

- µg by addition of nonrecombinant pCDNA3, as necessary. Results represent three to six different transfected cultures, with the experiments performed on at least two different days. For dark conditions, plates of NIH 3T3 cells were wrapped in foil immediately after transfection until the time of harvesting (48 hours), except for a single brief medium change performed under dim red light (Kodak Safelight, Wratten Series 1A) 24 hours before harvesting.
18. E. A. Griffin Jr., D. Staknis, C. J. Weitz, unpublished data.
  19. Transfected cells under dark (17) and light conditions were processed in parallel and incubated together. For light conditions, cells were illuminated (Fiber-Lite High Intensity Illuminator, Series 180; set at 10 relative illumination units) immediately after transfection until the time of harvesting. Plates were positioned 15 to 30 cm from the tips of the twin fiber-optic guides.
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## Negative Feedback Regulation of TGF-β Signaling by the SnoN Oncoprotein

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Smad proteins mediate transforming growth factor-β (TGF-β) signaling to regulate cell growth and differentiation. The SnoN oncoprotein was found to interact with Smad2 and Smad4 and to repress their abilities to activate transcription through recruitment of the transcriptional corepressor N-CoR. Immediately after TGF-β stimulation, SnoN is rapidly degraded by the nuclear accumulation of Smad3, allowing the activation of TGF-β target genes. By 2 hours, TGF-β induces a marked increase in SnoN expression, resulting in termination of Smad-mediated transactivation. Thus, SnoN maintains the repressed state of TGF-β-responsive genes in the absence of ligand and participates in negative feedback regulation of TGF-β signaling.

TGF-β signals through the Smad proteins to regulate cell growth and extracellular matrix production (1). In the absence of TGF-β, Smad proteins are distributed in the nucleus and cytoplasm (2–5). Upon stimulation by TGF-β1, Smad2 and Smad3 become phosphorylated by the activated TGF-β receptors and oligomerize with Smad4 (4–7). These Smad complexes then accumulate in the nucleus where they interact with other transcription factors (8), bind to DNA through their NH<sub>2</sub>-terminal Mad homology-1 (MH1) domains (9–11), and activate transcription of TGF-β-responsive genes through the COOH-terminal MH2 domains (3, 5, 7). As the common mediator, Smad4 complexes with various pathway-restricted Smads to transduce signals by TGF-β, bone morphogenic proteins, and activin (5, 7).

To identify new components of the Smad pathway, we used epitope tagging coupled with affinity purification to isolate Smad4-associated proteins (12). Flag-tagged full-length or truncated Smad4 was stably transfected into 293T cells. Cellular proteins associated with Smad4 were affinity-purified with anti-Flag agarose followed by elution

with the Flag peptide and visualization by silver staining (13). Two proteins of 80 and 97 kD associated predominantly with the MH2 domain of Smad4 (S4C) (Fig. 1A), less strongly with the full-length Smad4 (14), but not with the MH1 and linker domains (S4NL). Microsequencing analysis (13) identified the 80-kD protein as the human c-SnoN and the 97-kD protein as c-Ski (15, 16). SnoN and Ski are two closely related members of the Ski family of nuclear oncoproteins that were identified on the basis of homology with v-Ski, the transforming protein of the Sloan-Kettering virus (17). Human SnoN is a ubiquitously expressed nuclear protein of 684 amino acids (15, 18). Overexpression of SnoN leads to transformation of chicken and quail embryo fibroblasts as well as muscle differentiation of quail embryo cells (19). High levels of SnoN were also detected in carcinoma cells of the stomach, thyroid, and lung (15). SnoN is a transcriptional repressor and interacts with the nuclear hormone receptor corepressor N-CoR (20). Both oncogenic transformation and transcriptional repression require the NH<sub>2</sub>-terminal portion of SnoN (20, 21), which is highly homologous to c-Ski and v-Ski (15).

