



**HAL**  
open science

## **LSF/TFCP2c/LBP-1c is required for Snail1-induced Fibronectin gene expression**

Montserrat Porta-De-La-Riva, Jelena Stanisavljevic, Josue Curto, Clara Francí, Víctor Manuel Díaz, Antonio García de Herreros, Josep Baulida

► **To cite this version:**

Montserrat Porta-De-La-Riva, Jelena Stanisavljevic, Josue Curto, Clara Francí, Víctor Manuel Díaz, et al.. LSF/TFCP2c/LBP-1c is required for Snail1-induced Fibronectin gene expression. *Biochemical Journal*, 2011, 435 (3), pp.563-568. 10.1042/BJ20102057 . hal-00586472

**HAL Id: hal-00586472**

**<https://hal.science/hal-00586472>**

Submitted on 16 Apr 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## LSF/TFCP2c/LBP-1c is Required for Snail1-induced Fibronectin Gene Expression

Porta-de-la-Riva, Montserrat<sup>1,3</sup>, Stanisavljevic, Jelena<sup>1</sup>, Josue Curto<sup>1</sup>, Francí, Clara<sup>1</sup>,  
Díaz, Víctor Manuel<sup>1,2</sup>, García de Herreros, Antonio<sup>1,2</sup> and Baulida, Josep<sup>1</sup>

Corresponding Author: Josep Baulida  
IMIM-Hospital del Mar  
Parc de Recerca Biomèdica de Barcelona  
c/Doctor Aiguader, 88  
E-08003 Barcelona, Spain  
Tel: 34-93-3160436  
Fax: 34-93-3160410  
Email: [jbaulida@imim.es](mailto:jbaulida@imim.es)

<sup>1</sup>Programa de Recerca en Càncer, IMIM-Hospital del Mar, E-08003, Barcelona, Spain.

<sup>2</sup>Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, E-08003, Barcelona, Spain.

<sup>3</sup>Current address: Genetics and Functional Genomics in *C. elegans*, Cancer and Human Molecular Genetics, Catalan Institute of Oncology (ICO) - Bellvitge Institute for Biomedical Research (IDIBELL), Gran Via s/n, Km 2.7, l'Hospitalet 08907, Barcelona, Spain.

Running head title: TFCP2c Activity on Fibronectin Transcription

Key words: Snail1, TFCP2c, epithelial to mesenchymal transition, E-cadherin, Fibronectin

## Abstract

Fibronectins are cell-secreted glycoproteins that modulate cell attachment, spreading, migration, morphology, differentiation and oncogenic transformation. Fibronectin expression is activated during epithelial-mesenchymal transition (EMT) and is a hallmark of mesenchymal cells. Results presented in this article reveal that a transcription factor previously unrelated with EMT, LSF/TFCP2c/LBP-1c, was translocated to the nucleus and bound to the Fibronectin promoter upon EMT induction by Snail1. Consequently, the interference of TFCP2c/LSF/LBP-1c activity prevented Fibronectin expression. Moreover, TFCP2c/LSF/LBP-1c was detected in nuclei of embryonic dermal mesenchymal cells adjacent to the hair bud, a cell population that expresses endogenous nuclear Snail1 and Fibronectin. Therefore, our data indicate a new molecular role for TFCP2c/LSF/LBP-1c in Fibronectin expression.

## Introduction

Advanced tumour cells frequently show a down-regulation of epithelial markers accompanied by increased expression of Fibronectin (*FNI*) and other mesenchymal genes. This morphogenetic process, referred to as epithelial to mesenchymal transition (EMT), was first described to be essential during embryogenesis [1] and it provides epithelial tumour cells with properties that support cancer progression, such as migration, invasion, extravasation [2] as well as cell stemness associated to cancer [3]. Fibronectins are a class of high molecular weight cell-secreted glycoproteins that modulate cell-substrate adhesion as well as a set of cellular processes, among them cell attachment and spreading, migration, morphology, differentiation and oncogenic transformation [4]. Fibronectin expression is a feature of mesenchymal cells and correlates with stimuli (such as TGF- $\beta$ ) that induce Snail1 expression and EMT [5]. Snail1 is a member of SNAI genes that is expressed in the EMT processes analyzed [6]. This factor directly represses epithelial markers such as E-cadherin (the product of the *CDH1* gene) by binding to E-boxes in their promoters [7, 8].

This article provides evidence that Snail1-induced *FNI* gene expression requires the transcription factor TFCEP2c/LSF/LBP-1c [9] (TFCEP2c from now on), and that *SNAIL1* and *TFCEP2c* are coexpressed along with *FNI* during embryogenesis. Although this transcription factor is known to interact with cellular and viral promoters, any interaction with *FNI* or other mesenchymal genes has not been previously described. Therefore, our data indicate a new role for TFCEP2c in Fibronectin regulation in Snail1-induced EMT.

