

Article

# Nuclear Respiratory Factor-1, a Novel SMAD4 Binding Protein, Represses TGF- $\beta$ /SMAD4 Signaling by Functioning as A Transcriptional Cofactor

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## Supplementary Materials and Methods

### 1.1 Competition bimolecular fluorescence complementation (BiFC) assays

For the BiFC assays, HeLa cells were transiently co-transfected with 0.5  $\mu$ g Flag-VN173-tagged NRF1 domains (full length, NRF 1-108, NRF 108-304, or NRF 304-503), 0.5  $\mu$ g VC155-tagged SMAD4 domains (full length, MH1, Linker, or MH2) with or without 0.5  $\mu$ g of 3xFLAG-tagged full length NRF1. BiFC analysis was performed 24 h post-treatment. The detectable fluorescence signal indicates protein-protein interactions after co-transfection of the VC155 and VN173 plasmids [24]. The fluorescence signals were captured using a charge-coupled device (CCD) camera mounted on a TE2000-U inverted fluorescence microscope (Nikon, Melville, NY) with JP4 filters (Chroma, Rockingham, VT, USA).

### 1.2 DNA pull-down assays

DNA pull-down assays were performed as previously described [3]. Briefly, 5  $\mu$ g biotinylated double-stranded oligonucleotides corresponding to the NRF1 nuclear binding elements (NBEs) for the wild-type *SMAD4* promoter (Forward 5' -CTC CGC CCG CGC TGA GGC CCA GGC CC- 3' and Reverse 3' -GGG CCT GGG CCT CAG CGC GGG CGG AG -5'), and the mutant (Forward 5' -CTC CGC CCA TTA TGT AAT CCA GGC CC- 3' and Reverse 3' -GGG CCT GGA TTA CAT AAT GGG CGG AG- 5') were obtained by PCR amplification using synthetic biotinylated primers (Bioneer, Daejeon, Korea). DNA pull-downs were performed using 100  $\mu$ g total cell lysate (HeLa cells transfected

with 5 µg of 3xFlag-NRF1) in buffer containing 50 mM Tris-HCl, pH 7.5, 10% Glycerol, 140 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EDTA, and 10% Triton X-100; the buffer was supplemented with protease inhibitor at 1:1000 (all from Sigma-Aldrich, St Louis, MO, USA). Due to the affinity of Dynabeads Streptavidin (DynaL M270, Thermo Fischer Scientific Inc.) for biotinylated DNA, the DNA-protein complexes were pulled down with Dynabeads Streptavidin by centrifugation at 200 × g for 60 s. The pulled-down complexes were washed three times with 1 mL ice-cold Tris buffer (50 mM Tris, 100 mM KCl, 1mM EDTA, 5% glycerol, and 1 mM DTT), separated on an SDS-polyacrylamide gel, and visualized using western blot analysis.

The *p15INK4b* DNA pull-down assay was performed similarly using biotinylated oligonucleotides corresponding to the NBE in the WT (Forward 5' -CAG TCT CTG GCG CAT GCG TCC TAG CAT C -3' and Reverse 3' -GAT GCT AGG ACG CAT GCG CCA GAG ACT G -5') and mutant (Forward 5' -CAG TCT CTG ATT AAT TAT TCC TAG CAT C -3' and Reverse 3' -GAT GCT AGG AAT AAT TAA TCA GAG ACT G -5') *p15INK4b* promoter.

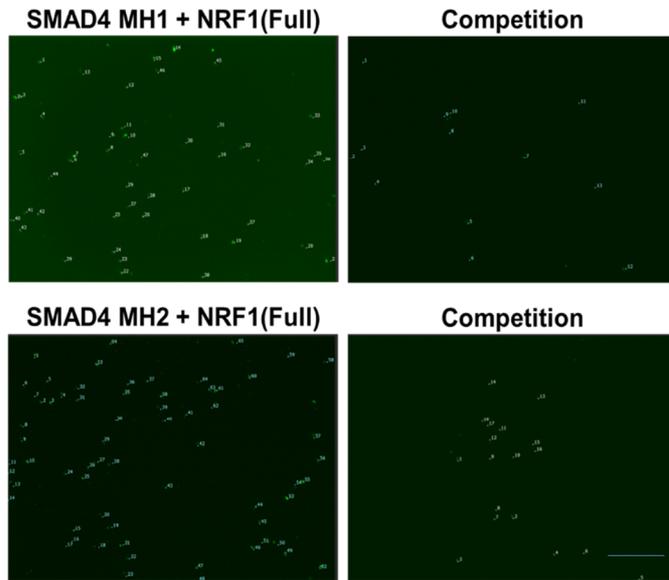
**Supplementary Table S1.** List of qRT-PCR primers and probes.

