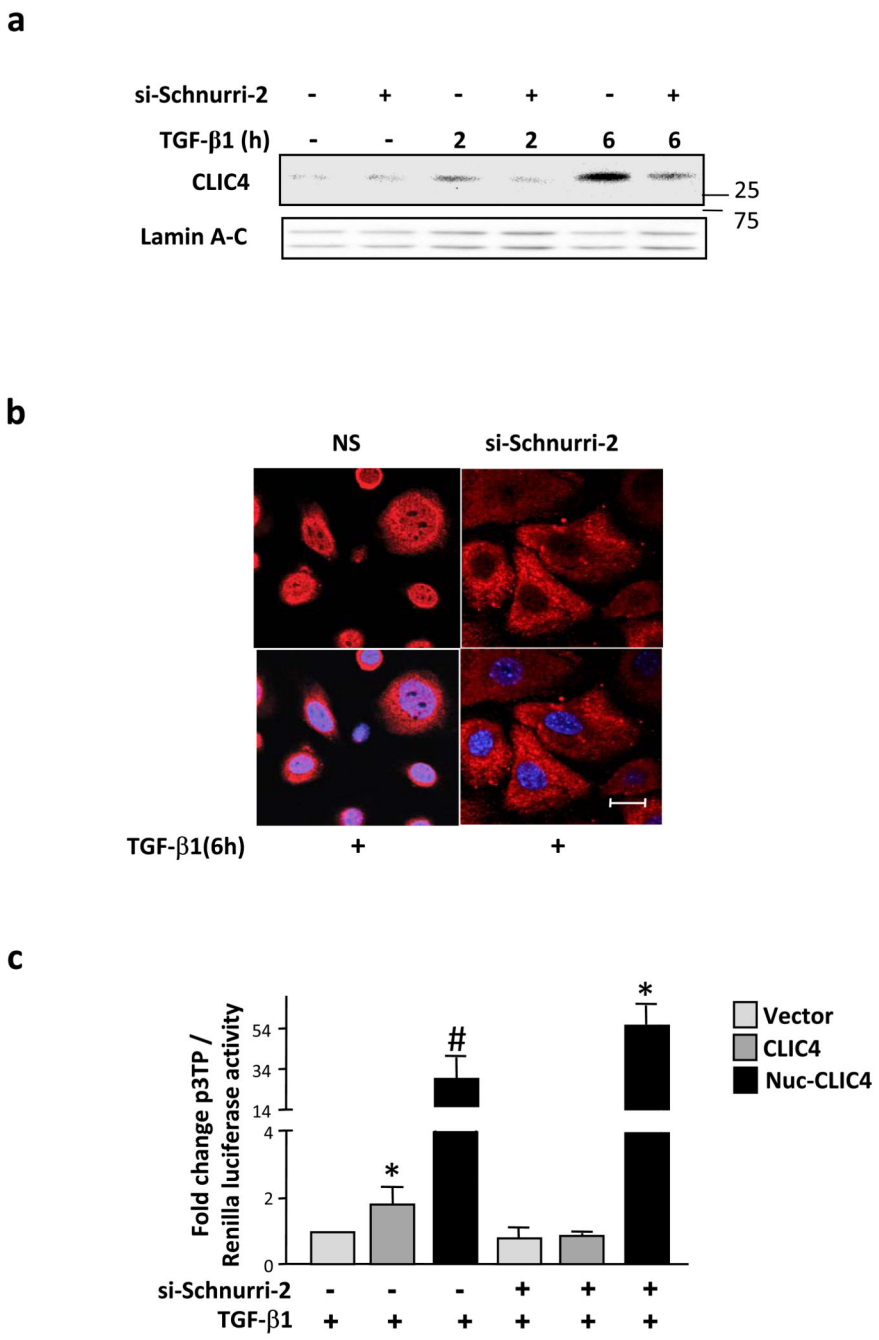

Figure 4. Schnurri-2 is essential for TGF-β dependent nuclear translocation of CLIC4

