

Meeting Report: Cellular Dependence—Old Concept, New Mechanisms

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A review of the meeting Dependence Receptors, Fondation des Treilles, France, 3 to 7 July 2003.

From 3 to 7 July, at Fondation des Treilles, near France's Côte d'Azur, scientists met to discuss the biology of dependence receptors. For more than half a century, it has been known that cells depend for their survival on stimulation that is mediated by various receptors and sensors. For example, cells may require specific soluble trophic factors, cytokines, hormones, extracellular matrix interactions, cell-cell interactions, or electrical activity for survival. For any given required stimulus, withdrawal leads to programmed cell death (PCD). It has generally been assumed that PCD induced by the withdrawal of supporting factors is due to the loss of the associated positive survival signals, such as Akt phosphorylation. Although such survival signals are clearly very important, data obtained over the past 10 years strongly suggest a complementary and novel form of signal transduction that induces PCD and is activated by stimulus withdrawal. This "negative signal transduction" is mediated by specific "dependence receptors" that induce PCD in the absence of the required stimulus (for example, when unbound by a trophic ligand), but block PCD in the presence of the required stimulus (for example, when bound by a trophic ligand). Thus, the expression of various dependence receptors creates states of dependence (or addiction) on their respective ligands. To date, 10 such receptors have been identified (1). When unbound by ligand, these receptors most often trigger type I PCD, which is apoptosis, as opposed to type II or type III PCD, which are autophagic and nonlysosomal vesiculate forms of PCD, respectively.

The ramifications of the dependence receptor theory are numerous. For example, the expression of dependence receptors ties cells to a specific context in which the ligand is available, and therefore may serve to block metastatic spread of neoplasms or growth beyond local ligand availability. Another example of dependence receptor effects is the mediation of cell death during development of cells that do not receive trophic factor support. In many cases, this

dependence receptor-mediated developmental cell death occurs during neuronal development. Thus, dependence receptors often play an apparently dual role as tumor suppressors and mediators of development. Mutations in dependence receptors have been associated with disease states that include tumor formation, metastasis, developmental neuronal loss, and adult-onset neurodegeneration (1).

The following pattern is emerging from mechanistic studies of dependence receptors (1). (i) Most dependence receptors are substrates for caspases, the cysteine proteases that mediate apoptosis (Figs. 1 and 2). (ii) Mutation of the caspase site(s) in dependence receptors results in a loss of the proapoptotic effect. (iii) In at least some cases, the monomeric dependence receptor

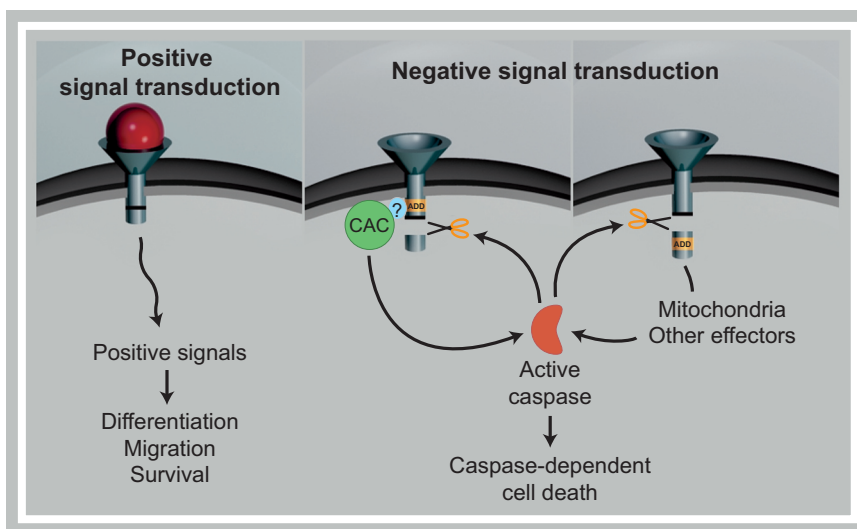


Fig. 1. Schematic of dependence receptor-mediated cellular effects. In the presence of their respective ligands, dependence receptors mediate differentiation, survival, and axon guidance (left). However, ligand withdrawal is associated with the mediation of programmed cell death, activated by conformational change, complex formation, caspase activation, and caspase cleavage of the dependence receptor. The proapoptotic addition/dependence domain (ADD) may lie either N-terminal to the cleavage site (middle) or C-terminal to the cleavage site (right). Cleavage of the receptor appears to mediate signal amplification. CAC, caspase-activating complex.

