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Article

Protein Association and Nucleocytoplasmic Distribution: An Experimental and Modeling Study

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Abstract: Protein abundance can shape signaling not only by changing how much of a signaling component is available, but also by altering the molecular states that component occupies. Here, we examined this principle in the Smad2/3 branch of the TGF-β superfamily. Using lentivirally expressed fluorescent R-Smads in HEK293T cells, we found that nuclear Smad2 accumulation increased disproportionately across the observed range of Smad2-GFP abundance. A minimal compartmental model reproduced this abundance-dependent behavior when concentration-dependent R-Smad dimerization was included, whereas a reduced model containing monomeric shuttling alone produced an approximately proportional response. FRET measurements further showed receptor-sensitive proximity between Smad2-GFP and Smad3-RFP, providing independent evidence for R-Smad association in the experimental system. A comparison nucleocytoplasmic shuttling system displayed a more nearly proportional abundance-localization relationship, and increasing the intracellular Smad pool preserved the characteristic Smad localization pattern. Taken together, these observations support a mechanism in which concentration-dependent R-Smad association links protein abundance to spatial redistribution between cytoplasm and nucleus. This provides a physical route by which variation in signaling-protein abundance can be converted into nonlinear intracellular localization dynamics.

Keywords: TGF-β superfamily signaling; R-Smad; Smad2; dimerization; nucleocytoplasmic transport; nonlinear dynamics

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1. Introduction

Signal transduction is determined by more than the presence or absence of pathway components. The amount of each component, the molecular partners it encounters, and the compartment in which it resides can all influence the state of a signaling network. Protein abundance is therefore potentially instructive: changing concentration can shift association equilibria and, when different molecular states move differently between compartments, alter the spatial organization of the signal itself [1].

The transforming growth factor-β (TGF-β) superfamily provides a suitable system in which to examine this problem. Signaling through this superfamily regulates proliferation, differentiation, survival, migration, tissue homeostasis, development, and pathological processes including cancer [2]. In the Smad2/3 branch, ligand-dependent activation of type I and type II receptor complexes leads to phosphorylation of receptor-regulated Smads (R-Smads). Activated R-Smads associate with other Smad proteins and accumulate in the nucleus, where they participate in transcriptional regulation. Nuclear

localization of R-Smads is therefore a central step linking receptor activity to downstream transcriptional control [3].

Among the principal R-Smads of the TGF-β/Activin branch, Smad2 and Smad3 are highly homologous and share several regulatory properties, yet their biological functions are not always equivalent. They can act redundantly in some contexts while displaying distinct or even opposing functions in others. These observations indicate that R-Smad signaling cannot be understood solely from the presence of individual pathway components and suggest that interactions among Smad molecules contribute to the way signaling information is processed [4].

R-Smad association offers a simple physical basis for such abundance dependence. Association reactions become increasingly favored as the concentrations of their interacting species rise. If monomeric and associated R-Smads differ in their nucleocytoplasmic behavior, an increase in total R-Smad abundance need not produce a proportional increase in nuclear signal. Instead, concentration can be translated into a change in the distribution of molecular states and consequently into a nonlinear change in nuclear accumulation [5]. Structural and biochemical studies showing activation-dependent Smad oligomerization make this mechanism plausible within the established biology of the pathway.

We tested this idea by combining quantitative fluorescence imaging with a minimal compartmental model. The experimental analysis first asks whether Smad2 abundance and nuclear accumulation remain proportional across individual cells. The model then asks whether concentration-dependent association is sufficient to account for the observed relationship and what behavior is expected when that association step is removed [6]. FRET measurements provide an independent readout of R-Smad proximity, while a comparison shuttling system and an increased intracellular Smad pool provide additional tests of the predicted abundance-localization behavior. This design allows the central mechanism to be evaluated through several complementary observations rather than through a single experimental readout [7].

2. Materials and Methods

2.1 Cell Lines and Lentiviral Transduction

Human embryonic kidney HEK293T cells and human osteosarcoma U2OS cells were used in this study. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator with 5% CO_2. Lentiviral transduction was used to generate stable expression of fluorescently tagged proteins through genomic integration. Smad2-EGFP, Smad2-RFP, and Smad3-RFP constructs were introduced to examine R-Smad localization and interaction under basal and activated conditions. U2OS cells expressing FoxO-GFP were used for comparative analysis of nucleocytoplasmic localization behavior.

