Signal Transduction (Medical Biotechnology)

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Publication date 2011

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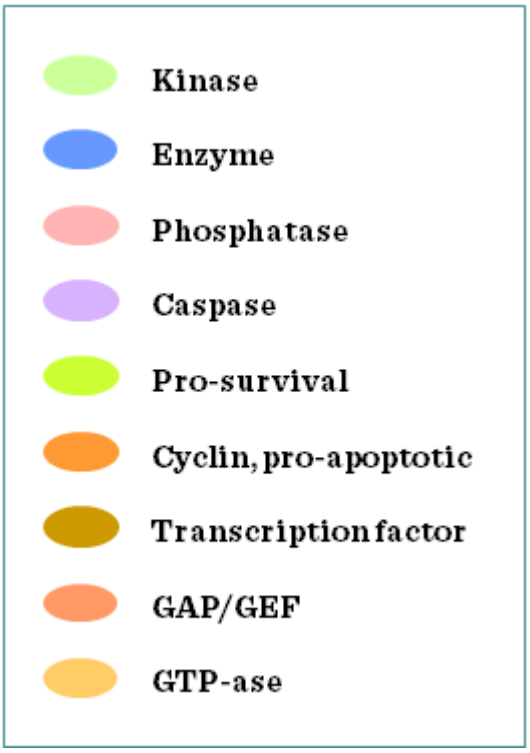


Chapter 2. I General signal transduction

1. I.1 Introduction, overview of extracellular signaling

Soluble mediators transmit information through the extracellular space over various distances in cell-to cell communication. In local (short distance) cell signaling, some cells may be in direct contact with each other in order to communicate. Cell-to-cell signaling means that mediators can pass from one cell to another cell through cell junctions, which are found in both animals and plants. Long distance signaling is mediated by hormones between animal cells (endocrine signaling), or growth factors between plant cells. Another general form of long distance signaling is synaptic signaling used mainly in the nervous system. In plants and animals extracellular signaling molecules control metabolic processes, growth and differentiation of tissues, synthesis and secretion of proteins, and the composition of intracellular and extracellular fluids.

1.1. Communication by extracellular signals usually involves sixsteps

(1) Synthesis and release of the extracellular mediator molecule by the signaling cell;

(2) Transport of the mediator to the target cell;

(3) Reception: detection of the signal by a specific receptor protein;

(4) Transduction: binding of the extracellular mediator molecule to a specific receptor on the target cell, and this signal is interpreted by a series of subcellular reactions called signal transduction events.

(5) Response: The signal triggers the desired reaction within the cell, for example a change in cellular metabolism, function, or development triggered by the receptor-signal complex.

(6) Termination: removal of the signal, which often terminates the cellular response.

1.2. Signaling molecules operate over various distances

Based on the distance over which extracellular, secreted molecules transmit the signal, cell-to-cell communication can be classified into three types: endocrine (long distance between the source of the mediator and the target cell – the mediator is transported by the circulation, sometimes bound to transport proteins), paracrine (the source of the mediator and the target cell are relatively close to each other – the mediator is transported by simple diffusion), or autocrine (in this case the mediator-producing- and the target cell is the same). In addition, certain membrane-bound proteins on a cell can directly transmit a signal to adjacent cells.

1.3. Receptor proteins exhibit ligand-binding and effector specificity

The cellular response to a particular extracellular signaling molecule depends on its binding to a specific receptor protein located on the surface or in the nucleus or cytosol of a target cell. The signaling molecule (a hormone, pheromone, or neurotransmitter) acts as a ligand, which binds to, or “fits” into a site on the receptor. Binding of a ligand to its receptor causes a conformational change in the receptor that initiates a sequence of reactions leading to a specific cellular response.

The response of a cell or tissue to specific hormones is determined by the particular hormone receptors it possesses and by the intracellular reactions initiated by the binding of any one hormone to its receptor. Different cell types may have different sets of receptors for the same ligand, each inducing a different response. Or the same receptor may appear on various cell types, and binding of the same ligand may trigger a different response in each type of cell (e.g. acetylcholine). Clearly, different cells respond in a variety of ways to the same ligand. On the other hand, different receptor-ligand complexes can induce the same cellular response in some cell types (e.g. glucagons and epinephrine).

Thus, a receptor protein is characterized by binding specificity for a particular ligand, and the resulting hormone-ligand complex exhibits effector specificity (i.e., mediates a specific cellular response).

1.4. Hormones can be classified based on their solubility and receptor location

Most hormones fall into three major categories: (1) small lipophilic molecules that diffuse across the plasma membrane and interact with intracellular receptors; and (2) hydrophilic or (3) lipophilic molecules that bind to cell-surface receptors (Figure I.1-1).

