Supplementary Information for

The hypoxia-controlled Fbxl14 Ubiquitin Ligase targets Snail1 for proteasome degradation

Rosa Viñas-Castells, Manuel Beltran, Gabriela Valls, Irene Gómez, José Miguel García, Bàrbara Montserrat-Sentís, Josep Baulida, Félix Bonilla, Antonio García de Herreros, and Víctor M. Díaz.

Supplementary methods

Antibodies and reagents. The following antibodies were used in this study: goat anti-ubiquitin, goat anti-CtBP, rabbit anti-β-TrCP1 (Santa Cruz Biotechnology); rabbit anti-GSK-3β, rabbit anti-phosphoGSK-3β (Ser9) (Cell Signaling); rabbit anti-HA, mouse monoclonal anti-Flag, goat anti-mouse/rabbit IgGs Alexa 488 or Alexa 586 (Sigma); mouse monoclonal anti-HIF-1α, rabbit anti-pyruvate dehydrogenase kinase 1, rabbit anti-Twist1, rabbit anti-Snail1 and rabbit anti-GFP antibodies (AbCam), goat anti-GST (Amersham), mouse monoclonal anti-E-cadherin (BD Transduction Laboratories) and a mouse monoclonal against Snail1 (1). Anti-Myc antibodies were obtained from supernatants of 9E10 hybridoma. Protein G-agarose beads were from Roche. MG132 and cycloheximide (CHX) were from Sigma and polyethylenimine (PEI) from Polysciences Inc.

RNA extraction and RT-PCR. Total RNA was extracted from transfected cells with the RNeasy mini kit (Qiagen). For Snail1 amplification, 0.5 μg of total RNA were used with the QIAGEN OneStep RT-PCR kit using specific primers. For Fbxl14 and HPRT amplification 0.5 μg of total RNA were reverse-transcribed with the SuperScript™ First-Strand kit using Oligo dT (Invitrogen) according to the manufacturer's instructions and cDNA was used in semiquantitative RT-PCR. Primers used were: FB-3, for human and murine Fbxl14 (200 ng cDNA, 34 cycles); SN-1, for murine Snail1 (37 cycles); SN-2, for human SNAIL1 (37 cycles); and HP-1, for human/murine HPRT (100 ng; 34 cycles) (Suppl. Table 1) as previously indicated (2).

Transcript analysis of tumor samples. Thirty-three samples of colon adenocarcinomas and their matched normal colon mucosa (taken, at least, 3 cm from the outer tumor margin) were obtained immediately after surgery, immersed in RNA *later*™ (Ambion Inc, Austin, Texas), snap-frozen in liquid nitrogen and stored at -80^{0}C until processing. The use of these samples for the study was approved by the Research Ethics Board of the Hospital Universitario Puerta de Hierro (Madrid, Spain). RNA was extracted from tumor cells lines and from about 30 mg of tumor and normal tissues using RNeasy Mini Kit (Qiagen). RNA samples were treated with a

RNase-free DNase, DNA-free™ (Ambion), as specified in the manufacturer's protocol and nucleic acids were quantified spectrophotometrically with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, Delaware, USA).

The analysis of these samples was carried out as previously described (3). 400 ng of RNA were retro-transcribed using the Gold RNA PCR Core Kit (PE Biosystems, Foster City, CA) and random hexamers. Real-time PCR was performed in a Light-Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart^{PLUS} DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany) and oligonucleotides corresponding to human Twist1 (TW-3), Fbxl14 (FB-4) and Carbonic Anhydrase 9 (CA9) (CA-1) (see Table 1).

Expression of TWIST1 RNA was only detected in 23 tumor samples and never in normal tissues. FBXL14 RNA levels were calculated in the tumor and in normal tissues by a relative quantification approach. CA9 was not detected in normal tissues; therefore, its expression was only determined in tumor tissues. An arbitrary value (0.001), corresponding to half the minimum value detected in the series, was assigned to three tumors in which CA9 expression was not detected. The amounts in the targets genes were expressed in relation to the expression of succinate dehydrogenase complex subunit A (SDHA; primers SDHA-1) (3). The relative concentrations of targets and reference genes were calculated by interpolation, using a standard curve of each gene generated with a serial dilution of a cDNA prepared from RNA extracted from SW480-ADH cells. The expression level of FBXL14 for each patient was calculated as the ratio of its expression in tumor (T) versus its expression in normal tissue (N) (T/N).

As the gene expression values was not normally distributed (according to Kolmogorov-Smirnov test), to carry out the statistical analysis we normalized data distribution by using $\log_{10}$. Expression levels of CA9 and FBXL14 were contrasted with presence or absence of TWIST1 expression in different tumor samples by ANOVA. CA9 and FBXL14 expression levels were studied by the Pearson test. Two-tailed p values ≤ 0.05 were considered statistically significant. Statistical analysis was carried out using the SPSS statistical package, version 13.0.

Immunocytochemical staining and confocal microscopy. Cells were grown on sterile coverslips, transfected with the indicated plasmids for 24 h and fixed with PBS-3% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were permeabilized with PBS-Triton X-100 0.5% [vol/vol] for 5 min and blocked with PBS-1% BSA for 1 h at RT. Coverslips were incubated with the indicated antibodies in blocking buffer (PBS-BSA 0.1%), rinsed with PBS and incubated with purified Alexa 488 and Alexa 586-conjugated goat anti-mouse or anti-rabbit IgGs, rinsed and mounted with Fluoromount G (Southern Biotech). Fluorescence was visualized using the inverted fluorescence microscope DM IRBE (Leica, Wetzlar, Germany) and captured in a TCS-NT Argon/Kripton confocal laser microscope

(Leica, Wetzlar, Germany). Incubation with matched mouse isotype IgGs, irrelevant rabbit IgGs or secondary antibodies always yielded negative results.

Pull-down assays with GST-tagged protein beads. Purification of GST fusion proteins and pull-down assays using GST-Snail1 or GST-Fbxl14 fusion proteins were performed as described (4).

References

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