In 293T cells cotransfected with various Flag-tagged Smad proteins and hemagglutinin (HA)-tagged SnoN, SnoN coprecipitated with Smad4 or Smad2 (Fig. 1B) but not with Smad1, Smad5, and Fast1 (22). A low level of SnoN associated with Smad3, partly as a

result of reduced SnoN expression in cells cotransfected with Smad3 (Fig. 1B). This Smad3-mediated reduction of SnoN expression is reproducible and important (see below). Purified recombinant Smads and SnoN can also interact in vitro (14), indicating a direct interaction between the two. Associations of SnoN with the Smads were mediated by the MH2, but not by the MH1 domains of the Smads (Fig. 1B). Binding of SnoN to Smad2 (Fig. 1C) and Smad3 (14) required the NH<sub>2</sub>-terminal 96 amino acids of SnoN, whereas residues 138 to 255 in SnoN mediated interaction with Smad4 (Fig. 1C).

SnoN binds to a specific DNA element, GTCTAGAC, but only with the help of an unidentified DNA binding partner (or partners) (21). Because this sequence is identical to the Smad-binding element (SBE) (11), we examined whether Smad4 is the DNA binding partner of SnoN. Whereas purified SnoN proteins failed to bind DNA in an electrophoretic mobility shift assay (EMSA) (10), the Flag-SnoN-HA-Smad4 complex bound to the SBE (Fig. 2A). Furthermore, anti-Flag or anti-HA supershifted the complex, confirming the presence of both SnoN and Smad4. Thus, Smad4 mediates binding of SnoN to SBE.

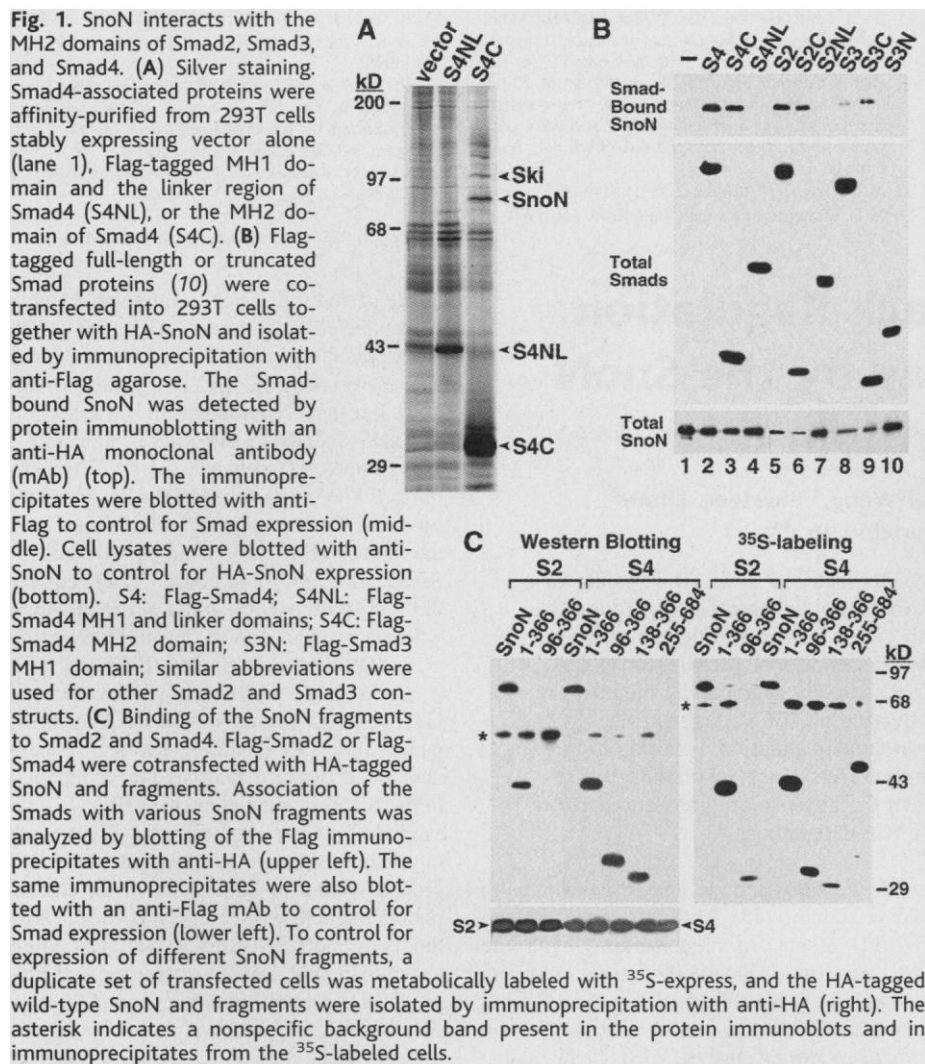
The effect of SnoN on TGF-β-induced and Smad-mediated transcriptional activation was examined in Hep3B cells. Expression of increasing amounts of SnoN resulted in a stronger inhibition of transcription from the 3TP-lux promoter, induced either by TGF-β (Fig. 2B) or by overexpression of Smad2 and Smad4 (Fig. 2C), but had little or no effect whenever Smad3 was also coexpressed. The NH<sub>2</sub>-terminal 366 amino acids of SnoN are sufficient for binding to the Smads, and this region also mediated transcriptional repression (22). On the other hand, a SnoN fragment containing residues 255 to 684 failed to bind to all three Smads (Fig. 1C) and did not repress TGF-β-induced transactivation (22).