(1) Lipophilic hormones with intracellular receptors: many lipid-soluble hormones diffuse across the plasma membrane and interact with receptors in the cytosol or nucleus. The resulting hormone-receptor complexes bind to transcription-control regions of the DNA thereby affecting expression of specific genes. Hormones of this type include the steroids (e.g., cortisol, progesterone, estradiol, and testosterone), thyroxine, and retinoic acid (Figure I.1-1 and Figure I.1-2).

(2) Water-soluble hormones with cell surface receptors: As water-soluble signaling molecules cannot diffuse across the plasma membrane, they all bind to cell-surface receptors. This large class of compounds is composed of two groups: (a) peptide hormones, such as insulin, growth factors, and glucagon, which range in size from a few amino acids to protein-size compounds, and (b) small charged molecules, such as epinephrine and histamine, that are derived from amino acids and function as hormones or neurotransmitters. Many water-soluble hormones induce a modification in the activity of one or more enzymes already present in the target cell. In this case, the effects of the surface-bound hormone are usually nearly immediate, but persist for a short period only. These signals also can give rise to changes in gene expression that may persist for hours or days. In yet other cases water-soluble signals may lead to irreversible changes, such as cellular differentiation.

(3) Lipophilic hormones with cell-surface receptors: The primary lipid-soluble hormones that bind to cell-surface receptors are the prostaglandins. There are at least 16 different prostaglandins in nine different chemical classes, designated PGA – PGI. Prostaglandins are part of an even larger family of hormones containing 20 carbon atoms called eicosanoid hormones. In addition to prostaglandins, they include prostacyclins, thromboxanes, and leukotrienes. Eicosonoid hormones are synthesized from a common precursor, arachidonic acid. Arachidonic acid is generated from phospholipids and diacylglycerol.

