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## SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

*Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo), Granulocyte Colony Stimulating Factor (G-CSF), which function as homomeric complexes and of heteromeric receptors such as receptors for interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which is transmitted via juxtamembrane and transmembrane domains to the cytosolic region and ultimately to members of the Janus family of tyrosine kinases (JAKs).*

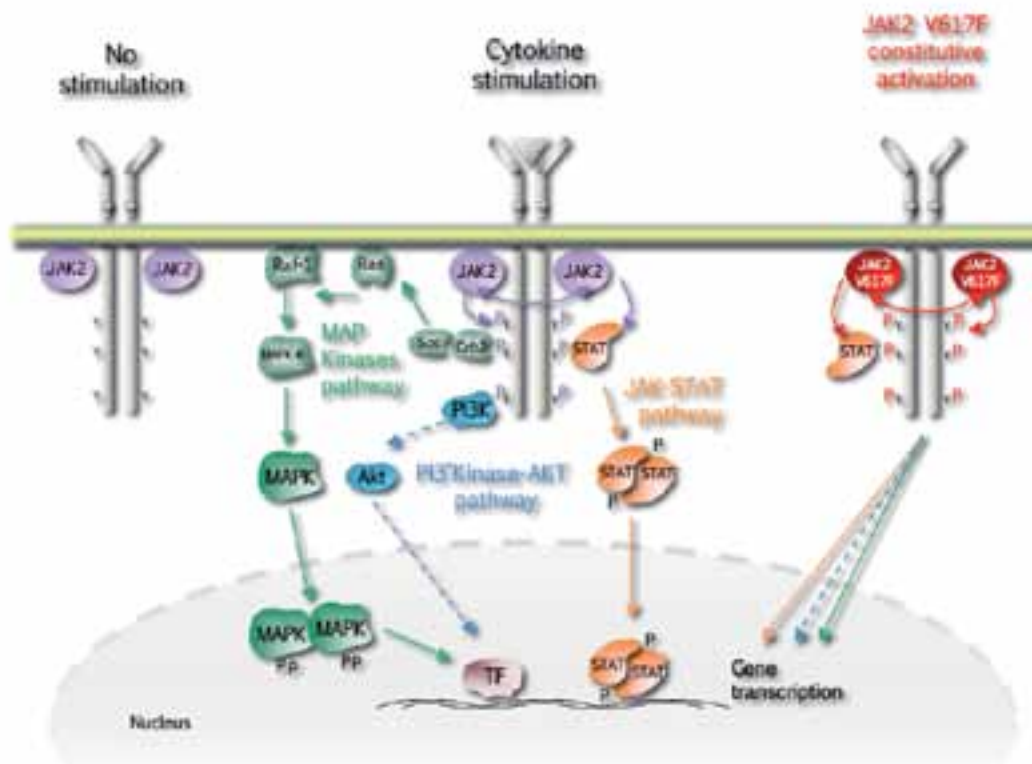
*Our key interests are: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; and ii) the mechanisms of JAK attachment to receptors, JAK activation and signaling. The laboratory identified constitutively active oncogenic mutants of JAK2, JAK1 and TYK2 and of cytokine receptors, with some being involved in human blood cancers. Specifically the mechanisms by which JAK2 V617F and TpoR W515 mutants induce, in humans, Myeloproliferative Neoplasms (MPNs), such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis are actively pursued. Furthermore, a microRNA was recently identified (miR-28) that appears to be a biomarker for a fraction of MPNs without known molecular cause, and that targets the mRNA for TpoR, inhibiting its translation. A major effort will be dedicated to the identification of molecular defects associated with the latter group of MPNs and with acute myeloid leukemia secondary to MPNs. A close collaborative structure has been created with clinicians and clinical biologists at St Luc Hospital for in-depth study of patient-derived cell*

## THE MECHANISMS BY WHICH THE MUTANT JAK2 V617F INDUCES POLYCYTHEMIA VERA AND OTHER MYELOPROLIFERATIVE NEOPLASMS IN HUMANS

*A. Dusa, C. Pecquet, J.-P. Defour*

The JAK-STAT pathway mediates signaling by more than 25 cytokine receptors and is constitutively activated in many cancers. Several mutations in genes coding for JAKs

have been identified in the past five years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudo-kinase domain, predicted to be inactive. Four Janus kinases are coded by the human genome JAK1, JAK2, JAK3 and TYK2. JAK2 is crucial for signaling by EpoR, TpoR, the G-CSFR, the interleukin 3 receptor and several other receptors. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.