SnoN repressed transactivation of Smads not by blocking the hetero-oligomerization of the Smads (14), but by recruitment of the transcriptional corepressor N-CoR. In nuclear extracts of 293T cells cotransfected with HA-Smad4 and Flag-SnoN, endogenous N-CoR was detected in the anti-HA immunoprecipitate (Fig. 2D). Thus, N-CoR formed a complex with Smad4 and Smad2 (14), but only when SnoN was coexpressed (Fig. 2D). Be-

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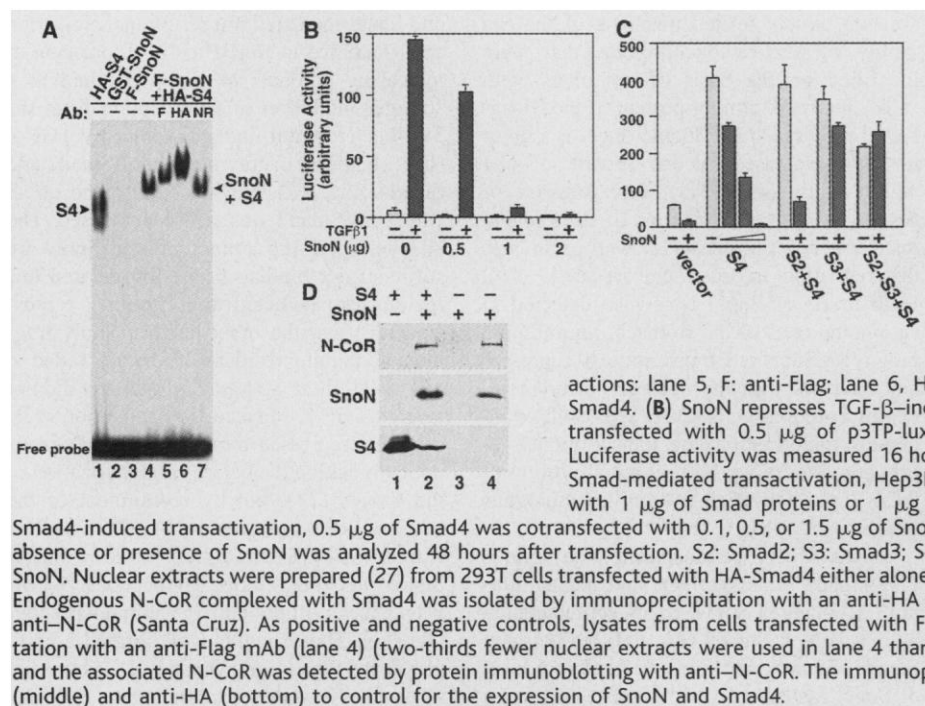
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cause N-CoR binds directly to mSin3 and the histone deacetylase complex (HDAC1) (23), SnoN may recruit a transcriptional repressor complex to the TGF- $\beta$ -responsive promoter to repress transcription.

Thus, SnoN can interact with Smad4 and Smad2 and inhibit their abilities to activate transcription. However, SnoN had little effect when Smad3 was also coexpressed (Fig. 2C). In earlier experiments it was difficult to detect SnoN in cells overexpressing Smad3, raising the possibility that Smad3 may down-regulate SnoN expression. Indeed, in cells transfected with a fixed amount of SnoN and varying amounts of the Smads, increasing levels of Smad3, but not Smad4, in the nuclear extracts were accompanied by a decrease in SnoN expression (Fig. 3A). Smad3-mediated down-regulation of SnoN was not caused by a decrease in the synthesis rate of SnoN (14), but by a reduction in the half-life of SnoN (less than 1 hour versus 4 to 5 hours in the absence of Smad3) (Fig. 3B) (24). The rapid degradation of SnoN appeared to require the COOH-terminal portion of SnoN, because a mutant SnoN containing only residues 1 to 366 was not degraded by Smad3 coexpression (22), although this molecule can still interact with Smad3 weakly (14). This mutant was also more potent than the full-length SnoN in repression of TGF- $\beta$ -induced transactivation (22), probably because of its relatively high stability.