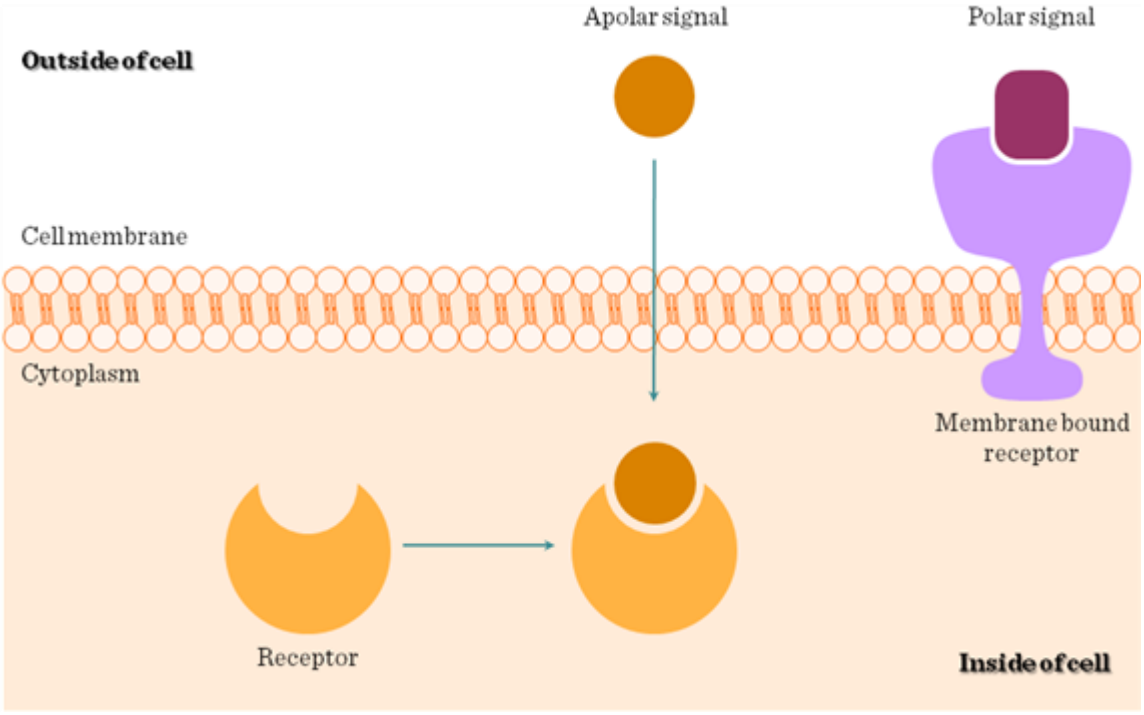


Figure I.1-1: Main types of receptors

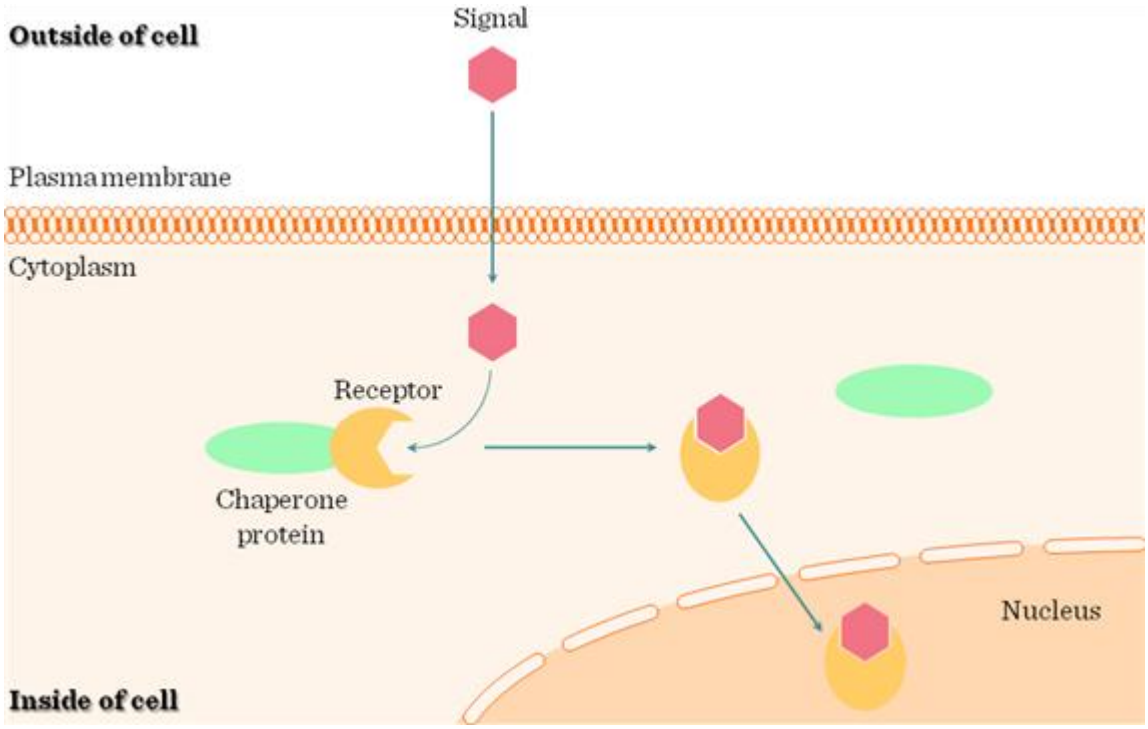


Figure I.1-2: Intracellular receptor signaling

2. I.2 Families of extracellular receptors

2.1. Introduction

Ligands act on extra- or intracellular receptors according to their hydrophilic or hydrophobic nature, respectively (Figure I.1-1). Hydrophobic/lipophilic molecules (e.g. steroid hormones, thyroid hormone, vitamin D) can diffuse through the plasma membrane lipid layer, thus reaching intracellular receptors (Figure I.1-2) (for details see Chapter II.2.3). Hydrophylic/water soluble ligands (e.g. peptide hormones, cytokines, chemokines, neurotransmitters), on the other hand, are unable to penetrate the lipid rich outer barrier of the cells; therefore, they need receptors protruding from the outer surface of the cell membrane.

2.2. Extracellular receptor groups

In case of extracellular receptors, the effect of ligand binding has to be transmitted into the cell. A number of signal transduction pathways have evolved to serve this purpose.

Extracellular receptors belong to 3 major categories (Figure I.2-1):

(1) Ion-channel receptors

(2) 7-transmembrane-spanning receptors (7-TM), also called G-protein-coupled receptors (GPCR)