forms an apoptosis-mediating complex, whereas multimerization leads to an apoptosis-inhibiting complex. (iv) Dependence receptors coimmunoprecipitate with specific caspases. (v) In at least some cases, apoptosis induction by dependence receptors proceeds through activation of caspase-9 (unlike death receptors, which mediate apoptosis following ligand binding through caspase-8 or -10), using a novel pathway that is independent of the previously described caspase-9 activator, Apaf-1. (vi) Caspase cleavage of dependence receptors results in fragments that include one or more proapoptotic addition or dependence do-

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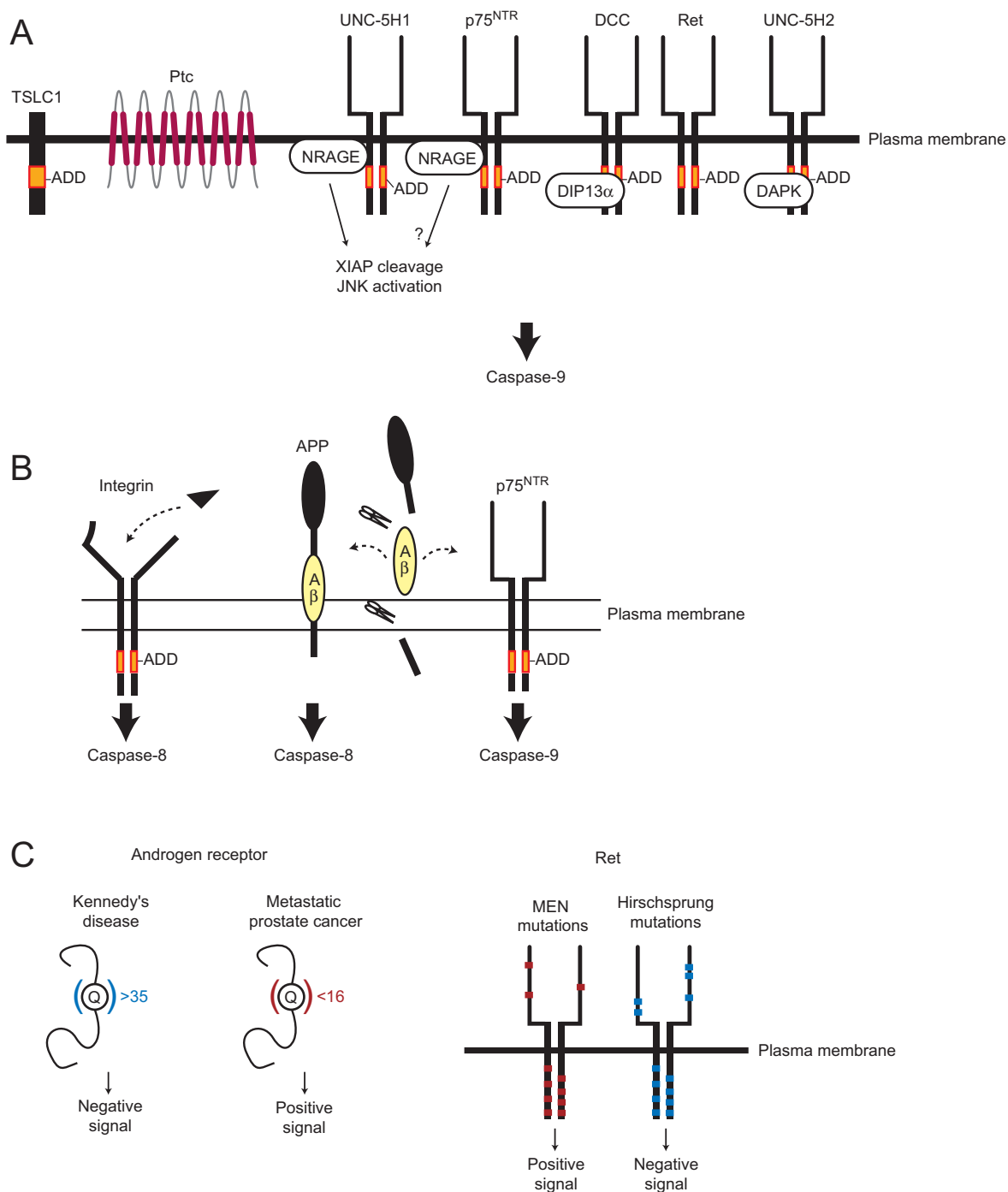