**Figure 1.** In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs to cross-phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become themselves phosphorylated and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatidylinositol-3-kinase, PI3K, and Akt). The mutant JAK2 V617F binds to the cytosolic domains of receptors and can trigger signaling in the absence of any cytokine binding to the extracellular receptor domain (right complex). As a consequence, signaling is induced permanently and myeloid progenitors survive, proliferate and differentiate in an uncontrolled manner. (Jean-Michel Heine).

Polycythemia Vera (PV), or the Vaquez-Osler disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Two other related diseases, Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are associated with excessive platelet production and fibrosis (scarring) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We postulated that JAK2 is involved in the pathogenesis of these diseases because we showed that JAK2 strongly promotes the maturation and cell-surface localization of TpoR (2), a process that was known to be highly defective in PV.

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation, that is responsible for >98% of Polycythemia Vera and for >50% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain and allows the mutated JAK2 to bind and activate EpoR, TpoR and G-CSFR in the absence of cytokines (Figure 1). Saturation mutagenesis at position V617 showed that not only Phe, but also Trp, Leu, Ile and Met can activate JAK2, although Trp is the only mutation that exhibits comparable activity with V617F (5). The homologous V617F mutations in JAK1 and TYK2 also enable these kinases to be activated without ligand-binding to cytokine receptors (4). Similarly, at least Trp, Leu and Ile can also activate JAK1, besides Phe, at the homologous V658 position. These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1). An example of such involvement is the identification of mutants in the pseudokinase domain of JAK1 in approximately 20% of adult T-lymphoblastic leukemia.

A major effort in the group is geared to-

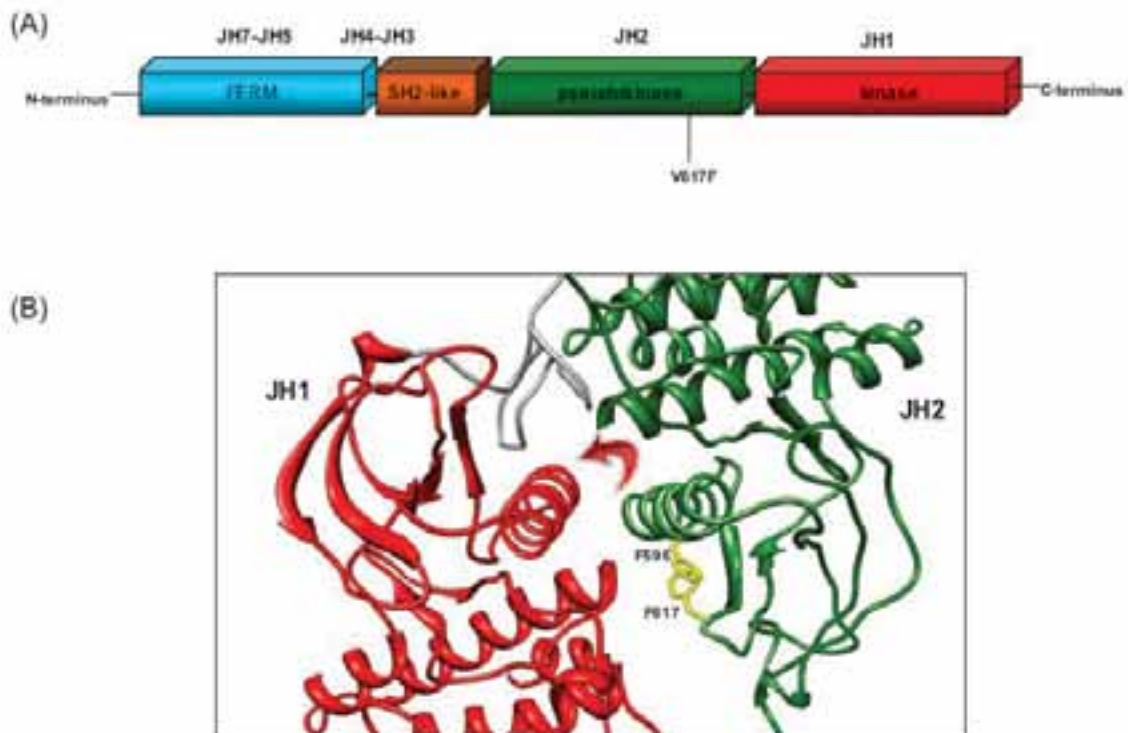
wards the understanding of how a pseudokinase domain mutation can induce kinase domain activation. The aim is to be able to specifically inhibit mutated JAK2 but not the wild type JAK2, which is crucial for several physiologic processes. Towards that end, we identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (6). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinase domain might be a target for specific JAK2 V617F inhibition (Figure 2).