(3) Enzyme-linked receptors

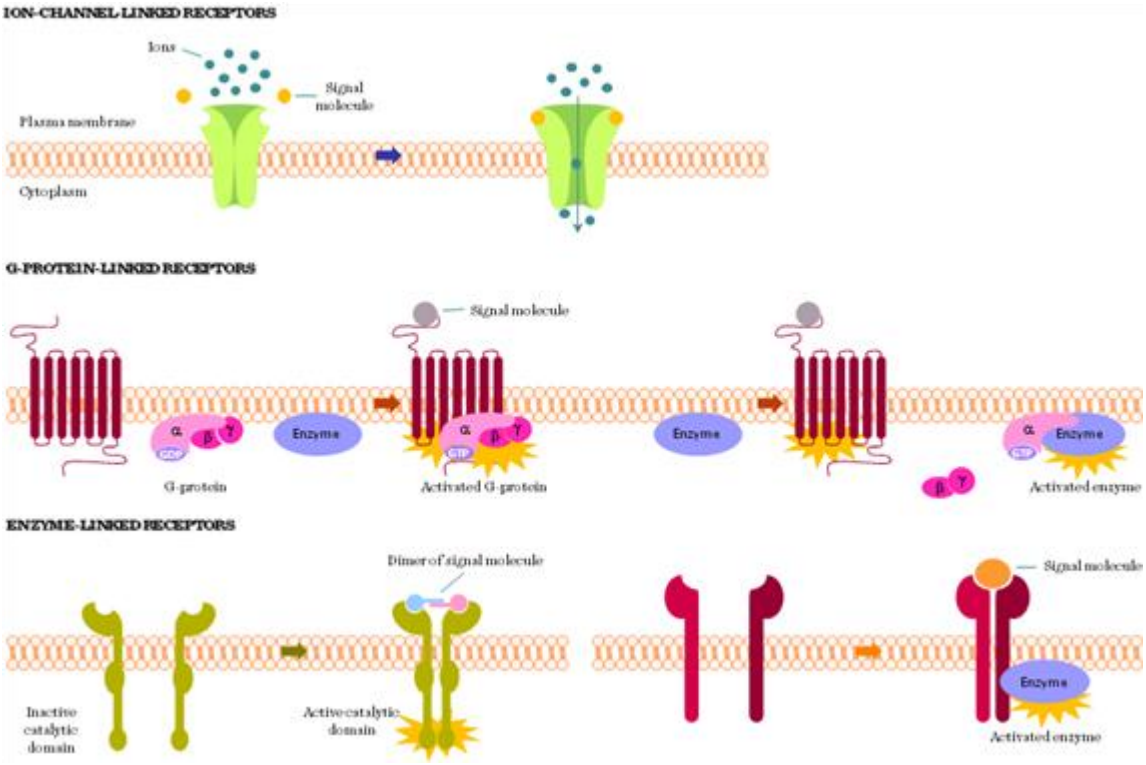


Figure I.2-1: Extracellular receptor types

2.3. I.2.1 Ion-channel receptors

Ligand-operated receptors in the plasma membrane (e.g. GABAA, GABAC, iGlu, Glycine, Serotonin, nicotinic Ach, P2X receptors) belong to this group. They are quite abundant in the nervous system and on contractile cells (smooth/striated/heart muscle). Their function is relatively simple: upon activation they open and ion currents occur as a consequence of the concentration gradient between the extra- and intracellular environment. The transient ion concentration changes lead to the contraction or depolarization of the target cells. There are 3 groups of ion-channel receptors:

(1) Cys-loop receptors: have pentameric structure with 4 trans-membrane (TM) regions in each subunit (e.g. Acetylcholin (Ach) Nicotinic Receptor – Na+ channel; GABAA, GABAC, Glycine – Cl- channels [inhibitory role in CNS]) (Figure I.2-2 and Figure I.2-3).

(2) Glutamate-activated cationic channels: have tetrameric structure with 3 TM regions in each subunit (e.g. iGlu) [excitatory role in CNS]

(3) ATP-gated channels: have three homologous subunits with two TM regions in each subunit (e.g. P2X purinoreceptor)