Because TGF- $\beta$  inhibits the growth of many cell types, we speculated that overexpression of SnoN may render these cells resistant to TGF- $\beta$ -induced growth arrest. However, because TGF- $\beta$ -induced activation



(A) SnoN binds to the SBE through interaction with Smad4. <sup>32</sup>P-labeled SBE oligonucleotide ( $2 \times 10^4$  cpm) was incubated with various purified proteins in EMSA reactions. Proteins used: lane 1: HA-Smad4 (HA-S4) isolated from 293T cells transfected with HA-Smad4 alone; lane 2: 0.8  $\mu$ g of GST-SnoN containing the full-length SnoN fused to GST; lane 3: Flag-SnoN (F-SnoN) purified from singly transfected 293T cells; lanes 4 to 7: Flag-SnoN-HA-Smad4 complex (F-SnoN + HA-S4) purified from cotransfected 293T cells with anti-Flag agarose. The amounts of HA-Smad4 and Flag-SnoN in the above reactions were normalized by protein immunoblotting. Antibodies used in supershift reactions: lane 5, F: anti-Flag; lane 6, HA: anti-HA; lane 7, NR: nonrelevant antibody. S4: Smad4.

(B) SnoN represses TGF- $\beta$ -induced transcriptional activation. Hep3B cells were transfected with 0.5  $\mu$ g of p3TP-lux and increasing amounts of SnoN as indicated. Luciferase activity was measured 16 hours after TGF- $\beta$ 1 stimulation. (C) For repression of Smad-mediated transactivation, Hep3B cells were cotransfected with p3TP-lux, together with 1  $\mu$ g of Smad proteins or 1  $\mu$ g of SnoN (or both), as indicated. For repression of

actions: lane 5, F: anti-Flag; lane 6, HA: anti-HA; lane 7, NR: nonrelevant antibody. S4: Smad4. (D) Recruitment of N-CoR to Smad4 by SnoN. Nuclear extracts were prepared (27) from 293T cells transfected with HA-Smad4 either alone (lane 1) or together with Flag-SnoN (lane 2). (Top) Endogenous N-CoR complexed with Smad4 was isolated by immunoprecipitation with an anti-HA mAb and detected by protein immunoblotting with anti-N-CoR (Santa Cruz). As positive and negative controls, lysates from cells transfected with Flag-SnoN alone were subjected to immunoprecipitation with an anti-Flag mAb (lane 4) (two-thirds fewer nuclear extracts were used in lane 4 than in lanes 1 to 3) or with an anti-HA mAb (lane 3), and the associated N-CoR was detected by protein immunoblotting with anti-N-CoR. The immunoprecipitates were also blotted directly with anti-Flag (middle) and anti-HA (bottom) to control for the expression of SnoN and Smad4.

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of Smad3 could down-regulate SnoN, the effect of the full-length SnoN, if not highly overexpressed, may not be apparent. Indeed, when introduced into a TGF- $\beta$ -responsive pro-B cell line, Ba/F3 (25), SnoN reduced the growth inhibitory responses only moderately (from 90 to 60% inhibition) (Fig. 3C). In contrast, overexpression of the more-stable SnoN(1-366) substantially impaired the ability of Ba/F3 cells to undergo growth arrest in response to TGF- $\beta$ 1 (30% inhibition) (Fig. 3C). These results are consistent with the previous observation that SnoN induces oncogenic transformation only when highly overexpressed (19).

Finally, the effect of TGF- $\beta$  on the level of SnoN mRNA was examined in Hep3B (Fig. 4A) and Ba/F3 cells (14) by Northern (RNA) blotting. A marked increase in SnoN mRNA was observed 1 to 2 hours after TGF- $\beta$ 1 stimulation and lasted for at least 24 hours (Fig. 4A), indicating that SnoN is an early TGF- $\beta$ -responsive gene. The induction of SnoN expression by TGF- $\beta$ 1 suggests that SnoN participates in the negative feedback control of TGF- $\beta$  signaling.