## INVOLVEMENT OF TpoR IN MYELOPROLIFERATIVE DISEASES

*C. Pecquet, J.-P. Defour, M. Girardot, E. Leroy*

TpoR appears to be central to MPNs: i) most MPN patients strongly down-modulate TpoR levels in megakaryocytes and platelets; ii) mutations in the TpoR intracellular juxta-membrane motif W515 lead to constitutive activation of the receptor, and severe in vivo MPN with myelofibrosis; iii) asparagine mutations in the transmembrane domain of TpoR also activate TpoR and one such mutation has been shown to be associated with ET; iv) alterations of traffic of TpoR to the cell surface can induce thrombocytosis due to insufficient clearance of Tpo and high sensitivity of early megakaryocytes to high Tpo.

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (Pecquet et al., submitted). In addition we discovered that Tpo can induce a strong antiproliferative effect in cells that express high JAK2 levels (Pecquet et al., in preparation). This effect can be detected in post-mitotic megakaryocytes (7). We are exploring the precise signaling mediators of this effect and showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F

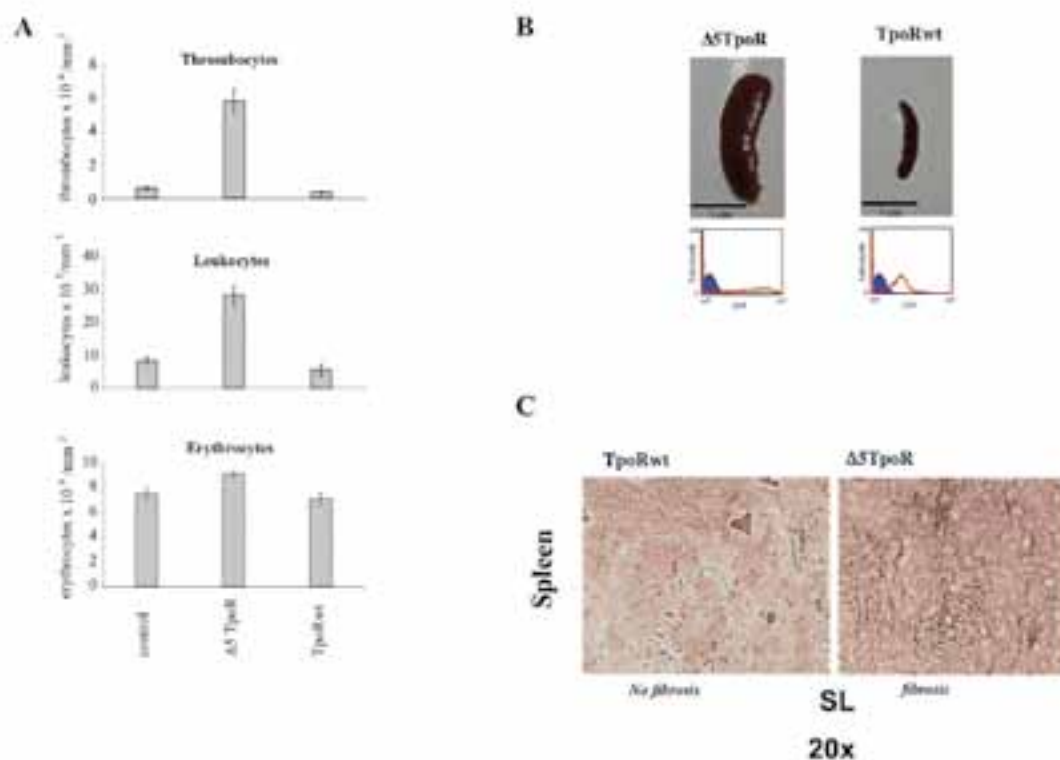


**Figure 2.** (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pseudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients. (B) The pseudokinase (JH2) and kinase (JH1) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other and leading to JH1 inhibition. Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 (red arrow) and eventually in activating the kinase activity of JH1. The region around V617F and the middle of JH2 helix C surrounding F595 could become the target of inhibitors that might specifically decrease constitutive activation of JAK mutants (Alexandra Dusa).

cells, and is partially responsible for TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells.

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR)

results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Mutagenesis of the RWQFP motif showed that W515 (W508 in the murine) is the key residue maintaining human TpoR normally inactive (6). In vivo, in bone marrow reconstituted mice, the delta5TpoR and TpoR W515A induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (Figure 3) (7). Why the