Fig. 2. Schematic representation of the dependence receptors. **(A)** Dependence receptors that activate caspase 9-dependent cell death. **(B)** Dependence receptors that trigger cell death through activation of caspase 8. The presence of p75^{NTR} sensitizes cells to Ab. **(C)** Mutations in the Ret or the androgen receptor can either promote or prevent cell death, making the receptors less responsive to the presence or absence of ligand, respectively.

mains (ADDs). (vii) Blocking receptor-mediated death by ligand binding of dependence receptors requires signaling through a ligand-response domain (LRD) (2). Because dependence receptors recruit caspase zymogens that are relatively but not

completely inactive and are cleaved to yield cytotoxic fragments, such receptors may serve as caspase amplifiers in the absence of ligand binding.

The purpose of the meeting at Fondation des Treilles was to

bring together scientists working on dependence receptors and related subjects, in order to discuss the pros and cons of the overall concept, potential new dependence receptors, and proposed mechanisms of action. Chantal Thibert (University of Lyon, France) described results supporting the designation of the sonic hedgehog receptor, Patched (Ptc), as a dependence receptor (3). Sonic hedgehog (Shh) has been well described as a morphogen that is liberated by the floor plate and notochord, forming a ventrodorsal gradient within the developing neural tube. Shh binds to Ptc, but its downstream signaling is mediated by smoothed (Smo), whose interaction with Ptc is altered when Shh binds Ptc. However, Shh is also a trophic factor: When it is withdrawn, massive cell death occurs in the neural tube (4-8). In addition, Ptc is a tumor suppressor, mutations in which are associated with multiple tumor types, including basal cell carcinoma, rhabdomyosarcoma, and medulloblastoma (9). Thibert and her colleagues found that Ptc expression in cultured cells and in the developing neural tube led to apoptosis that, in turn, could be blocked by the addition of Shh. Furthermore, the developmental abnormalities associated with Shh loss were partially corrected by a complementary loss of Ptc proapoptotic effects. Such an effect is difficult to reconcile with a model in which Ptc simply mediates the morphogenetic signaling of Shh and supports the notion of Ptc as a dependence receptor in which Shh binding blocks Ptc-induced apoptosis. Interestingly, Ptc-induced apoptosis did not appear to be mediated by Smoothed (Smo), unlike its well described morphogenic effects, suggesting that two separate pathways may be involved.

Lindsay Hinck (University of California, Santa Cruz, United States) and Fabien Llambi (University of Lyon) described the mediation of cell death by the UNC-5H family, the mammalian homologs of the *Caenorhabditis elegans* gene, *uncoordinated-5* (*Unc-5*). The UNC-5Hs (UNC-5H1, 2, and 3 in rat; UNC-5A, B, and C in human) are netrin-1 receptors that mediate the chemorepulsive activity of netrin-1 on various axons. They are also dependence receptors that mediate apoptosis that is, in turn, blocked by netrin-1. Hinck found that the proapoptotic effects of the UNC-5Hs decreased in the order UNC-5H1 > UNC-5H2 > UNC-5H3. Therefore, UNC-5H1 was used as bait in a yeast two-hybrid screen, and NRAGE (also referred to as Dixin-1 and MAGE-1) was identified as an UNC-5H1 interactor. NRAGE, a member of the MAGE (melanoma antigen) protein family, mediates apoptosis through at least two mechanisms, one involving the degradation of the caspase inhibitor XIAP (10) and the other involving the activation of the c-Jun N-terminal kinase (JNK) signaling pathway (11). NRAGE binding, for which UNC-5H1 was shown to have greater affinity than UNC-5H2 or UNC-5H3, required the juxtamembrane region of UNC-5H1, the same region required for apoptosis induction. This region includes a short PEST sequence (SSPTSEAEFVSRSLSTQNYFRSLPRGTS in human UNC-5H1) followed by a ZU-5 domain. Further support for the hypothesis that NRAGE mediates UNC-5H1-induced apoptosis came from a demonstrated correlation between down-regulation of NRAGE and resistance to UNC-5H1-mediated apoptosis in differentiating PC12 cells (a pheochromocytoma cell line that can be stimulated to differentiate into neurons or chromaffin cells). These results suggest a model in which UNC-5H1 undergoes a developmental switch from proapoptotic receptor to axon guidance receptor, in association with the down-regulation of NRAGE.