Technical support and author contributions. Technical support included assistance with fluorescence/FRET imaging equipment operation and image acquisition. The author was responsible for the scientific conception, experimental design, mathematical modeling, analysis and interpretation of the findings, and preparation and scientific revision of the present manuscript.

2.2 Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min. They were subsequently incubated for 10 min at room temperature in blocking solution containing PBS, bovine serum albumin (BSA), and Triton X-100 (3 g BSA per 100 mL PBS-Triton) to reduce nonspecific antibody binding. Samples were incubated for 24 h with primary antibodies against Smad3 (Abcam) or Smad2 (Cell

Signaling Technology) at 1:100 dilution, followed by fluorophore-conjugated secondary antibodies for at least 1 h. Nuclei were stained with Hoechst dye (1:2000, Invitrogen).

2.3 Activin A and SB-431542 Treatments

HEK293T and U2OS cells were seeded onto coverslips in 24-well plates and allowed to adhere for 24 h in DMEM supplemented with 10% FBS. Cells were treated with Activin A (20 ng/mL; R&D Systems, Bio-Techne) for 2 h to activate the pathway. Where indicated, cells were treated with the TGF-β receptor kinase inhibitor SB-431542 (10 μM; Sigma) for 24 h. Following treatment, cells used for fixed-cell analysis were fixed with 4% PFA for 15 min, stained with Hoechst (1:2000), and mounted in Fluoromount (SouthernBiotech).

2.4 Image Acquisition and Quantitative Analysis

Fluorescence images were acquired using a multiphoton confocal microscope. For each experimental condition and cell type, approximately 100 cells were imaged, with comparable cell size selected while retaining a range of fluorescence intensities. Regions of identical size were selected within the nucleus and cytoplasm of each cell. Fluorescence intensities were quantified and used to examine the relationship between total cellular abundance and nuclear accumulation. Image processing and fluorescence quantification were performed using Fiji.

2.5 FRET Analysis

Förster Resonance Energy Transfer (FRET) microscopy was used to assess close molecular association between fluorescently tagged R-Smads in living cells. FRET efficiency between Smad2-GFP and Smad3-RFP was measured under basal, Activin A-stimulated, and SB-431542-inhibited conditions using the same imaging platform. Energy-transfer efficiency was quantified using the microscope-associated analysis software. FRET was used as a molecular-proximity readout of R-Smad association within the experimental system.

2.6 Minimal Computational Model

A minimal computational model was developed to test whether concentration-dependent molecular association could generate the observed abundance-dependent nuclear accumulation. The model represents cytoplasmic monomers (C), cytoplasmic associated species (CC), nuclear associated species (NN), and nuclear monomers (N). The modeled reaction scheme is:

$$C + C \rightarrow CC \qquad (1)$$

$$CC \rightleftharpoons NN \qquad (2)$$

$$NN \rightarrow N + N \qquad (3)$$

$$C \rightleftharpoons N \qquad (4)$$

Equation (1) represents cytoplasmic dimerization; Equation (2) represents reversible translocation of the associated species; Equation (3) represents nuclear dissociation; and Equation (4) represents bidirectional monomeric nucleocytoplasmic transport. The corresponding differential equations are:

$$\frac{d[C]}{dt} = -k_1[C]^2 - k_4[C] + k_4[N] \quad (5)$$

$$\frac{d[CC]}{dt} = k_1[C]^2 + k_{-3}[NN] - +k_3[CC] \quad (6)$$

$$\frac{d[NN]}{dt} = +k_3[CC] - k_{-3}[NN] - k_2[NN] \quad (7)$$

$$\frac{d[N]}{dt} = k_2[NN] - k_{-4}[N] + k_4[C] \quad (8)$$

Here, k_1 represents the cytoplasmic association rate; k_2 the dissociation of nuclear associated species; k_3 and k_{-3} the forward and reverse transport rates of associated species; and k_4 and k_{-4} the monomeric import and export terms represented in the model formulation. The model was designed as a minimal mechanistic framework to determine whether molecular association coupled to differential compartmental transport is sufficient to generate the qualitative behavior observed experimentally.