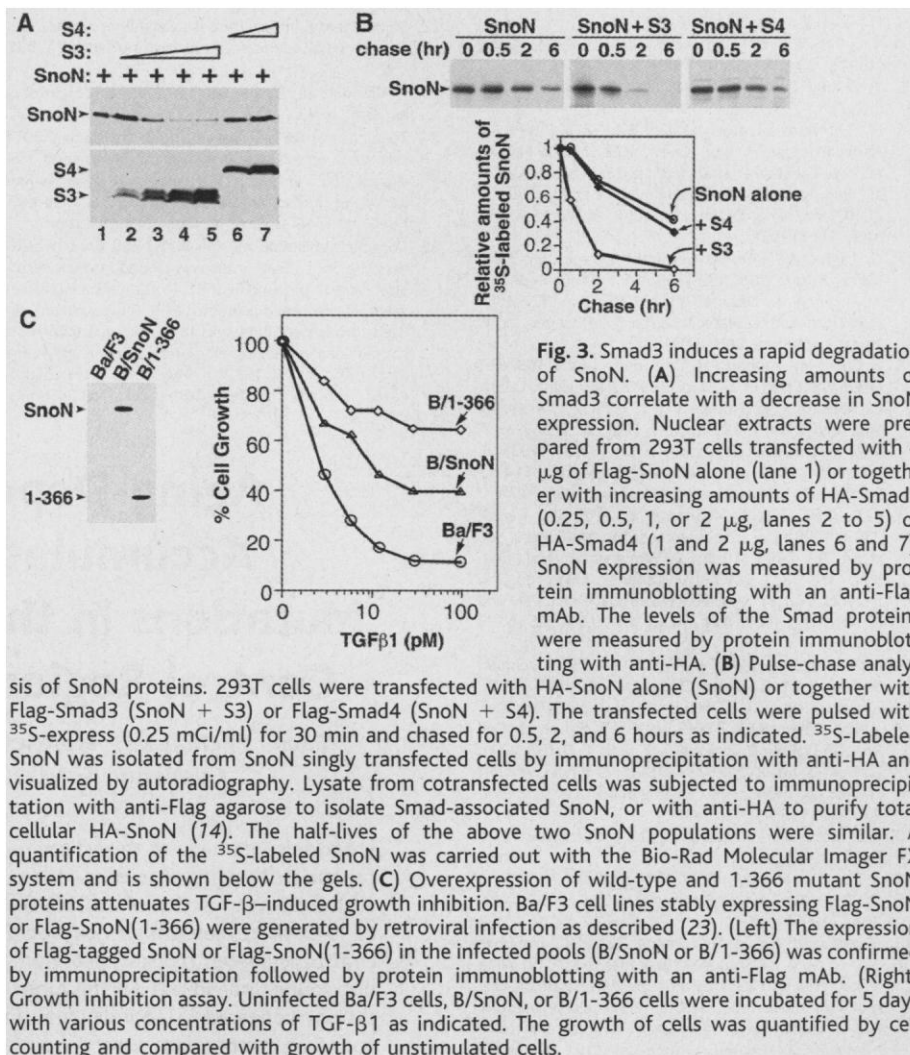
On the basis of these results, we propose a model of regulation of TGF- $\beta$  signaling by SnoN. In the absence of TGF- $\beta$ , SnoN binds to the nuclear Smad4 and represses TGF- $\beta$ -responsive promoter activity through recruitment of a nuclear repressor complex. TGF- $\beta$  induces activation and nuclear translocation of Smad2, Smad3, and Smad4. Smad3 causes degradation of SnoN, allowing a Smad2 and Smad4 complex to activate TGF- $\beta$  target genes. To initiate a negative feedback mechanism that permits a precise and timely regulation of TGF- $\beta$  signaling, TGF- $\beta$  also induces an increased expression of SnoN at a later stage, which in turn binds to Smad heteromeric complexes and shuts off TGF- $\beta$  signaling.