NRF1 (S)	CAG TCA CTA TGG CGC TTA ACA
NRF1 (A)	ATC TGT CCC CCA CCT TGT AA
TaqMan probe (S)	FAM – GGC TGA GG – Dark quencher
SMAD4 (S)	TGG CCC AGG ATC AGT AGG T
SMAD4 (A)	CAT CAA CAC CAA TTC CAG CA
TaqMan probe (S)	FAM - CTC CAG CT – Dark quencher
p15INK4b (S)	CAA CGG AGT CAA CCG TTT C
p15INK4b (A)	GGT GAG AGT GGC AGG GTC T
TaqMan probe (S)	FAM - TCC AGG TC – Dark quencher

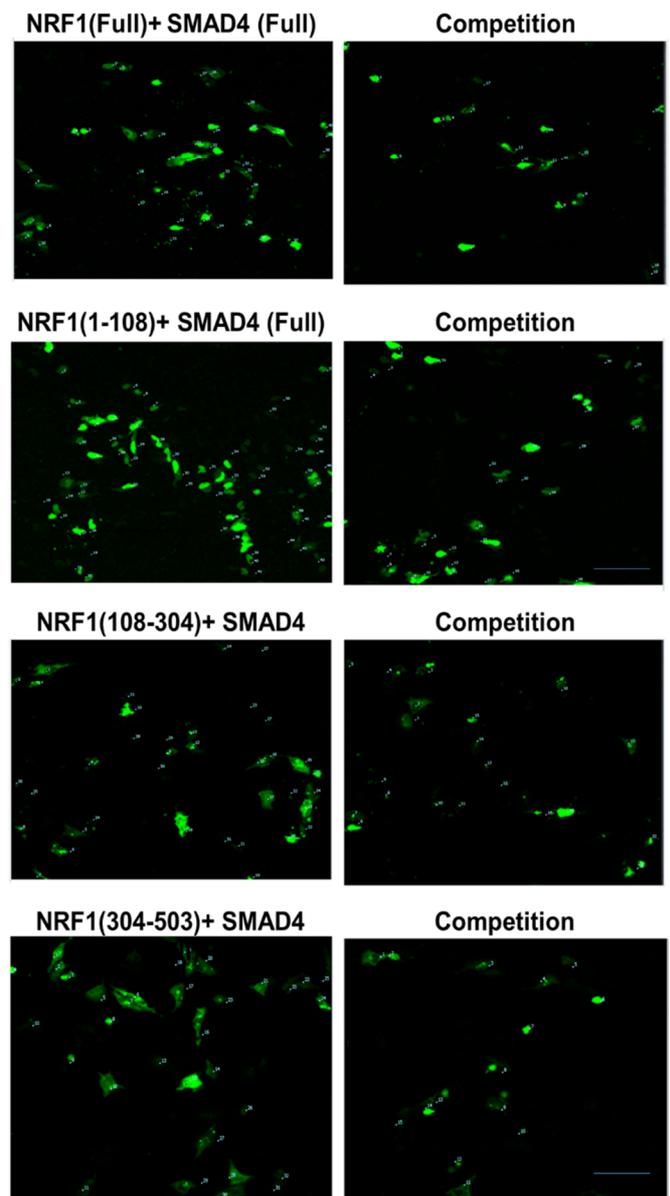
# Supplementary Figures (S1-S5)