## Materials and methods

### *Cell lines, transfection and infection*

RWP1, HT29 M6, MEFs and NMuMG cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) plus 10% FBS under standard conditions. For NMuMG cells medium was supplemented with 10  $\mu$ g/mL of Insulin. DNA transfection was performed with LipofectAMINE-PLUS (Invitrogen) reagent according to manufacturer's instructions and DNA transduction with lentivirus. These experimental procedures are detailed in the supplementary data.

### *Transcription activity and DNA constructs*

Luciferase-based reporter assays were performed as described elsewhere [10, 11]. DNA vectors used in the experiments are detailed in the supplementary data.

### *Analysis of RNA*

RNA was extracted with *Gene Elute™ Mammalian Total RNA Miniprep Kit* (Sigma-Aldrich). For quantitative analysis, 1 µg of RNA was retro-transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche) and 100 ng of the obtained cDNA was used as a template for quantitative SYBR-Green-based PCR. Correct product size was confirmed in agarose gels. The calculated RNA amount was systematically normalized by the Pumilio RNA levels. For the semi-quantitative analysis, 50 ng RNA was analysed with SuperScript One-Step RT-PCR with Platinum *Taq* (Invitrogen) and agarose gels. See the supplementary table S1 for specific oligonucleotides.

#### *Analysis of proteins*

**Immunofluorescence:** A standard IF protocol on cells grown for at least 48 hours on ethanol sterilized glass coverslips was applied. All steps were done at room temperature. Cells were fixed with 4% paraformaldehyde (PFA) for ten minutes. PFA auto-fluorescence was quenched with 50 mM NH<sub>4</sub>Cl/PBS for five minutes and cells were permeabilised with 0.2% Triton X-100 for five minutes. Blocking was carried out for 1 h with PBS containing 3% bovine serum albumin (BSA). Coverslips were then incubated in humid atmosphere for 1 h with a primary antibody (20 µg/ml for TFPC2c antibody) and, after extensive washing, for 45 min with the secondary antibody (in dark). Nuclear counterstaining was performed by incubating the coverslips for one minute with 5 µg/ml propidium iodide and 100 µg/ml RNase. Coverslips were mounted with fluoromont G and fluorescence was viewed and captured through a Leica TCS-SP2 confocal microscope.

**Analysis of proteins in the mouse embryo:** The protocol was reported by Franci et al. [12]. CD-1 pregnant mice (Harlan) were killed by cervical dislocation; the embryos were removed by Caesarean section, fixed in formalin and embedded in paraffin. Sections (4 µm) were dewaxed and rehydrated. Antigens were retrieved by boiling the samples in Tris-EDTA pH 9 for 15 min. Endogenous peroxidase activity was quenched with 4% hydrogen peroxide in PBS containing 0.1% sodium azide for 15 min. After several rinses with PBS, sections were incubated with PBS containing 1% BSA to block non-specific binding and were washed with PBS. Sections were incubated with either 10 µg/ml of purified anti-Snail1 MAb EC3, 5µg/ml anti-Fibronectin PAb or 20 µg/ml anti-TFPC2 PAb overnight at 4°C. After several further rinses with PBS, bound Ab was detected using anti-mouse or anti-rabbit Envision (Envision System Peroxidase, DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin.

#### *Chromatin Immunoprecipitation (ChIP)*

Cells seeded in 150 mm-diameter plates were washed twice with pre-warmed PBS (37°C) and cross-linked with 1% formaldehyde for 10 minutes at 37°C in DMEM. Reaction was stopped by adding 250 µl of glycine 2.5 M (0.125 M final concentration) and incubating for 2 more minutes. Cells were washed twice with cold PBS and 1ml of Soft Lysis Buffer (50 mM Tris pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol and protease and phosphatase inhibitors) was added to the plates on ice. After scrapping, lysates were transferred to Eppendorf tubes, incubated for 10 minutes on ice and centrifuged for 15 minutes at 3,000 rpm. Supernatant was discarded and pellet

resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris pH 8.0) and sonicated to generate fragments of DNA from 200 to 1,000 bp (40% amplitude in Branson DIGITAL Sonifier® UNIT Model S-450D sonicator, 10 pulses of 10 seconds each). Lysates were incubated for 20 minutes on ice and subsequently centrifuged at maximum speed for 10 minutes.

Protein concentration in the supernatants was determined by Lowry and 500 µg of protein per immunoprecipitation (IP) was diluted in Dilution Buffer (0.001% SDS, 1.1 Triton X-100, 16.7 mM Tris pH 8.0, 2 mM EDTA, 2 mM EGTA, 167 mM NaCl). Pre-clearing in order to reduce background was performed for 3 hours at 4°C with agitation using mouse IgG (Dako) and salmon sperm-BSA (bovine serum albumin) blocked agarose-protein G (Upstate). Samples were then centrifuged at 2,000 rpm to remove the beads, 10% of the samples was stored as the input and the rest of the samples was divided for IP and incubated with either specific TFCP2c antibody (ab42973, Abcam) or an irrelevant antibody of the same species overnight at 4°C with agitation.