This model predicts that the cellular level of SnoN should decrease shortly after TGF- $\beta$  stimulation, followed by an increase at a later stage. We therefore measured the level of endogenous SnoN in nuclear extracts of Hep3B cells that had been stimulated with TGF- $\beta$ 1 for various amounts of time (Fig. 4B). In the absence of TGF- $\beta$ 1, SnoN associated with endogenous Smad4. A decrease in SnoN occurred at 15 min after TGF- $\beta$ 1 addition and reached its lowest level by 30 min (Fig. 4B). SnoN was again detected 1 hour after stimulation and by 2 hours reached a level even higher than that in the absence of TGF- $\beta$ , because of an increase of the SnoN mRNA. Very high levels of SnoN were either not degraded efficiently by Smad3 (14), or Smad3 may be sequestered and unavailable for degradation of SnoN at a later time point.

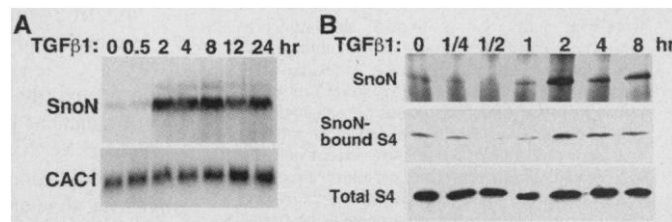
Thus, SnoN maintains the repressed state of TGF- $\beta$  target genes in the absence of ligand and participates in the negative feedback regulation of TGF- $\beta$  signaling. This

differs from the action of the closely related Ski. Although Ski also binds to the Smads and represses TGF- $\beta$  signaling, its expression was not affected strongly by TGF- $\beta$  or Smad3 (16). Furthermore, Ski strongly repressed transactivation by Smad3, whereas SnoN had little effect. Both Ski and SnoN are transcriptional repressors. By repressing TGF- $\beta$ -induced growth inhibition or retinoic

acid receptor signaling (26), they serve as positive regulators of cell growth. Because Smad proteins are important tumor suppressors, the ability of high levels of SnoN to repress TGF- $\beta$  signaling could be responsible, at least partially, for the transforming activity of SnoN. By cooperating with other proteins important in cell differentiation such as Mad and thyroid hormone receptor (20),



**Fig. 4. (A)** TGF- $\beta$ 1 activates SnoN expression. Hep3B cells were serum-starved for 16 hours and stimulated with 100 pM TGF- $\beta$ 1 for various amounts of time as indicated. Total cellular RNA was isolated from these cells, and SnoN expression was analyzed by Northern blotting. The human chromatin assembly complex-1 (CAC1) was used as a control for equal loading. **(B)** Regulation of endogenous SnoN expression by TGF- $\beta$ 1. Hep3B cells were stimulated with 200 pM TGF- $\beta$ 1 for 15 min, 30 min, or 1, 2, 4, and 8 hours as indicated. Nuclear extracts were prepared from these cells, and endogenous SnoN was isolated by immunoprecipitation with an anti-SnoN peptide antiserum (28) and analyzed by protein immunoblotting with anti-SnoN (top). SnoN-associated nuclear Smad4 was detected from the same immunoprecipitates with anti-Smad4 (middle). Total cellular Smad4 was purified by immunoprecipitation with anti-Smad4 and analyzed by protein immunoblotting with anti-Smad4 (bottom).



they may induce terminal differentiation of certain cell types. These are consistent with the previous observation that Ski and SnoN induce oncogenic transformation and muscle differentiation of quail embryo cells.