To isolate the mechanistic contribution of association, a reduced model was also considered in which dimerization was removed and proteins shuttled only as monomers. Comparison of the full and reduced models provides an internal mechanistic test of whether the association step is responsible for the transition from proportional to nonlinear abundance-localization behavior.

3. Results

3.1 Smad2-GFP Reports Activation- and Abundance-Dependent R-Smad Localization

We first assessed whether the fluorescent fusion proteins retained the characteristic nucleocytoplasmic response of endogenous Smads. In parental HEK293T cells, immunofluorescence showed that endogenous Smad2 and Smad3 were predominantly cytoplasmic under basal conditions and accumulated in the nucleus following Activin A stimulation (Figure 1). This behavior provided a reference against which the fluorescent constructs could be evaluated [8].

Smad2-GFP reproduced the characteristic activation-dependent redistribution of R-Smads and showed increased nuclear localization following Activin A treatment. On this basis, Smad2-GFP was selected as the principal reporter for quantitative localization analysis, providing a responsive fluorescent readout with which to examine how intracellular abundance relates to nuclear accumulation [9].

Inspection of Smad2-GFP-expressing cells revealed an additional abundance-dependent behavior. Even under basal conditions, cells with greater fluorescence intensity tended to display greater nuclear localization. Thus, variation in R-Smad abundance was associated with a systematic shift in intracellular distribution [10]. This observation defined the central mechanistic question of the study: can R-Smad abundance itself alter the balance between cytoplasmic and nuclear localization?