Blocked beads were added to each sample and incubated for one more hour at 4°C. Five washes were performed in MoBiTec columns with each of the buffers: Low Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0 and 150 mM NaCl), High Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0 and 500 mM NaCl), LiCl Buffer (250 mM LiCl, 1% Nonidet P-40, 1% Sodium deoxycholate, 1 mM EDTA and 10 mM Tris pH 8.0) and TE Buffer. Columns were centrifuged to eliminate traces of buffer (2 minutes, 2,000 rpm) and the remaining beads were incubated with Elution buffer (100 mM Na<sub>2</sub>CO<sub>3</sub> and 1% SDS) at 37°C for 30 minutes. DNA was recovered by centrifugation (5 minutes, 2,000 rpm).

4 µl of 5 M NaCl (250 mM final concentration) was added to each eluate and decrosslinking was performed by incubating samples at 65°C overnight. After 2-4 hours digestion with proteinase K (55°C), DNA was purified by the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences) and finally analyzed by quantitative SYBR-green PCR (Supplementary table S3).

For ChIP of exogenous promoters [13], 3.5x10<sup>6</sup> RWP1 cells were plated in 150 mm dishes and 5 µg of indicated *FNI* promoter was transfected with LipofectAMINE®-PLUS reagent (Invitrogen), according to manufacturer's instructions. 48 hours after transfection, cells were treated following the protocol previously described. The amount of protein used per IP was 100 µg.

## Results

### *TFCP2c binds to the FN1 promoter in Snail1 expressing cells*

Upregulation of mesenchymal genes, such *FNI*, is a characteristic molecular event related with EMT [10, 11]. Accordingly, Fibronectin RNA and protein levels increased when RWP1 and HT29 M6 epithelial cells were forced to transit towards a mesenchymal phenotype by ectopic Snail1HA expression (Fig. 1a). In line with this increment, the transcriptional activity of the -341/+265 proximal *FNI* promoter was

higher in Snail1 than in control cells (Fig. 1b). Whereas Fibronectin basal levels in HT29 M6 were lower than in RWP1 cells, the increase induced by Snail1 was higher. As expected for EMT, we detected reduced expression of the epithelial gene E-cadherin in Snail1 expressing cells (Fig. 1a and b).

To find out transcription factors mediating *FNI* activation by Snail1, we looked for transcription factor binding sites in the *FNI* proximal promoter. We narrowed our search to the initial 19 bp (-341/-323) sequence of the proximal promoter, since the exclusion of this region was sufficient to interfere *FNI* transcription by Snail1, as observed through reporter assays of 5'-deleted proximal *FNI* promoters (Fig. 1c). The transcription factor binding algorithm (TESS) detected three consensus binding motives for the transcription factor TFCP2c/LSF/LBP-1c, conserved among species (Fig. 1d); therefore, we decided to evaluate the possibility that TFCP2c was mediating Fibronectin activation in Snail1 cells.

We analyzed whether TFCP2c was binding to the *FNI* promoter by means of ChIP assays. We detected binding of TFCP2c to the proximal sequence of the *FNI* promoter in HT29 M6-Snail1HA but not in control cells. In contrast, Snail1 expression did not stimulate the binding of an irrelevant transcription factor (Fig. 2a). Taking advantage of the fact that the transfection efficiency of RWP1 is higher than that of HT29 M6, we tested whether the TFCP2c is binding to the exogenous proximal *FNI* promoter transfected into RWP1 control and Snail1 cells. Similarly to the endogenous promoter, we found higher binding of TFCP2c to the transfected proximal promoter (-341/+265) in RWP1 Snail1HA than in control cells; however, this was not the case for a promoter missing the sequence (-341/-332) including the TFCP2c binding motives (Fig. 2b). Next, we analyzed by immunofluorescence whether the increased TFCP2c binding to the *FNI* promoter in Snail1 cells was accompanied by an increment of the nuclear amount of TFCP2c. We detected higher nuclear TFCP2c staining in HT29 M6 Snail1 than in control cells (Fig. 2c). Since total levels of TFCP2c analysed by western blot were not increased in Snail1 cells, altogether, our data indicate that Snail1 expression triggers the nuclear localization of TFCP2c and its binding to the *FNI* proximal promoter.