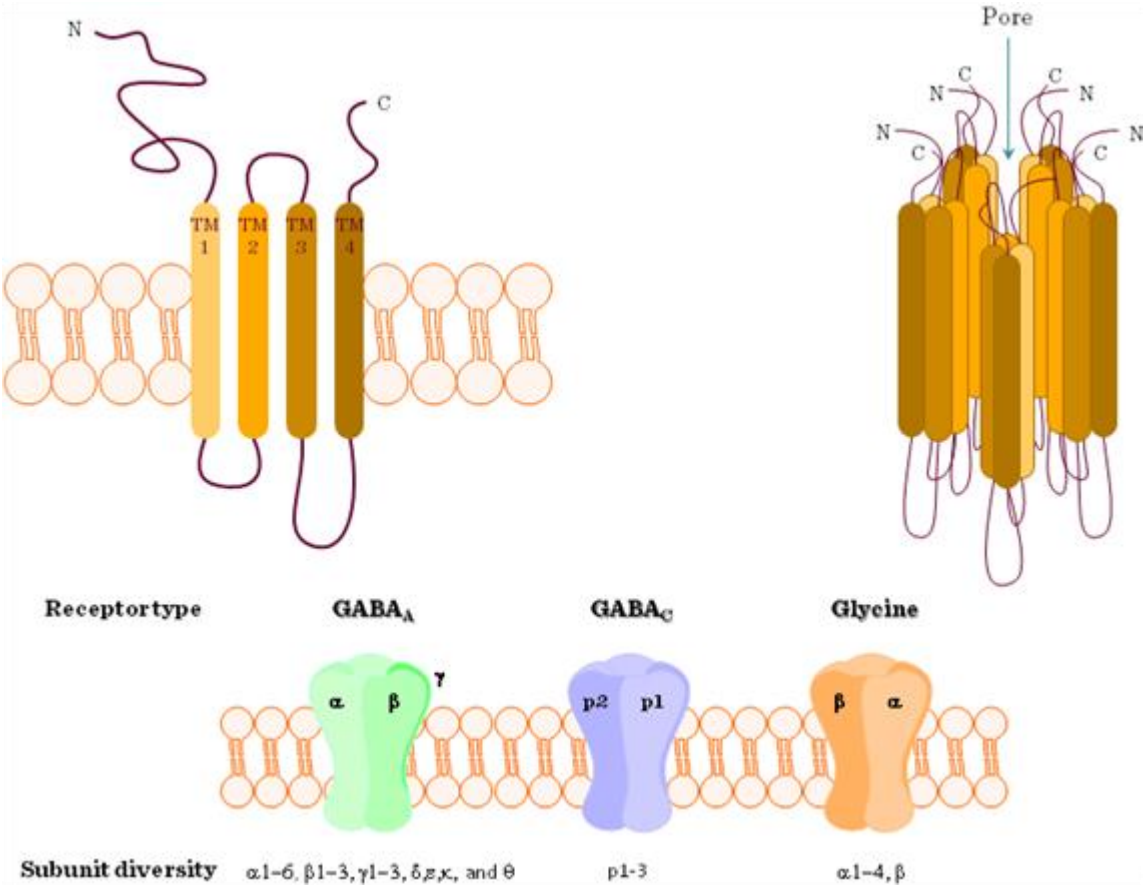


Figure I.2-2: Cys-loop ion-channel receptors

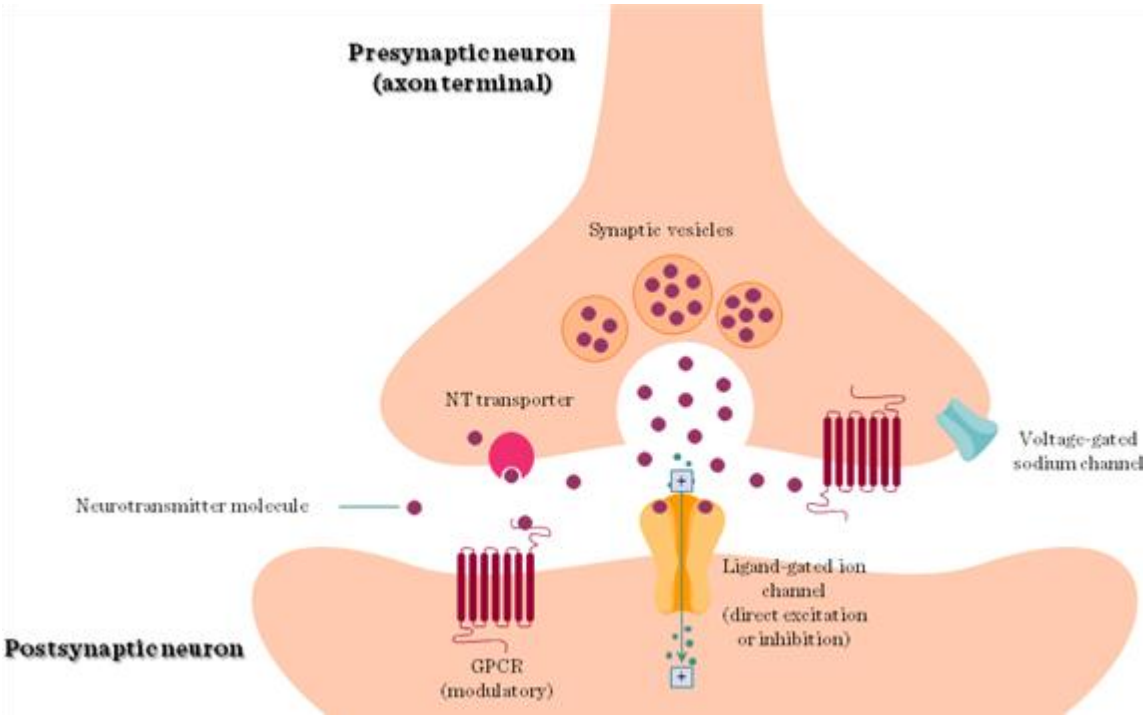


Figure I.2-3: Synapse between two neurons - neurotransmission

2.4. I.2.2 7-transmembrane-spanning receptors (7-TM)

2.4.1. Groups of the 7-transmembrane (7-TM) spanning receptors

The 7-TM receptor family (Table I.2-1) is one of the largest gene families in vertebrates comprising more than 700 members. They fall into:

(1) Class A: Rhodopsin-like (e.g. prostaglandins, thromboxanes, serotonine, dopamine, histamine, catecholamines, Ach (M), rhodopsin, melatonin, chemokines, bradykinin, somatostatin, opioid, vasopressin receptors)

(2) Class B: Secretin family (e.g. glucagon, GnRH, PTH, CRH receptors)

(3) Class C: Glutamate and GABA (metabotropic) (Glutamate, GABA, sweet taste, secretin receptors)

(4) Frizzled (e.g. Wnt, Hedgehog, bitter taste receptors)

(5) Adhesion family (e.g. chondroitin sulfate receptors) groups.

Despite the complexity of the already identified ligands, it has to be noted, that there are still more than 200 "orphan" receptors (= no identified ligand yet) in the 7-TM family.