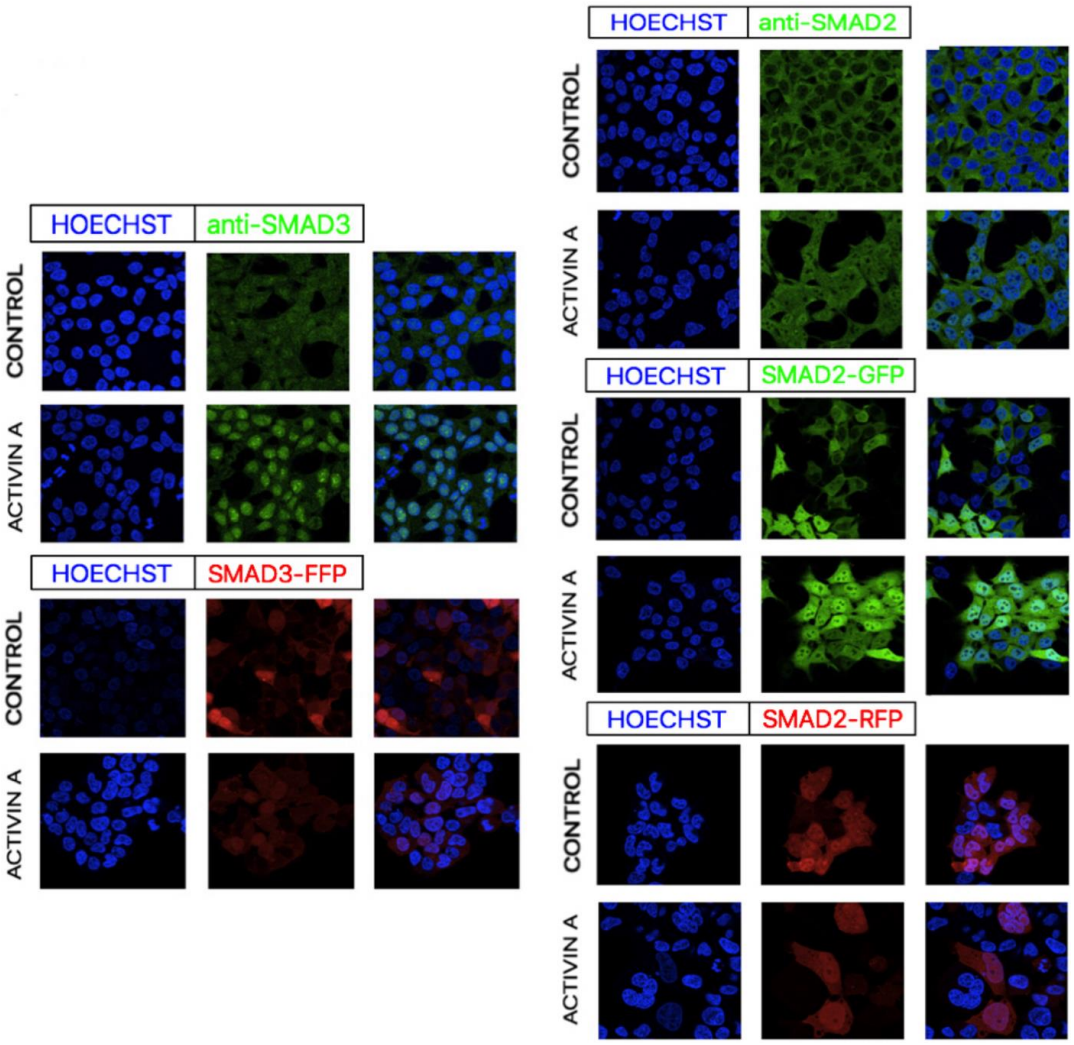


Figure 1. Validation of endogenous and fluorescent R-Smad localization in HEK293T cells. Endogenous Smad2 and Smad3 show increased nuclear localization following Activin A stimulation relative to control conditions. Smad2-GFP preserves the characteristic activation-dependent nuclear translocation and was selected as the principal reporter for quantitative localization analysis [11]. The images further show an abundance-dependent increase in nuclear localization, motivating quantitative analysis of the relationship between protein abundance and intracellular distribution. Hoechst marks nuclei.

3.2 Smad2 Abundance Is Nonlinearly Coupled to Nuclear Accumulation

To determine whether R-Smad abundance influences intracellular distribution, nuclear and cellular fluorescence were quantified across approximately 100 HEK293T cells spanning a range of Smad2-GFP abundance. A proportional localization regime would yield a corresponding proportional increase in nuclear fluorescence [12]. Instead, the measured relationship showed a systematic upward deviation from proportionality: cells with higher Smad2-GFP abundance accumulated disproportionately more protein in the nucleus (Figure 2A).

Thus, Smad2 abundance and nuclear accumulation do not behave as simply proportional quantities across the measured cells. The upward deviation becomes more apparent at higher fluorescence values, indicating that increases in cellular Smad2 are accompanied by progressively greater nuclear accumulation [13]. This abundance-dependent redistribution provides the experimental phenomenon addressed by the mechanistic model below.

3.3 Dimerization Provides a Minimal Mechanism for Abundance-Dependent Nuclear Accumulation

We next asked whether concentration-dependent R-Smad association could account for the abundance-localization relationship. Because the probability of molecular association increases with concentration, dimerization provides a direct means of converting changes in total protein abundance into changes in the relative amounts of monomeric and associated species [14]. In the model, these molecular states participate differently in nucleocytoplasmic transport, allowing abundance to influence compartmental distribution without introducing additional feedback regulation.

With the association step present, the minimal model generated the same qualitative departure from proportional nuclear accumulation seen experimentally (Figure 2B). At low abundance, monomeric species contribute strongly to the total pool; as abundance rises, the associated fraction increases and the balance of transport states shifts. The resulting change in state composition produces a progressively greater nuclear contribution [15]. The model therefore provides a compact mechanistic explanation for how concentration itself can influence R-Smad localization.