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- Flag-tagged full-length Smad4, Smad4C (residues 319 to 551), or Smad4NL (residues 1 to 318) were transfected into 293T cells by the Lipofectamine-Plus method (Gibco-BRL). Stable 293T cells expressing Flag-Smad4C were generated by cotransfecting pCMV5B-Flag-Smad4C with a construct expressing the puromycin resistance gene, followed by selection in the presence of puromycin (1  $\mu$ g/ml, Sigma). For large-scale purification of Smad4C-associated proteins,  $2 \times 10^9$  cells were lysed in buffer containing 50 mM Hepes (pH 7.8), 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 3 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Lysates were then applied to anti-Flag M2 agarose (Sigma) (30  $\mu$ l of anti-Flag agarose per milliliter of lysate). After extensive washing, Smad4C and its associated proteins were eluted with Flag peptide (0.4 mg/ml) (72). The proteins were resolved on a 10% low-Bis polyacrylamide gel, transferred to nitrocellulose membrane, and digested with Lys-C. Five peptides from the 80-kD protein were sequenced and showed a perfect match to c-SnoN: KILIEEMK (amino acid residues 345 to 452 in SnoN), KTDAPSGMELQS (residues 366 to 377), KTVSYPDVSLLE (residues 449 to 460), KVGIGLVAAASS (residues 503 to 514), and KLEMMIK (residues 663 to 669). Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; and Y, Tyr.
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- To generate stable murine Ba/F3 cell lines overexpressing SnoN, Flag-SnoN was cloned into the retroviral vector pMX-IRES-GFP (4) that also expresses green fluorescence protein (GFP). The construct was used to transfect Bing cells to generate amphotropic retroviruses expressing Flag-SnoN. Forty-eight hours after transfection,  $1 \times 10^7$  Ba/F3 cells were cocultivated with the transfected Bing cells for 24 hours, and the infected cells were selected by cell sorting on the basis of GFP expression.
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- Two polyclonal antibodies to SnoN were raised, one against a glutathione-S-transferase (GST) fusion protein containing residues 1 to 366 of the human SnoN protein [antibody (Ab) 1330], and the other against a peptide located at the COOH-terminus of human SnoN (KELKLIKSSKTAKE). For immunoprecipitation of endogenous SnoN, the peptide antibody was covalently coupled to protein A Sepharose and incubated with lysates from Hep3B cells stimulated with or without TGF- $\beta$ 1. SnoN bound to the antibody column was then eluted with an excess amount of immunizing peptide and analyzed by protein immunoblotting with anti-Smad4 (Santa Cruz) or with anti-SnoN (Ab 1330).
- We thank S. Pearson-White for providing the c-SnoN cDNA, P. Kaufman for the human CAC1 probe, and H. Nolla for help with the FACS analysis. Supported by U.S. Department of Energy (DOE)-LBNL grant DE-AC03-76SF00098, DOE/OBER grant DE-AC03-76SF00099, Wendy Will Case Cancer Fund, California breast cancer research program award, and March of Dimes research grant to K.L. S.L.S. was supported by a predoctoral fellowship from the National Science Foundation.

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## Aging-Dependent Large Accumulation of Point Mutations in the Human mtDNA Control Region for Replication

Yuichi Michikawa,<sup>1</sup> Franca Mazzucchelli,<sup>2</sup> Nereo Bresolin,<sup>2</sup> Guglielmo Scarlato,<sup>2</sup> Giuseppe Attardi\*<sup>1</sup>

Progressive damage to mitochondrial DNA (mtDNA) during life is thought to contribute to aging processes. However, this idea has been difficult to reconcile with the small fraction of mtDNA so far found to be altered. Here, examination of mtDNA revealed high copy point mutations at specific positions in the control region for replication of human fibroblast mtDNA from normal old, but not young, individuals. Furthermore, in longitudinal studies, one or more mutations appeared in an individual only at an advanced age. Some mutations appeared in more than one individual. Most strikingly, a T414G transversion was found, in a generally high proportion (up to 50 percent) of mtDNA molecules, in 8 of 14 individuals above 65 years of age (57 percent) but was absent in 13 younger individuals.

One postulated cause of aging is the accumulation of mutations in mtDNA (1). This notion is supported by the observation of an aging-related accumulation in human mtDNA of oxidative and alkylation derivatives of nucleotides (2), of small deletions and insertions (2), and of large deletions (3), although their low frequency (<1 to 2%) has raised questions about their functional significance. Furthermore, it has not been clear whether there is an accumulation of aging-dependent point mutations in human mtDNA (4), due in part to the lack of a reliable method for detecting heteroplasmic mutations (that is, mutations that occur together with wild-type mtDNA) and to the search having been largely limited to the protein- and RNA-coding regions of mtDNA.

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### <sup>8</sup> **Regulation of the Human p21/WAF1/Cip1 Promoter in Hepatic Cells by Functional Interactions between Sp1 and Smad Family Members**

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<sup>26</sup>**Transformation of Hematopoietic Cells by the Ski Oncoprotein Involves Repression of Retinoic Acid Receptor Signaling**

Richard Dahl; Matthias Kieslinger; Hartmut Beug; Michael J. Hayman

*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, No. 19. (Sep. 15, 1998), pp. 11187-11192.

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