Fabien Llambi reported that UNC-5H1, 2, and 3 all display proapoptotic effects, and that, like other dependence receptors, they all display caspase cleavage sites in their intracytoplasmic domains. Focusing then on UNC-5H2, he noted that the death domain is required for its proapoptotic activity and that enforced dimerization, using the FK-binding protein system (12), blocked cell death induction. He also noted that the death domain of UNC-5H2 strongly resembles the death domain of DAPK (death-associated protein kinase). Because the two death domains were similar, it was hypothesized that UNC-5H2 and DAPK interact, and this was subsequently demonstrated. Furthermore, DAPK expression increased PCD induced by UNC-5H2, and dominant-negative DAPK blocked UNC-5H2-induced PCD, supporting a role for DAPK in UNC-5H2-induced cell death. Finally, netrin addition led to a decrease in DAPK activity, and UNC-5H2 expression in the absence of netrin was associated with dephosphorylation of DAPK, which leads to its activation. These results suggest that the mechanism by which UNC-5H2 functions as a dependence receptor rests at least in part on a netrin-inactivated dephosphorylating effect of UNC-5H2 on DAPK.

Laetitia Mazelin (University of Lyon) reported that UNC-5H expression is lost in many tumors (13) and that its expression blocked anchorage-independent growth by inducing apoptosis. This effect was reversed by netrin-1 addition, supporting a role for UNC-5Hs as dependence receptors and tumor suppressors. Ongoing work with netrin-overexpressing transgenic mice should help to determine whether blocking the UNC-5H—and deleted in colorectal cancer (DCC) (see below)—proapoptotic effects with an increased concentration of ligand will lead to increased tumor formation.

Complementing the presentations on UNC-5Hs was an interesting presentation by Hirofumi Arakawa (National Cancer Center, Tokyo), who described novel p53 transcriptional targets. He pointed out that the total number of p53 transcriptional targets is likely to be about 100. One of the genes up-regulated by p53 was found to encode p53RLD1, which is identical to UNC-5B, the human homolog of rat UNC-5H2. Surprisingly, p53-induced apoptosis was blocked both by application of UNC-5B antisense oligonucleotides and by exposure of cells to netrin-1. Thus, p53 expression placed cells in a netrin-dependent state, implying that p53 may be viewed as a “dependence nonreceptor.”

Before publication of the initial results demonstrating that UNC-5Hs may function as dependence receptors, another netrin receptor, DCC (deleted in colorectal cancer), had been shown to function similarly (14). Patrick Mehlen and Yong Chen (Wake Forest University, Winston-Salem, NC) described the effects of DCC on cell death. DCC encodes a cell surface receptor that belongs to the immunoglobulin superfamily. Inactivation of the DCC gene has been implicated not only in colorectal cancer, as its name implies, but also in many other tumors. However, controversy remains as to whether DCC is indeed a tumor suppressor gene (15), and little is known about the function of the DCC protein in tumorigenesis. The expression of DCC in several different cell lines induced apoptosis, and this effect, like that of UNC-5Hs, was blocked by netrin-1. DCC displays a caspase cleavage site at Asp¹²⁹⁰, and mutation of this site completely suppressed the proapoptotic effect of DCC. Bcl-2 did not block DCC-induced apoptosis, which was shown to involve a novel, caspase-9-dependent but Apaf-1-independent pathway (16). A number of DCC interactors have been identified, and one of

these, dubbed DIP13 α (DCC-interacting protein 13 α), was shown to interact with DCC residues 1243 to 1264, which comprise the region of DCC required for PCD induction. DIP13 α knockdown by RNA interference blocked DCC-induced apoptosis, and deletion of the DCC-interacting domain of DIP13 α abolished its ability to increase DCC-induced apoptosis (17). Thus, DCC functions as a netrin-1 dependence receptor, and its proapoptotic effect is mediated by DIP13 α .

Patrick Mehlen described Ret as a receptor tyrosine kinase that mediates cellular dependence on glial-derived neurotrophic factor (GDNF) and potentially on the related trophic factors neurturin and persephin. As for other dependence receptors, Ret expression in cultured cells induces apoptosis; the addition of ligand (GDNF) blocks this effect. Unliganded Ret is cleaved by caspases (at two sites, Asp⁷⁰⁷ and Asp¹⁰¹⁷), and mutation of either caspase cleavage site inactivates the proapoptotic effect of the unbound receptor. Ret mutations are associated with both proliferative and developmental abnormalities. Some Ret mutations are associated with multiple endocrine neoplasia type 2 (MEN2), which features tumors that include medullary carcinoma of the thyroid, pheochromocytoma, and parathyroid adenomas. Other Ret mutations are associated with Hirschsprung's disease, a developmental disorder characterized by loss of ganglion cells in the hindgut, leading to intestinal dilation. The mechanism by which these two sets of mutations in Ret lead to two very different syndromes has been unclear and is thought to be related to gain or loss of tyrosine kinase activity in MEN2 and Hirschsprung's disease, respectively. However, expression of these mutants in cultured cells suggested a different interpretation: Expression of the MEN2-associated mutants failed to induce apoptosis, either in the presence or absence of ligand, whereas expression of five different Hirschsprung-associated mutants led to apoptosis induction both in the presence and absence of ligand (18). Thus, the MEN2-associated mutants were, in a sense, "stuck" in the "off" position with respect to cell death induction, whereas the Hirschsprung-associated mutants were "stuck" in the "on" position with respect to cell death induction (1).