Table I.2-1: Receptor types

Receptor properties	Ligands
Ligand binds in the core region of the 7 transmembrane helices	11-cis-retinal (in rhodopsin) acetylcholine catecholamines biogenic amines (histamine, serotomine, etc.) nucleosides and nucleotides leukotrienes, prostaglandins, prostacyclins, thromboxanes
Short peptide ligands bind partially in the core region and to the external loops	peptide hormones (ACTH, glucagon, growth hormone) parathyroid hormone, calcitonin
Ligands make several contacts with the N-terminal segment and the external loops	hypothalamic glycoprotein releasing factors (TRH, GnRH)
Induce an extensive reorganization of an extended N-terminal segment	metabotropic receptors for neurotransmitters (such as GABA and glutamate) Ca^{2+}-sensing receptors, for example on parathyroid cells, thyroidal C-cells (which secrete calcitonin) and on the renal juxtaglomerular apparatus
Proteinase activated receptors	receptors for thrombin amd thrypsin

2.4.2. Structure of the 7-transmembrane (7-TM) spanning receptors

As implicated by their name, the polypeptide chain of these receptors crosses the plasma membrane 7 times (Figure I.2-4); the N-terminus is extracellular, while the C-terminus is intracellular. The transmembrane (TM) α-helical domains are separated from each other by extracellular- and intracellular loops (EL and IL). These domains take on a "barrel-like" conformation in the membrane, with the ligand-binding site in the middle. While the extracellular ligand-binding region of the receptors show variation,, the transmembrane and intracellular parts, on the other hand are more conserved. Palmitoylation of cysteine residues at the C terminus establish the connection of the 7-TM receptors to cholesterol and sphingolipid rich membrane microdomains ("rafts"). IL2 and IL3 are important for the association with G-proteins (Figure I.2-5) (see next chapter).

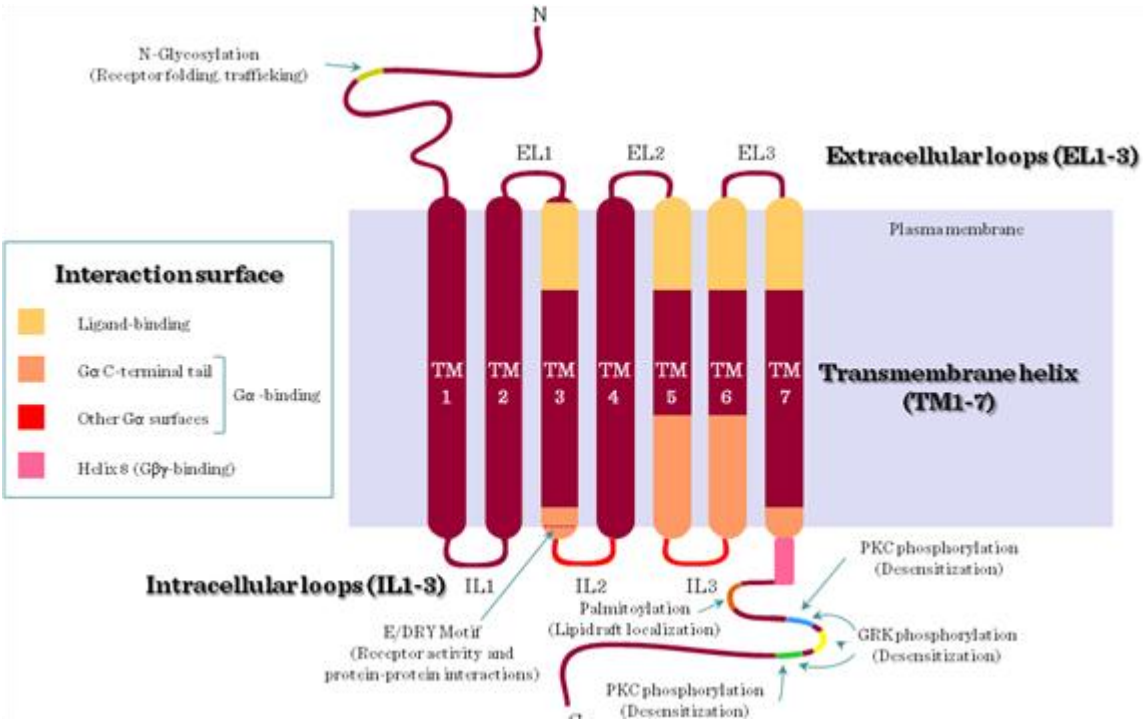


Figure I.2-4: 7-transmembrane-spanning receptors (7-TM)