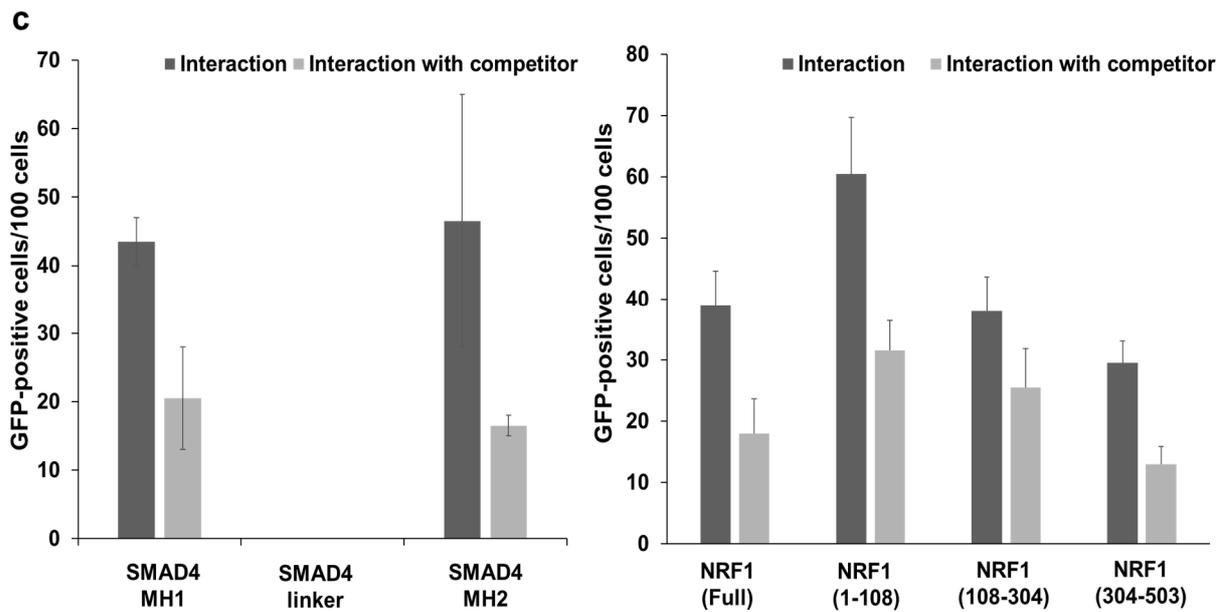
## Supplementary Figure S1.

**a**



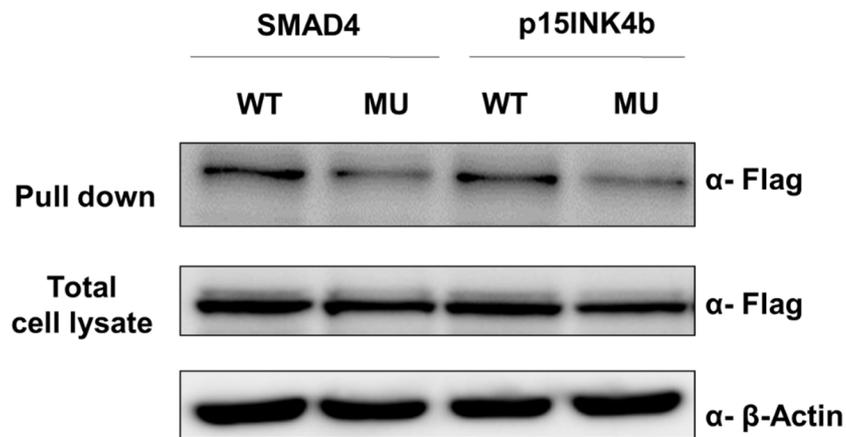
**b**





**Supplementary Figure S1.** Specific binding confirmation using the BiFC competition assay. HeLa cells transiently transfected with **a** *FLAG-VN173*-tagged NRF1 or its truncated constructs and **b** *HA-VC155*-tagged SMAD4 or its truncated constructs with NRF1 and SMAD4 without tagging as competitor. BiFC analysis was performed 24 h post-transfection. **c** for the competition assay, HeLa cells were co-transfected with plasmids encoding *FLAG-VN173*-tagged NRF1 and *HA-VC155*-tagged SMAD4 in the absence or presence of co-transfection with the 3xFLAG-tagged NRF1 plasmid (0.5 mg). The fluorescence signal was captured under fluorescence microscopy (x10) and counted in 5 random areas. GFP-positive cells were normalized with total cell counts and represented counts/100 cells. Scale bar, 100  $\mu$ m. Values are the means  $\pm$  SD.

Supplementary Figure S2.



**Supplementary Fig S2. DNA pull-down assay.** NRF1 can be recruited to the NRF1 binding elements (NBE) located on the *SMAD4* and *p15INK4b* promoters. The binding of 3xFLAG-tagged NRF1 on the NBE of *SMAD4* (WT), *SMAD4* (mutant), *p15INK4b* (WT), and *p15INK4b* (mutant) promoter sequences was confirmed by a DNA pull-down assay. The results indicate that introducing mutations in the NBE affects NRF1 recruitment.