**Figure 3.** Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active  $\Delta 5TpoR$  induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen.  $\Delta 5TpoR$  is a mutant where the amphipathic RW<sup>515</sup>QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by  $\Delta 5TpoR$ . (B) Splenomegaly was induced by  $\Delta 5TpoR$  at day 45 post reconstitution. The spleen size in TpoRwt mice was equivalent to that in control healthy mice. Green fluorescence protein (GFP) levels were equal after transduction, but enhanced migration to the spleen and proliferation explain the high GFP levels in  $\Delta 5TpoR$  spleens. (C) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates fibrosis of the spleen in the  $\Delta 5TpoR$  mice (Christian Pecquet and Judith Staerk).

phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group. We recently established that the myelofibrosis phenotype induced by TpoR W515 mutants depends on cytosolic Y112 (Y626) of TpoR, and appears to involve excessive STAT3 and MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 (Y626) might be useful in the treatment of myelofibrosis.

### CONSTITUTIVE ACTIVATION OF JAK-STAT SIGNALING AND GENES TARGETED BY STAT5 IN TRANSFORMED HEMATOPOIETIC AND PATIENT-DERIVED LEUKEMIA CELLS

*M. Girardot, J. Van Hees*

Cytokine stimulation of cytokine receptors induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a

continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1, 9). A similar picture has been noted in patient-derived leukemia cells. The critical question is which genes are specifically regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation (Chip) and sequencing of native promoters bound by STAT5 we noted that, in transformed cells, STAT5, and mainly STAT5B, can also bind to low affinity N4 (TTCNNNN-GAA) DNA sites, not only to the high affinity N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Preferred Partner (LPP) (10), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation (10). miR-28 is pathologically overexpressed in 30% of MPNs (10). Targets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders. Furthermore, miR-28 is specifically associated with megakaryocyte proliferation and induces a block in differentiation (10). We are studying the mechanisms of pathologic induction of LPP/miR-28 via constitutively active STAT5.

