

Meeting report – TGF- β superfamily: signaling in development and disease

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Journal of Cell Science 126, 4809–4813

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doi: 10.1242/jcs.142398

The latest advances on the transforming growth factor β (TGF- β) and bone morphogenetic protein (BMP) signaling pathways were reported at the July 2013 FASEB Summer Research Conference ‘The TGF- β Superfamily: Development and Disease’. The meeting was held in Steamboat Springs, Colorado, USA at 6700 feet above sea level in the Rocky Mountains. This was the seventh biannual meeting in the series. In attendance were investigators from a broad range of disciplines with a common interest in the mechanics of TGF- β and BMP signaling pathways, their normal developmental and homeostatic functions, and the diseases associated with pathway misregulation.

This conference was organized by Peter ten Dijke (Leiden University, The Netherlands) and Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) to cover various aspects of the transforming growth factor β (TGF- β) pathway, including signaling mechanisms, functions during development and homeostasis, roles in human disease and possible therapeutic implications. The TGF- β family of ligands, which includes bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activin, nodal and TGF- β s, regulate a broad range of cellular processes in both the adult and the developing embryo (for a review, see Massagué, 2012). Mechanisms operating outside the cell, at the cell surface and inside the cell regulate TGF- β family signaling pathways in a context-dependent temporal

(developmental) and spatial (tissue-specific) manner (Fig. 1).

The meeting brought together cell biologists, developmental biologists, biochemists, and geneticists from around the world to discuss recent advances made in our understanding of TGF- β signaling pathways, e.g. how ligand activation is controlled, how posttranslational modifications regulate receptor activity and the function of Smads (the major intracellular mediators of TGF- β signaling), and how TGF- β /Smad signaling intersects with other signaling pathways. Much excitement was generated in the conference and this meeting report provides a snapshot of what we learned during these five days.

Signaling mechanisms and regulation

Initiation of signaling starts with the activation of ligands and their release from latency. TGF- β family members are synthesized with a large N-terminal prodomain and a C-terminal growth factor domain. After intracellular cleavage by furin and secretion, a non-covalent association persists between the dimeric growth-factor domain and the prodomain. The prodomain of TGF- β is sufficient to confer latency and targets it for storage in the extracellular matrix, in complex with latent TGF- β -binding proteins (LTBPs). Timothy Springer (Harvard Medical School, Boston, MA) discussed the role of glycoprotein-A repetitions predominant protein (GARP) in regulating the bioavailability of TGF- β by outcompeting LTP1 for pro-TGF- β binding and by providing a cell surface platform for α v-integrin-dependent TGF- β activation (Wang et al., 2012). Jan Christian (University of Utah, Salt Lake City, UT) previously reported that tissue-specific cleavage of pro-BMP4 at a second upstream site is required for mature BMP4 activity (Goldman et al., 2006). Here, she reported that sequential cleavage of the two sites is also essential, because mice carrying a knock-in point mutation that leads to simultaneous cleavage of both sites die during embryogenesis. She also showed that fibrillin is required for stabilization of mature BMP4. As mutations in fibrillin underlie the human disease Marfan syndrome, her results imply that loss of BMP function could contribute to the pathology of this disorder.

The next step in the signaling of TGF- β family members is the presentation of receptors at the cell surface and activation of the receptor complexes upon ligand binding. Ye-guang Chen (Tsinghua University, Beijing, China) reported that the proto-oncoprotein c-Cbl neddylates TGF- β type II receptors, which promotes accumulation of the type II receptors in EEA1-positive endosomes, thereby preventing their endocytosis, ubiquitylation and degradation in lipid-raft compartments (Zuo et al., 2013). Previous studies have shown that the TGF- β type I receptor undergoes shedding mediated by TACE (also known as ADAM17), which decreases Smad2 signaling and generates a nuclear form of the type I receptor (Xu et al., 2012). Building upon this work, Rosemary Akhurst (University of California, San Francisco, CA) reported that a genetic variant of TACE, found only in C57BL/6 mice, is responsible for the genetic modifier effect of the *Tgfbm3* locus. This locus was originally identified as a suppressor of TGF- β 1-dependent vascular lethality, as well as a susceptibility locus for skin tumorigenesis (Freimuth et al., 2012). She now showed that there are enhanced levels of nuclear Smad2, but, paradoxically, also an elevation of nuclear type I receptor ICD in mouse embryonic fibroblasts from mice bearing the ‘hypoactive *Adam17*’ versus those with the wild-type *Adam17* allele. This study demonstrates the strong effect a natural protein variant can have on TGF- β signaling with distinct consequences for vascular development.