#### *TFCP2c is required for Snail1-induced Fibronectin expression*

The binding of TFCP2c to the *FNI* promoter in Snail1 cells suggests that TFCP2c activity is required for *FNI* transcription in these cells. Therefore, we analyzed the effect of a dominant negative for TFCP2c (TFCP2c Q234L/K236E) on Fibronectin expression. This mutant interferes with TFCP2c transcriptional activity by preventing the binding of the endogenous wild-type TFCP2c to DNA [14]. While expression of the dominant negative did not affect the low Fibronectin protein levels in RWP1 and HT29 M6 control cells, it clearly decreased the Fibronectin levels detected in Snail1 cells (Fig. 3a), especially in the case of HT29 M6 cells. Similar decrease was detected at the mRNA level, quantified as being of 50 or 60% in RWP1 Snail1HA or HT-29 M6 Snail1HA cells, respectively (Suppl. Fig. S1). In accordance with the effect of the dominant negative, we found that HT29 M6 Snail1 cells depleted of TFCP2c by infection of a shRNA specific for TFCP2c produced less Fibronectin than Snail1 cells infected with an irrelevant shRNA (Suppl. Fig. S1). The involvement of TFCP2c in

Fibronectin activation was also tested in epithelial cells forced to transit to mesenchymal by TGF- $\beta$ , which induces endogenous Snail1 levels. As shown in Fig. 3b, Fibronectin mRNA was increased at 8 h of TGF- $\beta$  treatment in control NMuMG sensitive cells but remained at basal levels in cells transduced with the dominant negative of TFCP2c.

To further analyze the relevance of TFCP2 on cells expressing endogenous Snail1 we transduced the dominant negative form of TFCP2 into mouse embryonic fibroblasts (MEFs) in which Fibronectin RNA expression depends of endogenous Snail1 (Supplementary materials and methods). Like in epithelial cells expressing exogenous Snail1, the expression of TFCP2c Q234L/K236E also decreased *FN1* expression (Fig. 3c). Other mesenchymal genes analyzed such as LEF1, Snail1 or ZEB1 were not altered by the dominant negative form. Since Fibronectin modulates the cell-substrate interaction we analyzed whether the dominant negative form also affected the migration of the MEFs. Wound healing (Fig. 3d) and single cell track assays (Fig. 3e) showed that the dominant negative form of TFCP2 reduced the migratory capacity of fibroblasts and the effect was rescued by providing exogenous fibronectin to the cells (Suppl. Fig. S2).

*Nuclear TFCP2c is expressed in embryonic dermal mesenchymal cells adjacent to the hair bud together with Snail1 and Fibronectin*

To verify that the effect of TFCP2c on *FN1* expression was not restricted to cultured Snail1 expressing cells, we analyzed the subcellular localization of TFCP2c *in vivo*. Expression of endogenous Snail1 is higher in embryonic than in adult tissues, but it is not constitutive and has a limited distribution in a subset of mesenchymal cells; for instance, Snail1 is expressed in embryonic mesenchymal cells adjacent to hair bud cells [12, 15]. In line with this data, by immunohistochemical analysis of whole mounted mice embryos at day E14.5, we found embryonic dermal mesenchymal cells with positive nuclear staining for Snail1 (Fig. 4a). Interestingly, we found a similar pattern of nuclear expression of TFCP2c (Fig. 4b) and Fibronectin was detected in the same areas (Fig. 4c). These data, therefore, suggest that the transcription factor TFCP2c is involved in the transcriptional activation of Fibronectin by Snail1 *in vivo*.

## Discussion

Our results indicate for the first time a role for the transcription factor TFCP2/LSF/LBP-1c in *FN1* transcription. LSF was known to regulate diverse cellular and viral promoters [9, 16] but none of them have a direct role in EMT regulation. Indirect relationship between TFCP2c targets and EMT can be established considering the fact that EMT and Snail1 expression generate cells with stem properties that hold potential for tumour initiation, tumour recurrence and metastasis [3, 17, 18]. Thus, it was described that TFCP2 activates the chicken Embryonic Stem 1 gene (cENS-1) expression early in embryonic development [19] and the osteopontin gene in the context of hepatocellular carcinoma [20]. We have observed that overexpression of

TFCP2c in epithelial HT26 M6 or RWP1 cells did not increase Fibronectin RNA levels (Suppl. Fig. S3). Moreover, the epithelial phenotype was maintained and no changes in E-cadherin RNA levels (Suppl. Fig. S3) were observed in these cells. Although an increase of TFCP2c was not sufficient to induce a complete EMT in the epithelial cells tested, our results directly involve TFCP2c in the molecular events triggering the expression of the mesenchymal gene *FNI*. Thus, we unveil a new molecular mechanism which could be targeted to prevent cancer cell properties associated to Fibronectin expression.

### Acknowledgements

We are grateful for the technical assistance of Dr. Raúl Peña, Alicia Riquel and Marta Garrido. This study was supported by a grant (PI071054) from the Fondo de Investigaciones Sanitarias of the ISCIII to JB and by grants from the Ministerio de Ciencia y Tecnología (SAF2006-00339) and the Fundació La Marató of TV3 to A.G. H. Partial support from the Instituto Carlos III (RD06/0020/0040) and from the Generalitat de Catalunya (2005SGR00970) is also appreciated.