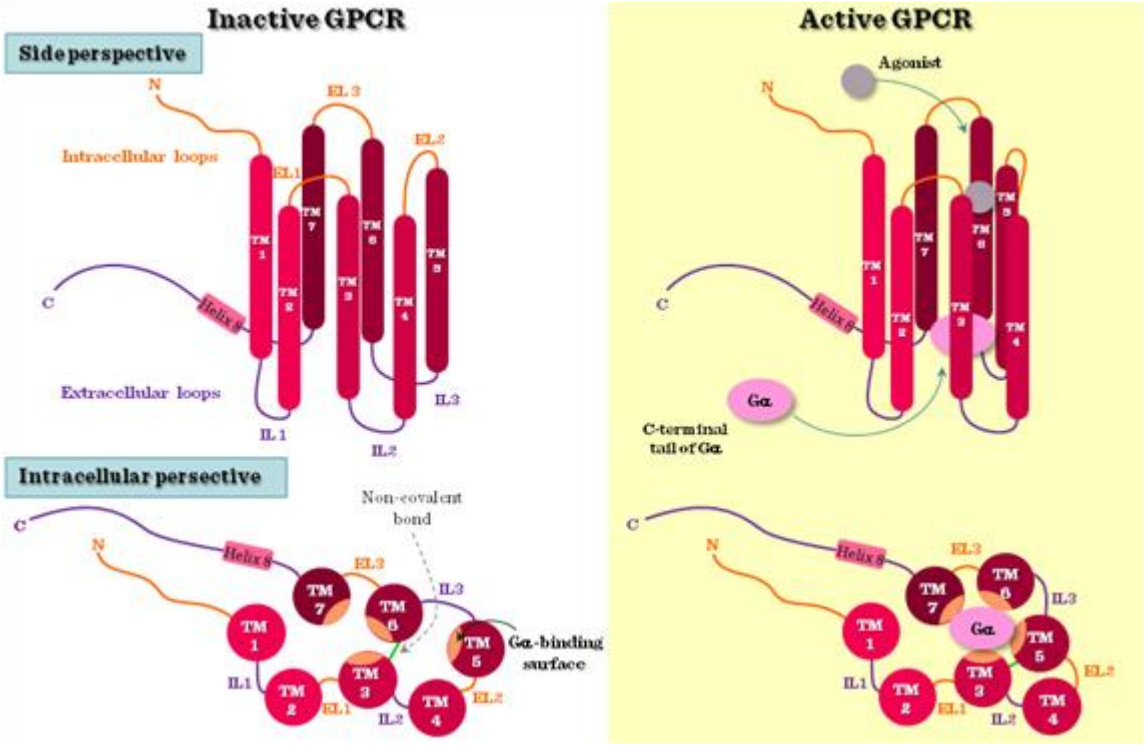


Figure I.2-5: Structure of 7-TM receptors

2.4.3. Regulation of 7-TM receptors (GPCR)

(1) 7-TM activation is regulated by the phosphorylation of the C terminus of the receptors by PKA (feedback phosphorylation) or G-protein receptor kinases (GRK1-7).

(2) Translocation: the active receptor with the surrounding membrane is internalized – dephosphorylated in acidic vesicles and recycled to the surface.

(3) Arrestin linking: binding of arrestin molecules inhibit the binding of Gs proteins to the receptors (e.g. rhodopsin in retina); + activation of alternative pathways: MAPK, PI3-K, PKB/Akt, Src (Figure I.2-6).

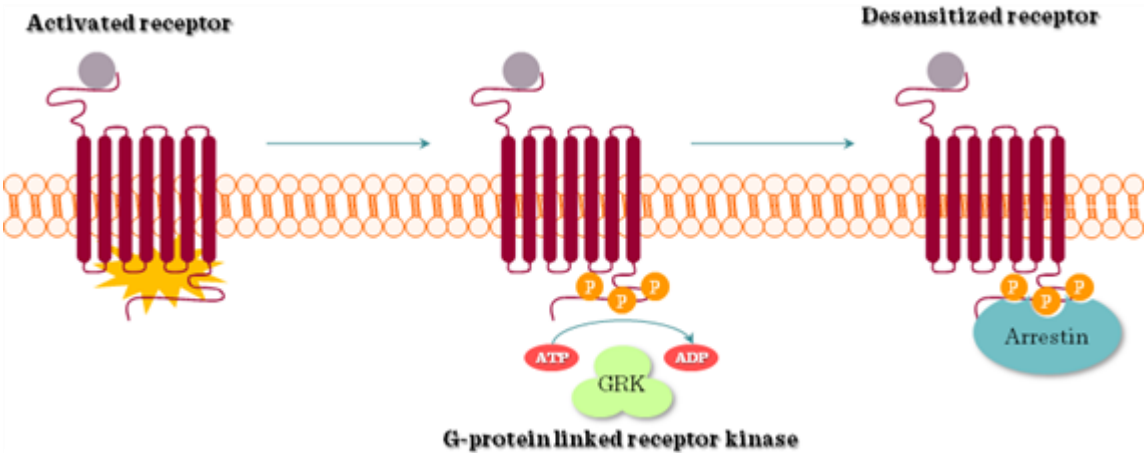


Figure I.2-6: Receptor desensitization

2.5. I.2.3 Enzyme-linked receptors

Enzyme-linked receptors are a group of multi-subunit transmembrane proteins that possess either intrinsic enzymatic activity in their intracellular domain or associate directly with an intracellular enzyme (Figure I.2-1). Generally, upon ligand binding a conformational change is transmitted via a transmembrane helix, which activates enzymatic activity initiating signaling cascades.