Rik Derynck (University of California, San Francisco, CA) presented his recent work that showed that BMP-induced receptor complex formation promotes the interaction of Smad6 with methyltransferase PRMT1 and arginine methylation of Smad6, which enables Smad6 dissociation from the receptors and activation of R-Smads (Xu et al., 2013). Peter ten Dijke identified an orphan nuclear receptor as a strong activator of TGF- β signaling through genome-wide cDNA screening. The receptor interacts with and facilitates Smad7 degradation, thereby promoting TGF- β /Smad signaling. Aris Moustakas discussed roles of liver kinase B1 (LKB1) in phosphorylating Smad4, thereby inhibiting it from binding to either TGF- β or BMP-responsive

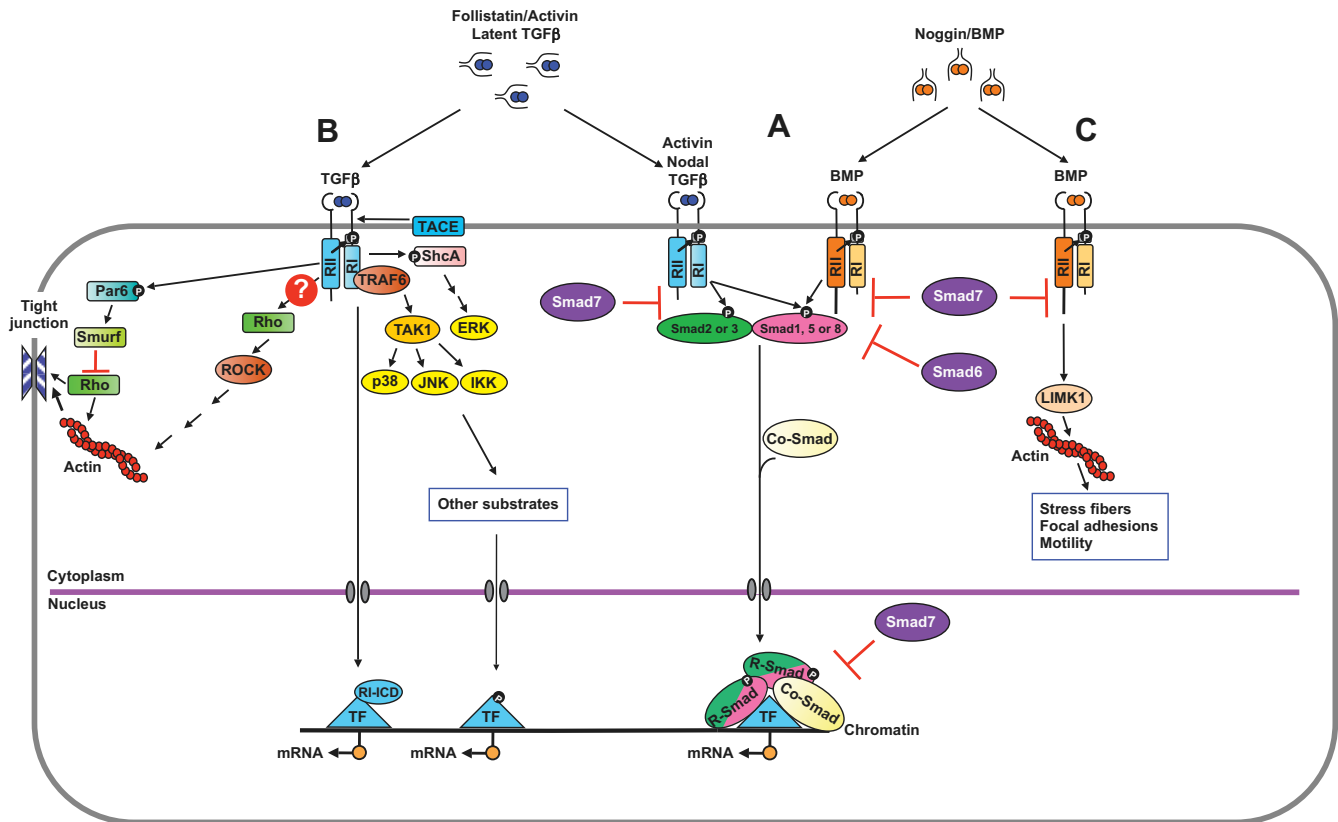


Fig. 1. Schematic overview of TGF- β signaling. The components of Smad and non-Smad pathways are shown, as well as the latent TGF- β complex and the extracellular antagonists, Follistatin (bound to Activin) and Noggin (bound to BMP). Ligand occupancy induces phosphorylation of the type II receptors (RII), which in turn activates type I receptors (RI). (A) Smad-dependent signaling mediated by TGF- β , Activin and/or Nodal and BMP, as well as the corresponding Smad proteins and their inhibition by the inhibitory (I)-Smads, Smad6 or Smad7. Note that TGF- β also activates BMP receptor-regulated (R)-Smads in certain contexts. The nuclear Smad complexes that regulate gene expression in concert with other transcription factors (TFs) most likely contain two R-Smad (identical or different) subunits and one co-Smad. (B) In TGF- β -associated non-Smad signaling, TGF- β RII and RI receptors signal through TRAF6 and TAK1 to activate the JNK, p38 and inhibitor of kappa B (I κ B) kinase (IKK) pathways, and through ShcA to activate the ERK pathway. TRAF6 also promotes cleavage of RI by TACE; the cleaved intracellular domain (ICD) of RI then associates with transcription factors to activate gene expression. The TGF- β type II receptor can directly phosphorylate PAR6 through recruitment by Smurf1 and target RhoA for degradation, which leads to the dissociation of tight junctions. (C) In BMP-associated non-Smad signaling, BMP type II receptors bind and activate LIMK1 to inhibit the actin-disassembling factor cofilin. Figure modified with permission from Moustakas and Heldin, 2009.

promoter sequences (Morén et al., 2011). He found that LKB1 also inhibits BMP signaling through interaction with Smad7 and the type I receptor ALK2. When overexpressed in adult wings of flies, LKB1 disrupts the BMP gradient and causes defects in vein morphogenesis.