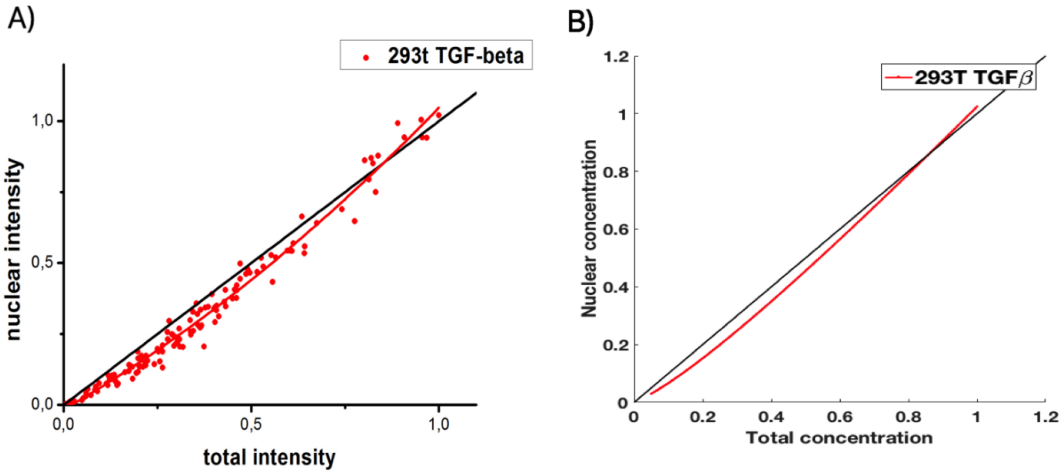


Figure 2. Experimental and model-based evidence for abundance-dependent nonlinear Smad2 nuclear accumulation. (A) Nuclear fluorescence plotted against total cellular fluorescence in approximately 100 HEK293T cells expressing Smad2-GFP. The measured relationship shows a systematic nonlinear deviation from the proportional reference trend. (B) The minimal R-Smad model incorporating cytoplasmic association, transport of associated species, nuclear dissociation, and monomeric shuttling reproduces the qualitative nonlinear trend [16]. Together, the experimental and computational results support concentration-dependent association as a mechanism linking R-Smad abundance to nuclear accumulation.

3.4 Removing Dimerization Restores an Approximately Linear Model Response

To determine whether this behavior depended on the modeled association reaction, we removed dimerization while retaining monomeric exchange between cytoplasm and nucleus. Under these conditions, the predicted relationship between total abundance and nuclear accumulation became approximately linear [17]. The contrast between the full and reduced models identifies concentration-dependent association as the element that generates the nonlinear behavior within this minimal framework.

This comparison generates a clear mechanistic prediction: a nucleocytoplasmic shuttling system lacking the R-Smad association step represented in the model should display a more nearly linear relationship between protein abundance and nuclear localization [18].

3.5 R-Smad Interactions Are Detected under Basal and Activated Conditions

To test whether R-Smad molecular association occurs in the experimental system, FRET was measured between Smad2-GFP and Smad3-RFP in living HEK293T cells. Detectable FRET was observed under both basal and Activin A-stimulated conditions, whereas the signal was markedly reduced following SB-431542 treatment (Figure 3B). These measurements provide an independent molecular readout of close R-Smad association and demonstrate its sensitivity to receptor inhibition [19].

The presence of FRET under basal conditions is noteworthy because abundance-dependent nuclear localization was also evident without exogenous Activin A. The reduction produced by receptor inhibition links the measured proximity to pathway state and is consistent with the existence of R-Smad association across the conditions in which the localization phenotype was observed [20]. In this study, FRET is therefore used as a molecular readout complementary to the model rather than as a substitute for the localization measurements.

3.6 A Comparison Nucleocytoplasmic System Shows a More Linear Abundance-Localization Relationship

The reduced model predicts that nucleocytoplasmic transport without the modeled association step should produce a more nearly proportional response. To examine this prediction experimentally, Smad2-GFP behavior in HEK293T cells was compared with FoxO-GFP localization in U2OS cells [21]. FoxO-GFP exhibited an approximately linear relationship between protein abundance and nuclear localization over the measured range, in contrast to the nonlinear trend observed for Smad2-GFP (Figure 3C).

This comparison provides an experimental counterpart to the reduced-model prediction. FoxO showed a more nearly proportional relationship between abundance and nuclear localization than Smad2, whereas Smad2 displayed the characteristic upward deviation. The result is consistent with the broader prediction that the molecular processes coupled to nucleocytoplasmic transport can determine whether increasing protein abundance is translated proportionally or nonlinearly into nuclear accumulation [22].

3.7 Increased Smad Abundance Preserves the Characteristic Localization Relationship

We next examined the abundance-localization relationship after increasing the intracellular Smad pool. Cells expressing Smad2-GFP alone were compared with cells co-expressing Smad2-GFP and Smad2-RFP, thereby extending the total intracellular Smad abundance represented in the experiment.