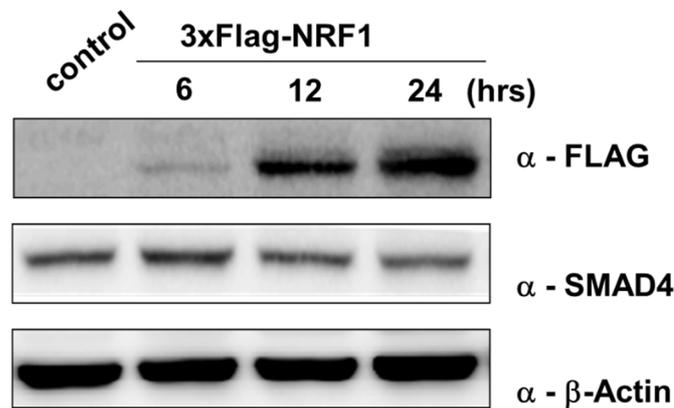
### Supplementary Figure S3.

- 432

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ctacaacctagccacctctccccagagctgtcgactggctgttgaaggccaatTTTTgtgcctacgcaggctctcaacaca  
gaacaaaacaaaaaacaacaaaggccgggctaataagctattataaacacttactggacgccactctacgccgag  
ctctcccgcgctccttgatactTTTTgcaacgagatgccaattccccggcgaccactccctcaaacaggccttcgctcc  
gccc gcgctgagggc ccaggcccagggtccagattcagagccgccgcccggctggcgctgccctgtag gcgcctgcgc  
agagcgaccctccccgtcactcggagcgggagggcgggggcagccgggagaaaggaaagctgcgggggaaaaggg  
ccaaaccctgaaattaccgggatgtggtcc ccgcgcgcgcgc at gctcagtggc tctcgacaagttggcagcaac.....  
↑  
+1 (Transcription start site)
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**Supplementary Figure S3.** NRF1 binding sites within the *SMAD4* promoter region - 500bp to the transcription start site was identified using MatInspector professional software and the internet-based TFSEARCH database.

### Supplementary Figure S4.



**Supplementary Figure S4.** SMAD4 expression is affected by NRF1 overexpression. Western blot results show changes in SMAD4 expression with an incremental increase in NRF expression.

Supplementary Figure S5.

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-716 GGATCTCAGATTCTTAAAAGTATAAATTTTTTTTTGTCTTATGTGTGCCAGGTTGCCACTCT -657
-656 CAATCTCGAACTAGTTTTTTTCTCTTTTAAGGGTTGTATCCATAATGCAAAAATGGAAAG -597
-596 AATTA AAAAGCACACGCAAAACATGATTCTCGGGATTTTTCTCTATTTTTATGGTTGACT -537
-536 AAT TCAAACA GAAA GACA CATCC AAGAGAAAATTGCTAAGTTTGATACAAGTTATGAAAC -477
-476 TTGTGAAGCC CAAGTACTGCCTGGGGATGAATTTAACTTGTATGACAGGTGCAGAGCTGT -417
-416 CGCTTTC AGACAT CTTAAGAAA CA CGGAGTTATTTTGAATGACTTTCCTC GGTCACAAG -357
-356 GGAGCCACCAACGTCTCCACAGTGAAACCAACTGGCTGGCTGAAGGAACAGAAATCCTCT -297
-296 GCTCCGCCACTGCGGGATTAGGAGCTGAGGGCAGTGGTGAACATTCCAAAAATATTAGCC -237
-236 TTGGCTTTACTGGACATCCAGCGAGCAGTGCAGCCAGCATTCTGGCGGCTCCCTGGCCC -177
-176 AGTCTCT GGCGCAT GCGTC CTAGCATCTTTGGGCAGGCTTCCC CGCCCTCGTGACGCGTC -117
-116 GGCCCGGGCTGGCCTCCCGGC GATCACAGCGGACAGGGGGCGGAGCCTAAGGGGGTGGG -57
-56 GAGACGCCGGCCCTTGGCCAGCTGAAAAC GGAATTCTTTGCCGGCTGGCTCCCCACTC 3
 4 TGCCAGAGCGAGCGGGGCGAGTGAGGACT CCGCGACGCGTC CGCAC CCCTG CGGC CAGAGC 63
 64 GGCTTTGAGCTCGGCTGCGT CCGCGCTAGGCG CTTTT TCCAG AAGCAAT CCAGGCGCGC 123
124 CCGCTGGT TCTTGAGCGCCAGGAAAAGCC CGGAGCTA CGACCGG CCGCTCGG CCACTGC 183

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**NRF1**

**NT 158-169**

**SMAD4**

**NT 523-520, 406- 409**

Supplementary Figure S5. The distal region of the p15INK4b promoter. SMAD4 and NRF1 binding sites within the p15INK4b promoter are highlighted as indicated.