Several talks provided new information on the regulation and modulation of Smad signaling. Xin-Hua Feng (Baylor College of Medicine, Houston, TX, and Zhejiang University, Hangzhou, China) reported that PPM1H, a member of PPM/PP2C phosphatase family, dephosphorylates the C-terminal SXS motif of Smad1 and Smad5 in the cytoplasm, thereby inhibiting Smad complex formation, nuclear accumulation and BMP-induced

transcription. He also presented new findings and showed that RanBP3L interacts with Smad1 and promotes nuclear export of Smad1. In the context of transcription regulation, Kohei Miyazono (University of Tokyo, Japan) described a role for thyroid transcription factor-1 (TTF1, also known as homeobox protein Nkx-2.1) in Smad-mediated transcriptional responses. Using genome-wide analyses by ChIP-Seq, he found that TTF1 colocalizes with Smad3 on chromatin and alters the binding patterns of Smad3. Moreover, TTF1 disrupts the Smad3–Smad4 complex and competes with Smad4 when Smad3 is bound to chromatin. In TTF1-expressing lung cancer cells, TTF1

inhibits TGF- β -mediated epithelial–mesenchymal transition (EMT) and suppresses tumor progression.

Some new functions for Smad proteins were also presented. Alan Mullen (Massachusetts General Hospital, Boston, MA) noted that in addition to regulating coding gene expression, Smad3 induces expression of long-noncoding RNAs (lncRNAs) in response to TGF- β during early endodermal differentiation. Many of these lncRNAs are divergently transcribed from active protein-coding genes, suggesting that they are co-regulated. Ying Zhang (National Cancer Institute, Bethesda, MD) discussed a role for Smad3 in the regulation of alternative splicing that

could contribute to the cell-context-dependent role of TGF- β in tumor progression.

Crosstalk with insulin signaling

Three presentations explored the interactions of TGF- β with the insulin pathway. Michael O'Connor (University of Minnesota, Minneapolis, MN) described his discovery of a role for the TGF- β subfamily member Dawdle in regulation of insulin signaling and metabolic function in *Drosophila*. By following clues from the Dawdle mutant phenotype, he identified a role for this ligand in glucose metabolism that was rescuable by ectopic expression of insulin-like proteins (*Drosophila* has no direct counterpart to mammalian insulin, but there are seven family members that together fulfill many of the same roles). He could trace the site of the interaction to insulin-producing cells in the brain and the fatbody, and his current studies are devoted to identifying the molecular basis for pathway crosstalk.