The abundance-localization pattern remained qualitatively similar after expansion of the intracellular Smad pool (Figure 3D). Rather than producing a separate high-abundance localization regime, the additional Smad preserved the characteristic relationship observed with Smad2-GFP alone. This observation supports the view that the measured behavior reflects the abundance-dependent organization of the Smad transport system across the examined range.

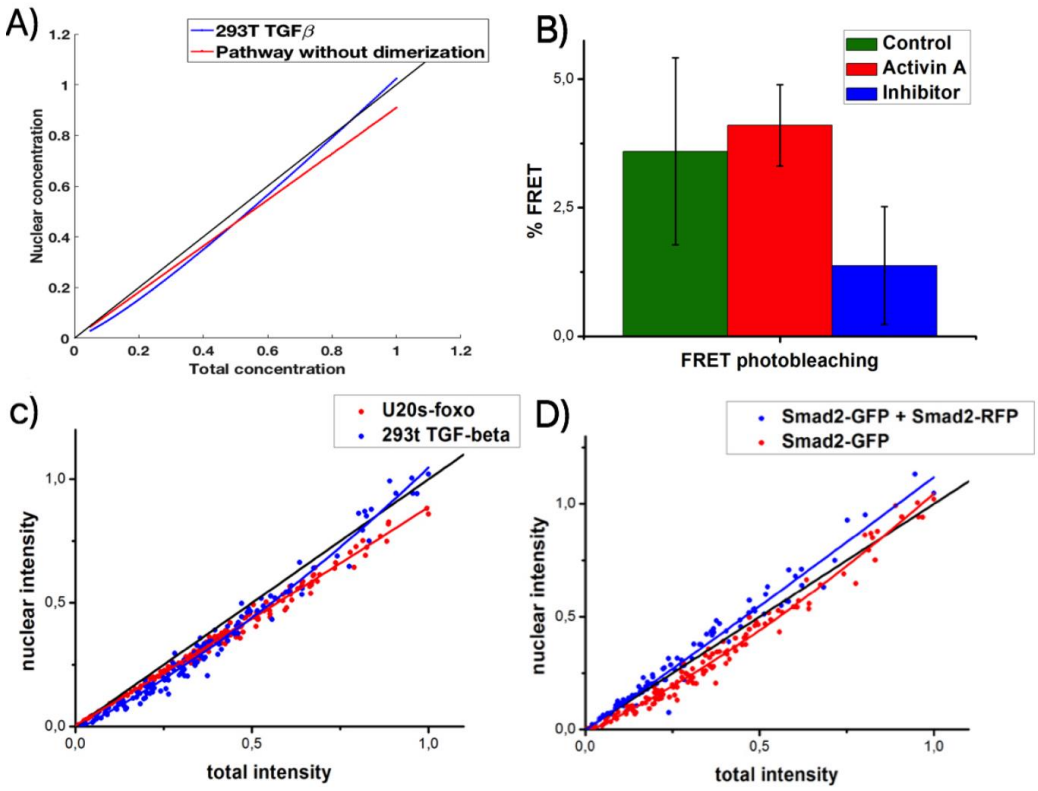


Figure 3. Complementary mechanistic tests of association-dependent nonlinear R-Smad accumulation. (A) Computational comparison of the full association model and a reduced model without dimerization; removal of dimerization restores an approximately linear abundance-localization relationship. (B) FRET between Smad2-GFP and Smad3-RFP under basal, Activin A-stimulated, and SB-431542-inhibited conditions; the receptor-sensitive FRET pattern supports R-Smad association. (C) Experimental comparison of Smad2-GFP with FoxO-GFP, showing the more nearly linear behavior predicted for the comparison shuttling system. (D) Smad2-GFP alone versus Smad2-GFP plus Smad2-RFP; the characteristic localization trend is preserved as the intracellular Smad pool is increased. Together, panels A-D provide convergent experimental and computational support for the proposed association-based mechanism.