### References

- 1 Nieto, M.A. (2002) The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell. Biol.* **3**, 155-166
- 2 Thiery J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nature Rev. Cancer*, **2**, 442–54
- 3 Hollier, B.G., Evans, K. and Mani, S.A. (2009) The Epithelial-to-Mesenchymal Transition and Cancer Stem Cells: A Coalition Against Cancer Therapies. *J. Mammary Gland. Biol. Neoplasia*. **14**, 29–43
- 4 Yamada, KM. (1983) Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* **52**, 761-99
- 5 Thuault, S., Tan, E.J., Peinado, H., Cano, A., Heldin, C.H. and Moustakas, A. (2008) HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. *J. Biol. Chem.* **283**, 33437-46
- 6 Barrallo-Gimeno, A. and Nieto, M.A. (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development*. **132**, 3151-61
- 7 Battle, E., Sancho, E., Francí, C., Domínguez, D., Monfar, M., Baulida, J. and García de Herreros, A. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**, 84-89

- 8 Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F. and Nieto, M.A. (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83
- 9 Veljkovic, J. and Hansen, U. (2004) Lineage-specific and ubiquitous biological roles of the mammalian transcription factor LSF. *Gene* **343**, 23-40
- 10 Guaita, S., Puig, I., Francí, C., Garrido, M., Domínguez, D., Batlle, E., Sancho, E., Dedhar, S., García de Herreros, A.G. and Baulida, J. (2002) Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. *J. Biol. Chem.* **277**, 39209-39216
- 11 Solanas, G., Porta-de-la-Riva, M., Agustí, C., Casagolda, D., Sánchez-Aguilera, F., Larriba, M.J., Pons, F., Peiró, S., Escrivà, M., Muñoz, A., Duñach, M., García de Herreros, A.G. and Baulida, J. (2008) E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. *J. Cell Sci.* **121**, 2224-2234
- 12 Francí, C., Takkunen, M., Dave, N., Alameda, F., Gómez, S., Rodríguez, R., Escrivà, M., Montserrat-Sentís, B., Baró, T., Garrido, M., Bonilla, F., Virtanen, I. and García de Herreros, A. (2006) Expression of Snail protein in tumor-stroma interface. *Oncogene* **25**, 5134-5144
- 13 James, M.A., Lee, J.H. and Klingelutz A.J. (2006) HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. *Int. J. Cancer.* **119**, 1878-1885
- 14 Shirra, M.K., Zhu, Q., Huang, H.C., Pallas, D. and Hansen, U. (1994) One exon of the human LSF gene includes conserved regions involved in novel DNA-binding and dimerization motifs. *Mol. Cell Biol.* **14**, 5076-5087
- 15 Jamora, C., Lee, P., Kocieniewski, P., Azhar, M., Hosokawa, R., Chai, Y. and Fuchs, E. (2005) A Signaling Pathway Involving TGF- $\beta$ 2 and Snail in Hair Follicle Morphogenesis. *PLOS Biol.* **3**, e11
- 16 Hansen, U., Owens, L. and Saxena, U.H. (2009) Transcription factors LSF and E2Fs: Tandem cyclists driving G0 to S?. *Cell Cycle* **8**, 2146-2151
- 17 Moody, S.E., Perez, D., Pan, T.C., Sarkisian, C.J., Portocarrero, C.P., Sterner, C.J., Notorfrancesco, K.L., Cardiff, R.D. and Chodosh, L.A. (2005) The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* **8**, 197-209
- 18 Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., Campbell, L.L., Polyak, K., Briskin, C., Yang, J. and Weinberg, R.A. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell.* **133**, 704-15

19 Aclouque, H., Mey, A., Birot, A.M., Gruffat, H., Pain, B. and Samarut, J. (2004) Transcription factor cCP2 controls gene expression in chicken embryonic stem cells. *Nucleic Acids Res.* **32**, 2259-71

20 Yoo, B.K., Emdad, L., Gredler, R, Fuller, C., Dumur, C.I., Jones, K.H., Jackson-Cook, C., Su, Z.Z., Chen, D., Saxena, U.H., Hansen, U., Fisher, P.B. and Sarkar D. (2010) Transcription factor Late SV40 Factor (LSF) functions as an oncogene in hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 8357-62

### Figure legends

#### Figure 1. The *FNI* promoter contains a responsive element at -341/-323 including TF2CP2c binding elements.

(a) *CDH1* and *FNI* gene expression in HT29 M6 and RWP1 control and Snail1HA cells. The left panel shows the protein levels detected by Western blot using indicated antibodies from total cell extracts. The right panel shows the relative mRNA levels for *FNI* and *CDH1* genes. *HPRT* was used for normalization. Bars represent an x-fold RNA increase in Snail1HA relative to control cells. (b) *CDH1* and *FNI* promoter activity in HT29 M6 and RWP1 control and Snail1HA cells. Transcription activity was calculated by luciferase reporter assays in control and Snail1HA cells. Cells were transiently co-transfected with 150 ng of a firefly luciferase promoter reporter plasmid, including the -341/+265 *FNI* promoter or the -178/+92 *CDH1* promoter, and 10 ng of a renilla luciferase TK vector for normalization. Bars show the increase of normalized luciferase activity in Snail1HA in relation to control cells. Error bars show the standard deviation of at least three independent experiments. (c) Transcription activation of *FNI* promoter fragments by Snail1. Reporter experiments were performed by transiently cotransfecting RWP1 parental cells with 100 ng of an indicated *FNI* promoter fragment cloned into a firefly luciferase vector, either 5 ng of pcDNA3-Snail1HA or an empty vector, and 10 ng of renilla TK vector for normalization. Bars represent the activity of the promoters in presence of Snail1HA relative to pcDNA3. Error bars show the standard deviation of four independent experiments. (d) The -341/-314 sequence of the *FNI* promoter includes a consensus site for TF2CP2c binding. Putative TF2CP2c binding motives detected by TESS are boxed. Consensus sequence is annotated on the top, where N = T, C, A or G; and R = A or G.