Two studies from the nematode *Caenorhabditis elegans* also focused on this interaction, one during development and the second during aging. Cathy Savage-Dunn (City University of New York, NY) reported a microarray analysis of worms with mutations in DBL-1 (a BMP-family member), which led to the identification of a number of metabolism genes that were already known targets of DAF-2 (an insulin-like receptor) signaling. Her current studies examine epistatic relationships between these pathways. Coleen Murphy (Princeton University, NJ) presented results that build upon her previous findings that TGF- β pathway mutations extend reproductive aging independently of established lifespan-extending mutations in the insulin-like receptor pathway. Reproductive aging is measured as increases in the timespan of fertility in mutants when compared to wild type. Her initial studies showed that SMA-2 (a receptor-associated Smad downstream of DBL-1) mutants have extended reproductive lifespan, but unaltered total lifespan, and that SMA-2 mutants display improved reproductive capacity due to, among other features, improved oocyte morphology and reduced chromosomal non-disjunction (Luo et al., 2010). Her new microarray analyses of aged oocytes show that the genes that are upregulated in SMA-2 mutant oocytes, such as those

involved in chromosomal integrity and cell cycle regulation, are the same ones that decline in aging oocytes in worms, mice and humans. This suggests that reproductive aging mechanisms are evolutionarily conserved and that the oocyte reproductive longevity program is distinct from the somatic cell longevity program.

Morphogen gradients and cell fate

The ability of TGF- β ligands to induce different cell fates in a concentration-dependent manner has fascinated developmental biologists for years. Two talks focused on distinct aspects of the BMP morphogen gradient that regulates cell fate along the ventral-to-dorsal axis in zebrafish. Mary Mullins (University of Pennsylvania, Philadelphia, PA) presented evidence that heterodimers of ligands and heteromeric complexes of their respective type I receptors are fundamental features of this gradient (Little and Mullins, 2009). Her studies utilize sophisticated genetic methods that combine mutants with precisely calibrated doses of injected morpholinos and/or rescue constructs. The data showed that BMP2–BMP7 heterodimers constitute the morphogen, whereas homodimers cannot function in this context. Furthermore, she showed that BMP2–BMP7 heterodimers signal through a heteromeric type I receptor complex of Alk3 or Alk6 (BMP2 type I-1A and IB) and Alk8 (orthologous to the Alk2 BMP7 type I receptor in mammals).

Anming Meng (Tsinghua University, Beijing, China) tackled the BMP morphogen gradient from a gene expression perspective. *BMP2* transcripts are initially expressed in a broad ventrolateral domain with their expression becoming progressively restricted to the ventral region by the blastula stage. This fits well with the fact that the highest concentration of BMP protein in the gradient induces ventral tissues. However, a widely noted but poorly understood feature of *BMP2* transcription is transient expression in the dorsally located 'organizer' region during gastrulation. His work addressed the question of whether *BMP2* expression in the organizer contributes to the ventral-to-dorsal gradient. By using an organizer-specific knockout of *BMP2* signal transduction via an Alk3 dominant-negative construct, he found an expansion of the secreted BMP

antagonist Chordin and a decrease of the BMP activity gradient, as well as dorsalization of the embryo. Therefore, *BMP2* organizer expression is important for gradient formation by restraining the distribution of Chordin.

A morphogen gradient of the BMP family member Dpp is an essential feature of wing development in flies. The simplicity and accessibility of wing primordia together with the powerful genetic toolkit of *Drosophila* makes it a useful model system that has produced numerous advances in our understanding of gradients. Markus Affolter (University of Basel, Switzerland) discussed a new tool for gradient analysis he recently developed in his lab that, in principle, could also work in vertebrate systems (Caussinus et al., 2012). Although small single-domain antibody fragments from camels (commonly known as nanobodies) have been used in biotechnological and medical applications for almost ten years, his modification of adding an F-box signal for proteosomal destruction to the anti-GFP nanobody now allows the rapid destruction of GFP fusion proteins. A different modification is the addition of a CD8 membrane-binding domain to the anti-GFP nanobody, which he expressed in developing *Drosophila* wings that express a Dpp–GFP fusion protein. As a proof-of-principle, he showed that this nanobody–CD8 fusion can effectively trap Dpp–GFP on the membrane, thus generating phenotypes that are similar to *dpp* mutations.