4. Discussion

4.1 Protein Abundance Can Influence Signaling through Spatial Redistribution

The central result of this study is that R-Smad abundance is linked to intracellular distribution in a non-proportional manner. Cells containing more Smad2-GFP showed progressively greater nuclear accumulation, indicating that abundance and localization are coupled rather than independent properties [23]. This finding extends the usual view of protein abundance as a quantitative scaling factor: in a compartmental signaling system, concentration can also change the molecular-state composition of the protein pool and thereby alter where that pool accumulates.

4.2 Dimerization Links Abundance to Localization

The experimental observations and the minimal model converge on dimerization as a plausible physical link between abundance and localization. Smad proteins are known to form activation-dependent oligomeric assemblies and Smad nucleocytoplasmic shuttling is itself dynamically regulated [24]. In the present model, bringing these two established properties together is sufficient to produce the observed qualitative behavior: increasing abundance favors the associated state, and the altered balance of molecular

states changes nuclear accumulation. Removing the association step eliminates this feature of the model and restores an approximately proportional response.

4.3 Molecular Association as a Source of Signaling Nonlinearity

Nonlinearity in signaling networks is often discussed in terms of feedback, cooperative regulation, enzymatic saturation, or multistep network structure. The present results point to a more elementary physical source that can operate alongside those mechanisms. A concentration-dependent association reaction can make the composition of a protein population itself abundance dependent. When the resulting molecular states differ in compartmental behavior, a change in abundance is converted into a change in spatial distribution. This mechanism requires no additional feedback loop to generate a departure from proportionality.

4.4 FoxO Comparison Supports the Model Prediction

The FoxO experiment tests the qualitative prediction generated by the reduced model from a second nucleocytoplasmic system. Its more nearly proportional abundance-localization relationship contrasts with the Smad2 pattern and shows that increasing fluorescence does not inevitably produce the same upward nuclear redistribution in every shuttling protein examined. In the context of the model, this comparison strengthens the interpretation that pathway-specific molecular interactions can shape how protein abundance is translated into compartmental localization.

4.5 Increased Smad Abundance Supports Continued Nucleocytoplasmic Transport

Expanding the intracellular Smad pool did not create a qualitatively distinct localization regime. Instead, the characteristic abundance-dependent pattern was retained. This behavior is consistent with continued nucleocytoplasmic exchange across the examined abundance range and complements the modeling and FRET results by showing that the observed relationship persists when the total Smad pool is increased experimentally.

4.6 Basal R-Smad Association and Pathway State

R-Smad proximity was detectable both in Activin A-stimulated cells and under basal conditions, and receptor inhibition markedly reduced the FRET signal. This pattern indicates that the association state of R-Smads is sensitive to pathway activity while also being detectable in the absence of exogenous ligand [25]. In conjunction with the basal abundance-localization relationship, the result suggests that the molecular-state distribution of R-Smads can contribute to spatial signaling organization across more than one receptor-activity state.

4.7 Protein Abundance as a Potential Component of Signal Processing

These findings suggest a broader principle for compartmental signaling. Protein abundance can become a regulatory variable when concentration changes the molecular states available to a signaling component and those states differ in their intracellular transport or retention. Under such conditions, abundance is not merely converted into more signal of the same kind; it can alter the spatial form of the signal. For R-Smads, concentration-dependent association provides a parsimonious route connecting cellular abundance to nuclear accumulation and therefore to the physical organization of pathway activity.

5. Conclusions

This study identifies a nonlinear relationship between R-Smad abundance and nuclear accumulation in the Smad2/3 branch of TGF-β-superfamily signaling. Across individual cells, increasing Smad2-GFP abundance was accompanied by progressively greater nuclear accumulation. A minimal model reproduced this behavior when

concentration-dependent dimerization was included, whereas monomeric shuttling alone generated an approximately proportional response.

The modeling result is reinforced by receptor-sensitive FRET between fluorescent R-Smads, the more nearly proportional behavior of a comparison nucleocytoplasmic shuttling system, and preservation of the characteristic Smad localization relationship after expansion of the intracellular Smad pool. Together, these findings support a physical mechanism in which R-Smad abundance alters the balance of associated molecular states and thereby changes compartmental distribution. More generally, they illustrate how protein concentration can be translated into spatial signaling behavior through concentration-dependent molecular association.

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