The *Homo sapiens* -341/-314 *FNI* promoter sequence was aligned with that of the indicated species.

#### Figure 2. TF2CP2c binds to the *FNI* promoter in Snail1HA cells.

(a) TF2CP2c binds to the *FNI* promoter in HT29 M6 cells. ChIP assays were performed in HT29 M6 control and stably expressing Snail1HA cells. Endogenous *FNI* promoter was amplified with specific primers covering the -375/-320 region. Levels of *FNI* promoter precipitated with specific antibody against TF2CP2c or WT1 were compared with those precipitated with non-specific antibody. Error bars show the standard deviation of four independent experiments. In Snail1 cells, the binding of TF2CP2c to the proximal *FNI* promoter was increased over 2.8 +/- 0.5 fold when compared with that to a -2kb (-1958/-1870; supplementary table S3) distal sequence. (b) TF2CP2c binds to the *FNI* promoter in RWP1 Snail1HA cells. RWP1 control and stably expressing Snail1HA

cells were transiently transfected with the -341/+265 or -322/+265 *FNI* promoter and 48 hours later a ChIP assay was performed using a TF2CP2c specific antibody. The amount of exogenous *FNI* promoter and an irrelevant DNA were analyzed by qPCR. Bars represent the *FNI* promoter/irrelevant DNA ratio in Snail1HA relative to control cells obtained from four independent experiments (mean +/- standard deviation). (c) Subcellular localization of TF2CP2 in HT29 M6 control and Snail1HA cells. TF2CP2c sublocalization was analyzed by immunofluorescence. Propidium iodide was used for nuclear staining.

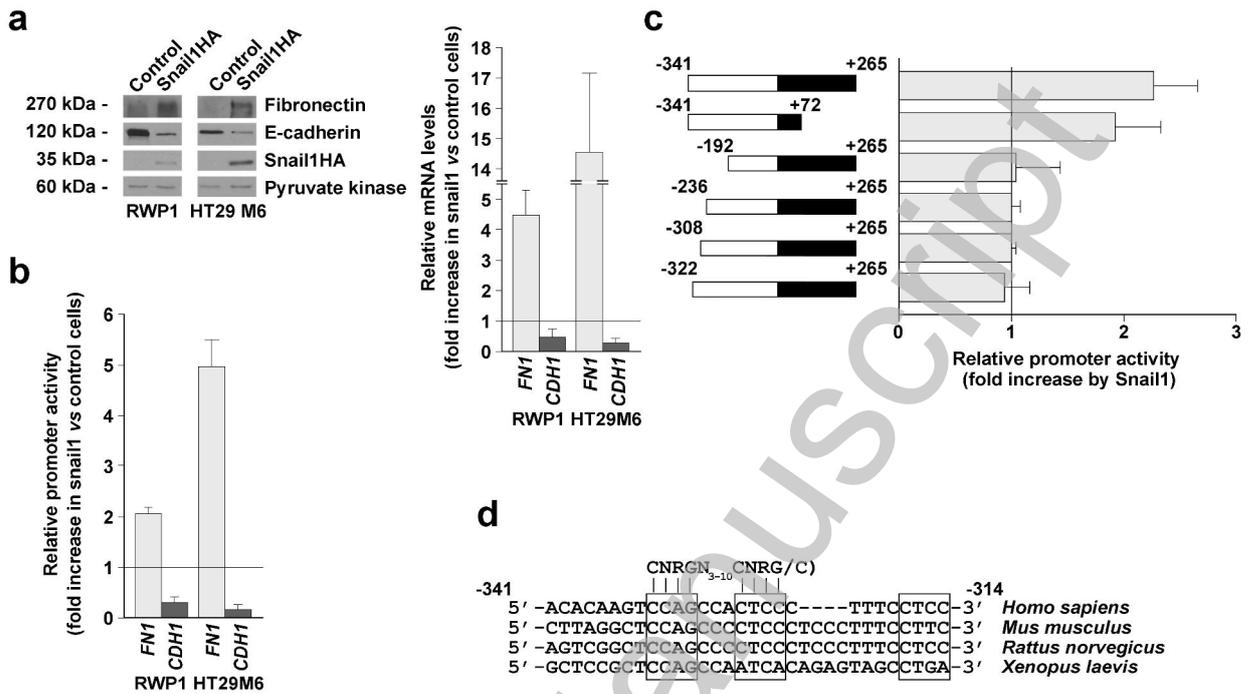
**Figure 3. TF2CP2c is required for Snail1-induced Fibronectin expression.**