Another theme of this meeting was the role of TGF- β family members in the induction of cell fate, beyond the generation of gradients. Elizabeth Robertson (University of Oxford, UK) discussed studies of the earliest phase of embryonic development (primary body axis formation) and the first stage of tissue-specific differentiation (formation of mesoderm) in mice. Her lab has a longstanding interest in the role of Nodal signaling in these two processes. Using tissue-specific Nodal knockouts, she identified common mechanisms that act in primary body axis and mesoderm formation (Nowotschin et al., 2013). In the early embryo induction of the anterior visceral endoderm and in older embryos induction of mesoderm, both require Nodal signals that act through Smad2 and Smad3 for the activation of the transcription factor *eomesodermin* (Eomes). Eomes then directly activates

the homeobox transcription factor *Lhx1* in both locations, which is sufficient for anterior visceral endoderm formation. However, in the epiblast, *Lhx1* activation leads to formation of mesendoderm and, in parallel, *Eomes* directly activates the transcription factor *Mespl* to facilitate the acquisition of cardiac cell fate. Thus, the same signaling pathway activates distinct transcriptional programs depending upon the context.

Three talks presented mice-knockout studies to analyze the role of TGF- β signaling in the formation of white or brown adipose tissue. Chester Brown (Baylor College of Medicine, Houston, TX) presented data from mice with a global knockout of *GDF3*, which resulted in protection from both diet-induced obesity and adipocyte hypertrophy. He further found an antagonistic role for *GDF3* during basal and β -adrenergic-stimulated lipolysis in an adipocyte cell line (3T3-L1) and under high-fat diet conditions in the mice. He also presented results from a related study and showed that when activin- β A and - β B are both lost in adipose tissue, there is a marked reduction in body weight, adipose tissue mass and an apparent 'beiging' of white adipose depots, irrespective of diet. Therefore, it appears that, in white adipose, *GDF3* and activin- β A and activin- β B have substantial roles in both mature adipocyte functions and adipocyte fate decisions. Ke Ma (Methodist Hospital Research Institute, Houston, TX) presented data from a study of adipose cells of *Smad3*-knockout mice that showed that TGF- β signals have a role in brown adipose differentiation. In wild-type mice, the core circadian clock transcription factor *Bmal1* activates the expression of TGF- β pathway components in adipocytes, which in turn suppresses the differentiation of brown adipocytes. In *Bmal1*-knockout cells, TGF- β signaling is attenuated, leading to upregulation of BMP signaling and enhanced brown adipose formation. Therefore, in brown adipocytes, the decision of a cell to differentiate appears to result from a balance between TGF- β and BMP signals, which is subjected to circadian clock modulation. Sushil Rane (National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD) also examined adipocytes of *Smad3* knockouts and found that these cells show increased mitochondrial biogenesis,

basal respiration rates and increased features of brown adipose tissue (Yadav et al., 2011).

One talk addressed how cell fate decisions are executed. Mihaela Serpe (National Institute of Child Health and Human Development, Bethesda, MD), presented the role of BMP signaling in the assembly of the neuromuscular junction, a glutamatergic synapse in *Drosophila*, and specifically, their work on a conserved transmembrane protein called *Neto* that is essential for the clustering of glutamate channels and for synapse functionality. *Neto* contains extracellular BMP-binding domains and selectively influences a retrograde BMP signal (from the muscle to the neuron) that regulates synapse growth. In particular, *Neto* modulates synaptic but not nuclear phosphorylated Mad (pMad) accumulation in motor neurons. Synaptic pMad mirrors the activity of type-A glutamate channels and thus constitutes an effective sensor for synaptic activity. *Neto* appears to localize BMP signaling and promote formation of a trans-synaptic complex that contains postsynaptic channels and presynaptic BMP signaling complexes. In turn, these localized BMP signaling complexes modulate the stable incorporation of glutamate channels, thereby sculpting synapse development.