(a) Primary Balb/c keratinocytes were transfected with nonsilencing (−) or Schnurri-2 (+) siRNA 24h prior to treatment with TGF-β1 (1 ng/ml) for 2 or 6h. Nuclear fractions were immunoblotted for CLIC4. Lamin A–C was used as control for loading. Uncropped images of scan is shown in Supplementary Information, Fig. S11. **(b)** Keratinocytes were transfected with nonsilencing (NS) or Schnurri-2 siRNA 24h prior to treatment with TGF-β1 for 6h. Cells were immunostained with anti-CLIC4 primary and rhodamine labelled secondary antibody and visualized by confocal microscopy with or without DAPI. Scale bar,

20 μm. **(c)** Keratinocytes were transfected with p3TP-lux and control plasmid pRLTK and non-silencing (−) or Schnurri-2 (+) siRNA prior to transduction with adenoviral constructs expressing control (vector), wild-type CLIC4 or nuclear targeted CLIC4 and treatment with TGF-β1 (50 pg/ml) for 14h. Luciferase assays were performed and data presented as fold change of non-silencing siRNA transfected vector sample. Values significantly different from control, *p=0.023 and #p=0.0068 compared to non-silencing siRNA transfected vector sample, *p=0.0005 compared to Schnurri-2 siRNA transfected vector sample. Each data point is an average of quadruplicates (n=4) from a representative experiment.