This same general profile—two sets of receptor mutants, one associated with a disease featuring increased cell numbers and the other associated with a disease featuring decreased cell numbers—is also seen with the androgen receptor, discussed by Lisa Ellerby (Buck Institute, Novato, CA) (19, 20). In this case, however, rather than point mutants, the disease-associated mutants under discussion involved expansions and (relative) contractions of the polyglutamine tract, which lies in the N-terminal region of the androgen receptor. As has been shown for the other dependence receptors, the androgen receptor induces cell death that is blocked by ligand binding, and mutation of the receptor's caspase cleavage site (Asp¹⁴⁶) inhibits the PCD induction by the unliganded receptor (19). The proapoptotic ADD domain was mapped to the polyglutamine region and juxta-polyglutamine residues. An increase in polyglutamine length increased the proapoptotic effect in the absence of testosterone, but did not affect the blocking effect of the ligand (except when the polyglutamine region was increased to over 100 glutamines, at which point the ligand no longer blocked PCD induction). A decrease in polyglutamine length decreased the proapoptotic effect. In vivo, polyglutamine expansion in the androgen receptor is associated with Kennedy's disease (also referred to as spinobulbar muscular atrophy), a chronic motor neuron degen-

erative disease. In contrast, short polyglutamine stretches (<16 glutamines) are associated with an increased likelihood of developing prostate cancer, especially metastatic prostate cancer (21, 22). Comparing the cell culture results with the in vivo effects of these androgen receptor mutations suggests that the association of androgen receptor with short polyglutamine stretches and metastatic prostate cancer may be due to a decrease in androgen dependence (1).

Dwayne Stupack (Scripps Research Institute, La Jolla, CA) addressed the question of whether integrins may function as dependence receptors, and Urzula Hibner (CNRS, Montpellier, France) discussed downstream signaling of the integrins. Integrins have traditionally been considered to be pro-survival mediators, promoting survival of cells that display anchorage dependence. Integrin-mediated adhesion supports the formation of cytoskeletal and contractile elements, which in turn promotes cellular resistance to exogenous insults that would otherwise induce apoptosis. Most cells also require integrin-mediated adhesion to respond to trophic factors. Whereas these positive effects are mediated by ligand-bound integrins, at least some integrins, such as the β 1 and β 3 integrins, mediate proapoptotic signals in the absence of ligation (or when occupied by a soluble antagonist). As for other dependence receptors, caspases may be coimmunoprecipitated with these integrins. However, differences between integrins and other dependence receptors were also noted. No caspase cleavage site was noted within the cytoplasmic tails of these integrins, and, rather than inducing a caspase-9-dependent PCD, they induced caspase-8-dependent PCD. Integrins functioning as anchorage-dependence receptors may conceivably play important roles in matching cells with their "correct" environments, and when this role is disrupted, may allow metastasis to an "unmatched" extracellular matrix (ECM) environment.

Intriguing similarities were noted between the characteristics of integrin-mediated anchorage dependence and PCD mediated by exposure of cells to cleavage products derived from the β -amyloid precursor protein (APP), the latter of which was described by Ed Koo (University of California, San Diego) and Filipe Calheiros (University of Lyon). Both integrins and APP may mediate PCD initiated by the binding of soluble ligands— β -amyloid peptide in the case of APP (23), and soluble antagonists in the case of integrins. Both may induce PCD mediated by caspase-8 (24). Both display short (fewer than 50 residues) intracytoplasmic domains that include an NPXY sequence. Finally, both may interact with specific proteins in the ECM that block the proapoptotic effects. Viewed in this context, Alzheimer's disease may be seen as a state of altered dependence, in which a soluble ligand activates a signaling pathway that includes caspase-dependent PCD, and potentially other effects, such as synapse loss and neurite retraction, as well.