(a) TF2CP2c Q234L/K236E expression reduces Fibronectin protein levels in Snail1HA cells. RWP1 and HT29 M6 control and Snail1HA cells were infected with empty pBABE (-) or pBABE-TF2CP2c Q234L/K236E-myc (DN), and protein levels were analyzed by Western blot from total protein extracts; Annexin II was used as loading control. (b) TF2CP2c Q234L/K236E mutant reduces Fibronectin RNA levels in MEFs. RNA levels corresponding to the indicated genes were analyzed by RT-PCR from MEFs transduced with pBABE-TF2CP2c Q234L/K236E-myc or with empty pBABE vector. (c) TF2CP2c Q234L/K236E mutant reduces Fibronectin activation by TGF- $\beta$  in NMuMG cells. mRNA levels corresponding to the indicated genes were analyzed by RT-PCR or RT-qPCR from TGF- $\beta_1$ -treated (8h, 50 ng/mL) NMuMG cells previously transduced (48 h) with pBABE-TF2CP2c Q234L/K236E-myc or with empty pBABE vector. (d) TF2CP2c Q234L/K236E mutant reduces migration of MEFs assayed by wound healing. Pictures were taken 48 hours after performing the scratch. Borders of the scratch at time zero are indicated with dashed lines. (e) TF2CP2 Q234L/K236E mutant reduces migration of MEFs assayed by single cell track. Movement of three representative cells is plotted.

**Figure 4. Nuclear TF2CP2c is expressed in embryonic dermal mesenchymal cells adjacent to the hair bud together with Snail1 and Fibronectin.**

(a) Snail1, (b) TF2CP2c and (c) Fibronectin expression in dermal mesenchymal cells of E14.5 embryos. Protein expression was detected by immunohistochemistry, as described in Supplementary Data. Nuclei were counter-stained with hematoxylin. Red arrowheads indicate positive cells. Electronic amplification (2.5x) is shown in boxes. All mice involved in this study were maintained in a rodent barrier facility in order to guarantee the specific pathogen free health status of the animals and the protocol was previously approved by the Animal Research Ethical Committee from the PRBB.

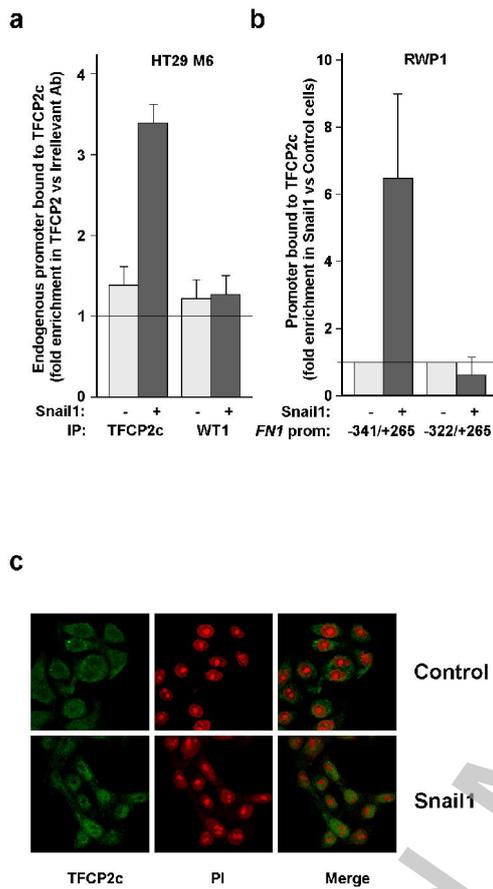
Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102057