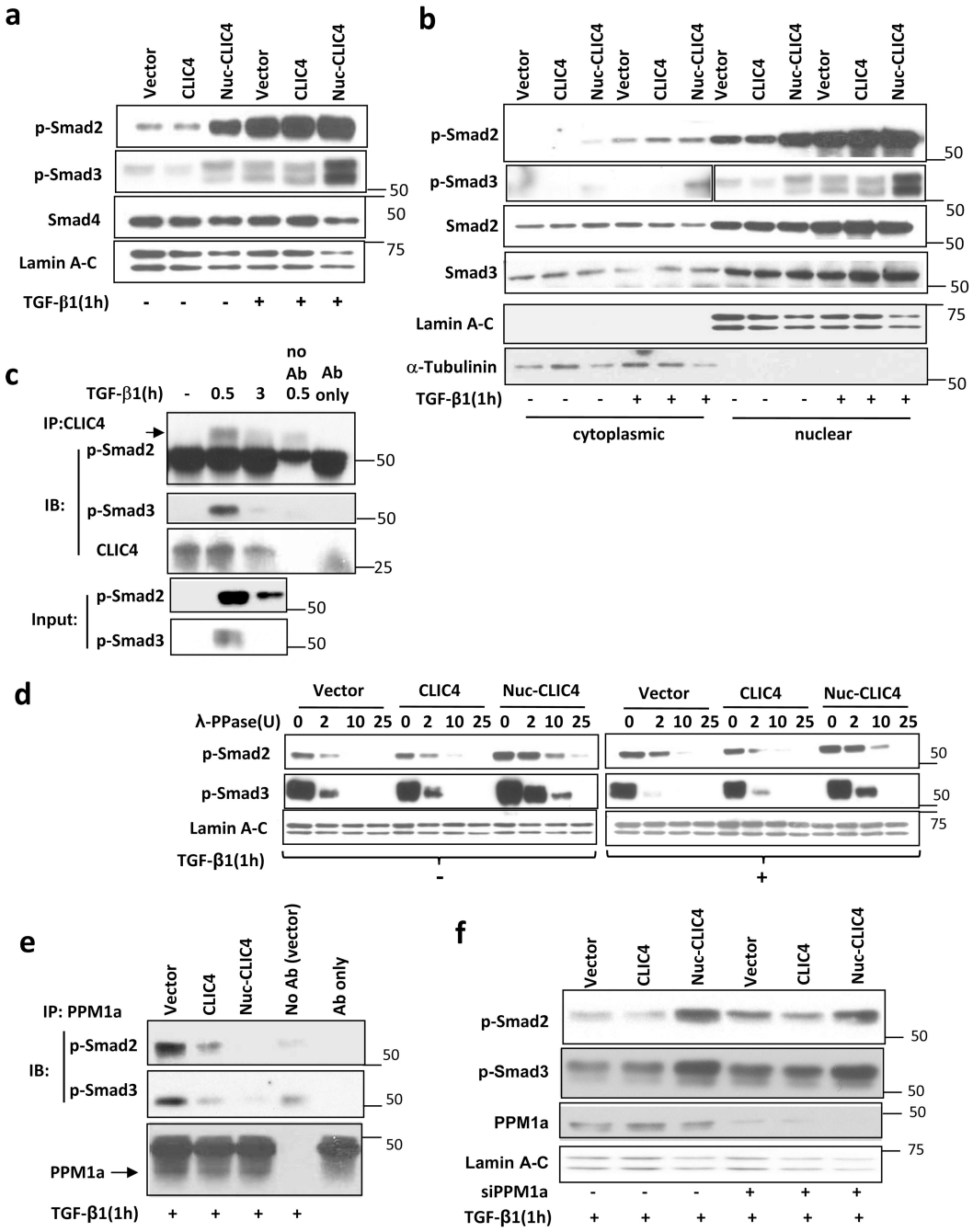

Figure 5. Nuclear CLIC4 associates with and stabilizes phospho-Smad2 and phospho-Smad3

(a) Nuclear lysates from primary keratinocytes transduced with adenoviral control (vector), wild-type CLIC4 or nuclear targeted CLIC4 expressing constructs for 16h and treated with TGF-β1 for 1h (1 ng/ml) were immunoblotted for the indicated proteins. Lamin A–C was used as loading control. **(b)** Cytoplasmic and nuclear lysates from primary keratinocytes transduced and treated as in (a) were immunoblotted for phospho-Smad2, phospho-Smad3 and total Smad2 and Smad3. α-Tubulin and Lamin A–C were used as controls for loading and subcellular fraction purity. The separated parts of the p-Smad3 blot are from the same

gel but adjusted for exposure to visualize both fractions optimally. **(c)** Nuclear lysates of untreated keratinocytes or treated with TGF-β1 for indicated times were immunoprecipitated with anti-CLIC4 antibody and immunoblotted for phospho-Smad2, phospho-Smad3 and CLIC4. Anti-CLIC4 antibody without cell lysate and lysate alone without antibody were subjected to immunoprecipitation and used as control. An aliquot of the lysate used for immunoprecipitation was used as input. **(d)** Primary keratinocytes were transduced and treated as in **(a)**. Nuclear lysates were dephosphorylated using indicated amounts of λ-PPase and immunoblotted for phospho-Smad2 and phospho-Smad3. Lamin A–C was used as loading control. Exposure time for TGF-α1 treated samples was adjusted to compensate for the higher phospho-Smad2 and 3 levels. The intensities of the phospho-Smad2 and phospho-Smad3 bands normalized to their respective Lamin A–C intensities were quantitated using ImageJ software. **(e)** Nuclear lysates of keratinocytes transduced and treated as in **(a)** were immunoprecipitated with anti-PPM1a antibody and immunoblotted for phospho-Smad2, phospho-Smad3 and PPM1a. Anti-PPM1a antibody without cell lysate and lysate alone without antibody were subjected to immunoprecipitation procedure and used as control. **(f)** Primary Balb/c keratinocytes were transfected with nonsilencing (−) or PPM1a (+) siRNA 48h prior to transduction with adenoviral control (vector), wild-type CLIC4 or nuclear targeted CLIC4 for 16h followed by treatment with TGF-β1 (1 ng/ml) for 1h. Lysates were separated by SDS-PAGE and immunoblotted for phospho-Smad2, phospho-Smad3 and PPM1a. Lamin A–C was used as loading control. Uncropped images of scans are shown in Supplementary Information, Fig. S11.