## **INTERACTION WITH ST LUC HOSPITAL CLINICIANS AND CLINICAL BIOLOGISTS: IDENTIFICATION OF THE MOLECULAR BASES OF MPNS WITHOUT KNOWN MOLECULAR CAUSE**

Under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Cédric Hermans, Prof. Augustin Ferrant, Dr. Laurent Knoops), Clinical Biology (Prof. Dominique Latinne, Dr Hélène Antoine-Poirel) and groups of de Duve Institute (Prof. Mark Rider, Prof. Jean-Baptiste Demoulin) our laboratory is performing a large study on the presence and signaling of JAK2, TpoR, and growth factor receptor mutations in patients with myeloproliferative neoplasms. Next generation sequencing will be employed for well-investigated patients, using primary cells that are characterized for functional defects and that do not harbor known mutations in order to unravel novel molecular defects in MPNs and leukemias.

## **DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EpoR, TpoR AND G-CSFR DIMERS**

*J.-P. Defour, C. Pecquet, E. Leroy*

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change, which results in the activation of cytosolic JAK2 proteins (8).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of  $\alpha$ -helices (9). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil  $\alpha$ -helices is imposed on the downstream TM  $\alpha$ -helix and

intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (9). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

Similar studies are undertaken for the related TpoR and G-CSFR. Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells. We have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the normal dimeric interface that leads to formation of platelets, other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Staerk et al., submitted).

## **STRUCTURE AND FUNCTION OF JUXTA- AND TRANS-MEMBRANE SEQUENCES OF CYTOKINE RECEPTORS**

*R.-I. Albu, A. Dusa, J.-P. Defour, J. Van Hees, C. Mouton*

We have previously shown that the EpoR as well as a fraction of IL2/IL9 receptors exist on the cell surface as a preformed ligand-independent inactive dimers (homomeric and

heteromeric in the case of IL2/IL9 receptor complexes). For the EpoR, transmembrane domain interactions stabilize the inactive dimer at the surface and the EpoR TM sequence is an example of TM dimer based on purely hydrophobic sequences (Proc. Natl Acad. Sci USA 2001, 98, 4379-84; EMBO J., 1999, 18, 3334-47). We study potential transmembrane interactions in the context of other transmembrane proteins, such as TpoR, G-CSFR. We use cell surface immunofluorescence co-patching of differentially epitope tagged receptors and protein ligation assays in order to determine the ligand-independent state of cell surface complexes. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand. In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the group of Prof. Jean-Noel Octave. We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these motifs promote amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733)

## **TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE**

*R.-I. Albu, C. Pecquet*

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine receptors (i.e. EpoR TpoR, IL9R, IL2R,  $\gamma$ ). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus, while for others, such as the TpoR, JAK2 and TYK2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope

tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. Furthermore, the extracellular fibronectin type III modules of TpoR (D1, D2, D3 or D4) appear to be critical for efficient cell surface localization of TpoR, which in turn is essential for clearance of Tpo by circulating platelets. We have identified critical determinants in the extracellular domain of TpoR traffic. While a receptor lacking D1D2 (D3D4-TpoR) is not efficiently localized at the cell surface, introducing the juxtamembrane W515K activating mutation and selection for cytokine-independent growth leads to enhanced expression and detectable cell surface localization of the N-terminally truncated D3D4-TpoR. These results support the notion that cell surface localization is a prerequisite for constitutive signaling by TpoR W515 mutants. The roles of precise extracellular domain glycosylation motifs in TpoR traffic and signaling are also determined.

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## LINKS

Group:  
Ludwig Institute for Cancer Research Ltd.  
NewsLink Sept 2005 of our group (<http://www.licr.org/12124501528/newslink/0509/>)

European Commission Marie Curie Research  
Training Network ReceptEUR ([www.recepteur.org](http://www.recepteur.org))

## RESEARCH

Whitehead Institute for Biomedical Research,  
MIT, Lodish Lab (<http://www.wi.mit.edu/lodish/>)

SUNY Stony Brook, Structural Biology, Smith  
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## LEARNING

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