Accepted Manuscript

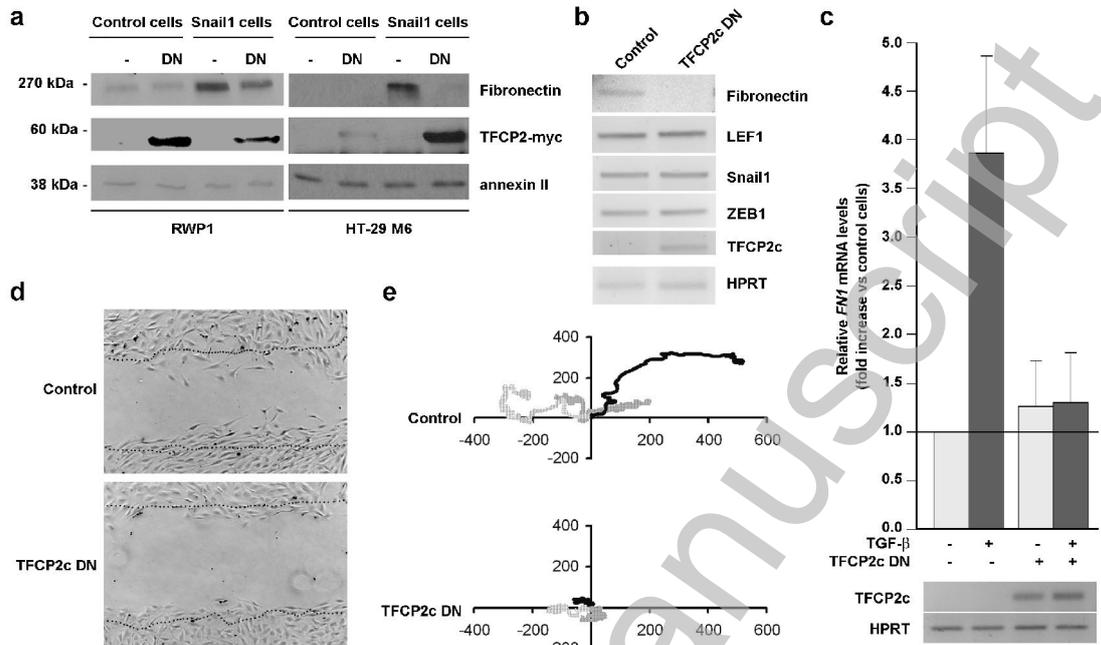
**Figure 2**



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102057

Accepted Manuscript

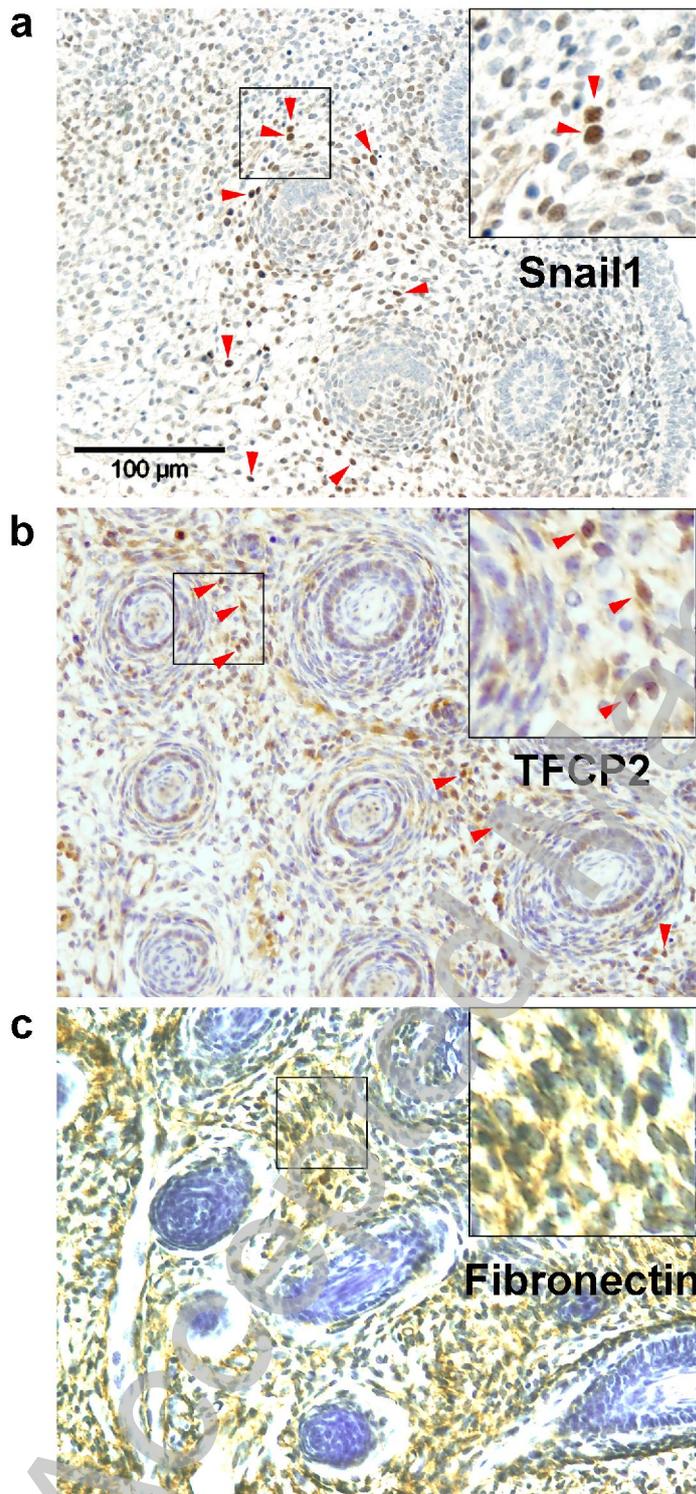
**Figure 3**



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102057

Accepted Manuscript

**Figure 4**



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102057