Cancer and disease treatment

It is known that TGF- β has dual roles during tumor progression. Prior to tumor initiation and early during progression, it acts as a tumor suppressor; however, at later stages in cancer it is often a tumor promoter. Lalage Wakefield (National Cancer Institute, Bethesda, MD) presented studies using the pan-TGF- β neutralizing antibody 1D11 in a panel of nine mouse models of metastatic breast cancer. She found that, although 1D11 treatment effectively inhibited metastasis in three models, it caused an undesirable stimulation of metastasis in three others. She suggested that the heterogeneity of the response to 1D11 was due to a neutralization of residual tumor suppressive effects of TGF- β in certain breast cancer models, which emphasizes the need for good biomarkers to select patient populations that might benefit from anti-TGF- β therapy. Keiji Miyazawa (University of Yamanashi, Chuo, Japan) showed that oligodendrocyte transcription factor 1 (*Olig1*) is a Smad cofactor that is

involved in TGF- β -induced cell motility (Motizuki et al., 2013). The interaction between *Smad2* or *Smad3* with *Olig1* requires the activity and binding of *Pin1*, a peptidyl-prolyl cis-trans-isomerase. Importantly, both *Pin1* and *Olig1* are only required for a subset of TGF- β -mediated responses (e.g. cell migration) but not other aspects of TGF- β -induced cytotaxis (e.g. growth inhibition), suggesting that it might be possible to selectively manipulate certain aspects of TGF- β signaling.

Li Yang (National Cancer Institute, Bethesda, MD) reported the genetic deletion of *Tgfb2* in myeloid cells inhibited tumor metastasis in several tumor models (Pang et al., 2013). This finding is in contrast to observations that *Tgfb2* deletion in epithelial cells and fibroblasts results in more malignant and invasive tumor phenotypes. Hal Moses (Vanderbilt University, Nashville, TN) presented his new findings that suggest that myeloid-derived cells that are recruited by increased chemokine expression in *Tgfb2*-knockout carcinoma cells are an underlying cause for increased metastasis of polyoma middle T-induced mammary carcinoma (PyMT). His group found that TGF- β that is secreted by myeloid cells induces the expression of lysyl oxidase in carcinoma-associated fibroblasts, which in turn alters collagen matrix remodeling and focal adhesion formation to promote invasion and metastasis (Pickup et al., 2013). Thus, the data presented by both Li Yang and Hal Moses suggest that TGF- β signaling in myeloid cells can be a therapeutic target.

Oral mucositis is one of the most severe side effects in cancer patients that are treated with upper-body radiation. Xiao-Jing Wang (University of Colorado Medical School, Denver, CO) showed that topical application of recombinant *Smad7* protein with a cell-permeable Tat tag (Tat-Smad7) to oral mucosa had preventive and therapeutic effects on radiation-induced oral mucositis in mice (Han et al., 2013). Her group found that *Smad7* dampened both the TGF- β and nuclear factor κ B (NF κ B) pathways to attenuate inflammation, growth inhibition, and apoptosis. In addition, *Smad7* also promoted oral epithelial migration by activating the expression of *Rac1*. These results suggest a possible new therapeutic strategy to block multiple pathological processes of excessive

inflammation-related wound-healing defects, such as those seen in oral mucositis.

Conclusions

The TGF- β research community continues to reveal new features of this signaling pathway. Many of the talks at this meeting suggest that the TGF- β field is entering a new, more mature, phase. Investigators now realize that understanding, and more importantly exploiting, genetic heterogeneity in individual responses to kinase inhibitors to achieve therapeutic benefit requires population level understanding. In the future, we expect more population studies of the incidence and penetrance of TGF- β mutations, a greater emphasis on modeling TGF- β responses and an increase in employing quantitative concepts, such as robustness, pleiotropy and haploinsufficiency. We look forward to joining the organizers Aris Moustakas and Akiko Hata (University of California, San Francisco, CA) at the meeting in 2015.

Acknowledgements

We thank Peter ten Dijke and Aris Moustakas for organizing an exciting and wonderfully interactive meeting. We also acknowledge the numerous talks and posters that were not discussed owing to space restrictions. Charlotte Konikoff (Arizona State University) and Liu-Ya

Tang (National Cancer Institute) assisted with manuscript preparation.

Funding

Y.E.Z. is supported by the intramural research program of National Institutes of Health (National Cancer Institute Center for Cancer Research); S.J.N. is supported by the National Human Genome Research Institute, National Institute of General Medical Sciences and the National Institute of Neurological Disorders and Stroke.

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