The A β peptide also binds to p75^{NTR} (25, 26), the first dependence receptor described (27-29). This neurotrophin receptor, p75^{NTR}, binds the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) with similar nanomolar affinities. Various aspects of neurotrophin receptors were discussed by Shahrooz Rabizadeh (Buck Institute, United States), Graham Barrett (University of Melbourne, Australia), Nina Schor (University of Pittsburgh, PA), and Roberto Bernardoni (University of Bologna, Italy). A member of the tumor necrosis factor receptor superfamily, p75^{NTR} has proven to be a remark-

ably complex and interesting receptor with respect to its functional effects. It cooperates with the Trk receptors to create high-affinity binding sites for neurotrophins, as well as increasing the specificity of ligand-receptor interactions. It also displays the properties of a dependence receptor in postnatal sensory neurons, where it promotes apoptosis in the absence of ligand (28). In the presence of nerve growth factor, p75^{NTR} enhances survival. Somewhat reminiscent of the studies of the UNC-5H family, mutagenesis studies have identified two distinct regions required for cell death induction: one juxtamembrane region [dubbed chopper in the case of p75^{NTR} (30)] that interacts with NRAGE (31), and a second region that is a type II death domain (32). Graham Barrett noted that the differentiation of PC12 cells is accompanied by an up-regulation of p75^{NTR} and down-regulation of NRAGE; this sets up a dependent state, because the subsequent withdrawal of NGF from these differentiated PC12 cells results in up-regulation of NRAGE followed by NRAGE-dependent PCD.

In addition to mediating neurotrophin withdrawal-induced PCD, p75^{NTR} also mediates NGF-induced PCD (33, 34). In most cases, this has been associated with the presentation of a neurotrophin to p75^{NTR} in the presence of a mismatched Trk (for example, NGF may induce PCD in cells expressing p75^{NTR} and TrkB). Nina Schor reported that NGF may also induce PCD through TrkA in PC12 cells. In that case, however, the PCD did not appear to be typical apoptosis. The PCD was blocked by the tyrosine kinase inhibitor K252a, but not by a monoclonal antibody that prevented binding of NGF to p75^{NTR}.

The expression of p75^{NTR} also sensitizes cells to toxicity of the Alzheimer's-associated A β protein (35), which is of potential interest in light of the close correlation between selective vulnerability and p75^{NTR} expression in the basal forebrains of patients with Alzheimer's disease (35). Roberto Bernardoni reported that p75^{NTR}-mediated A β sensitivity requires the extracellular domain and death domain, but not the chopper domain. This PCD was shown to proceed through activation of caspase-8 and caspase-3.

Yoshinori Murakami (National Cancer Center Research Institute, Tokyo) and Michèle Allouche (INSERM, Toulouse, France) reported on two potential new dependence receptors: TSLC1 (tumor suppressor in lung cancer) and ALK (anaplastic lymphoma kinase). TSLC1 is a tumor suppressor in human non-small cell lung cancers that is a type I transmembrane glycoprotein containing three immunoglobulin C2-type loops and that is expressed at cell attachment sites, where it forms homodimers. It has been proposed to function in the transduction of signals deriving from cell adhesion. Murakami reported that TSLC1 expression in cells in suspension culture induced apoptosis, whereas cell-cell interaction blocked this effect. Furthermore, apoptosis was associated with cleavage of TSLC1, although it is not yet clear whether this occurs at a caspase cleavage site or whether such cleavage is required for the cell death effect of TSLC1. These results suggested the interesting possibility that TSLC1 is at once dependence receptor and associated ligand, because its homodimerization in association with cell-cell interaction may block TSLC1-induced cell death. Ongoing experiments should indicate whether this is indeed the case.

ALK is a receptor tyrosine kinase expressed in the central nervous system both during and after development. It was discovered as part of the NPM (nucleophosmin)-ALK fusion protein associated with the t(2;5)(p23;q35) translocation frequently

observed in human anaplastic large cell lymphomas. Expression of the NPM-ALK fusion protein inhibits apoptosis triggered by anticancer chemotherapeutics, but not Fas-induced apoptosis. Although studies to assess whether ALK may function as a dependence receptor are not as far along as are those for the other receptors discussed at the conference, preliminary results support the notion that ALK may indeed function as such.

Overall, the meeting served to emphasize the emerging similarities between different putative dependence receptors, the potential involvement of these receptors in disease states, and the need for extending the in vivo studies to additional dependence receptors.

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