Groups of receptors that have intrinsic enzymatic activities include:

(1) Receptor Tyrosine Kinases (RTK) (e.g. PDGF, insulin, EGF, VEGF and FGF receptors) (Figure I.2-7);

(2) Receptor Tyrosine Phosphatases (e.g. CD45 [cluster determinant-45] protein of T cells and macrophages) (Figure I.2-7 and Figure I.2-8);

(3) Receptor Guanylate Cyclases (e.g. natriuretic peptide receptors) (Figure I.2-9);

(4) Receptor Serine/Threonine Kinases (e.g. activin and TGF-β receptors).

(5) Tyrosine-Kinase Associated Receptors: Receptors that associate with proteins that have tyrosine kinase activity (Cytokine Receptors, T- and B cell receptors, Fc receptors)

Receptors with intrinsic tyrosine kinase activity are capable of autophosphorylation as well as phosphorylation of other substrates. Additionally, several families of receptors lack intrinsic enzyme activity, yet are coupled to intracellular tyrosine kinases by direct protein-protein interactions (see below).

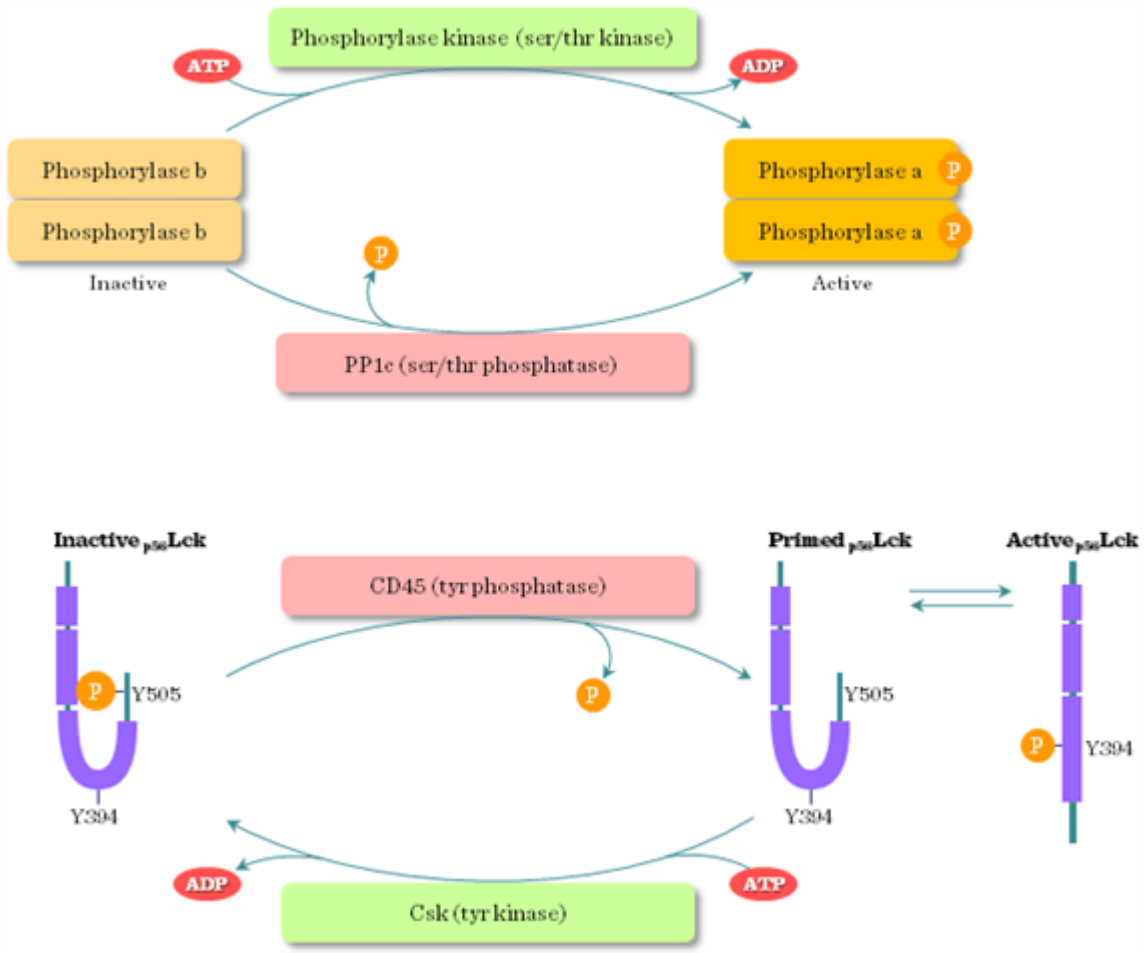


Figure I.2-7: Kinase-phosphatase balance

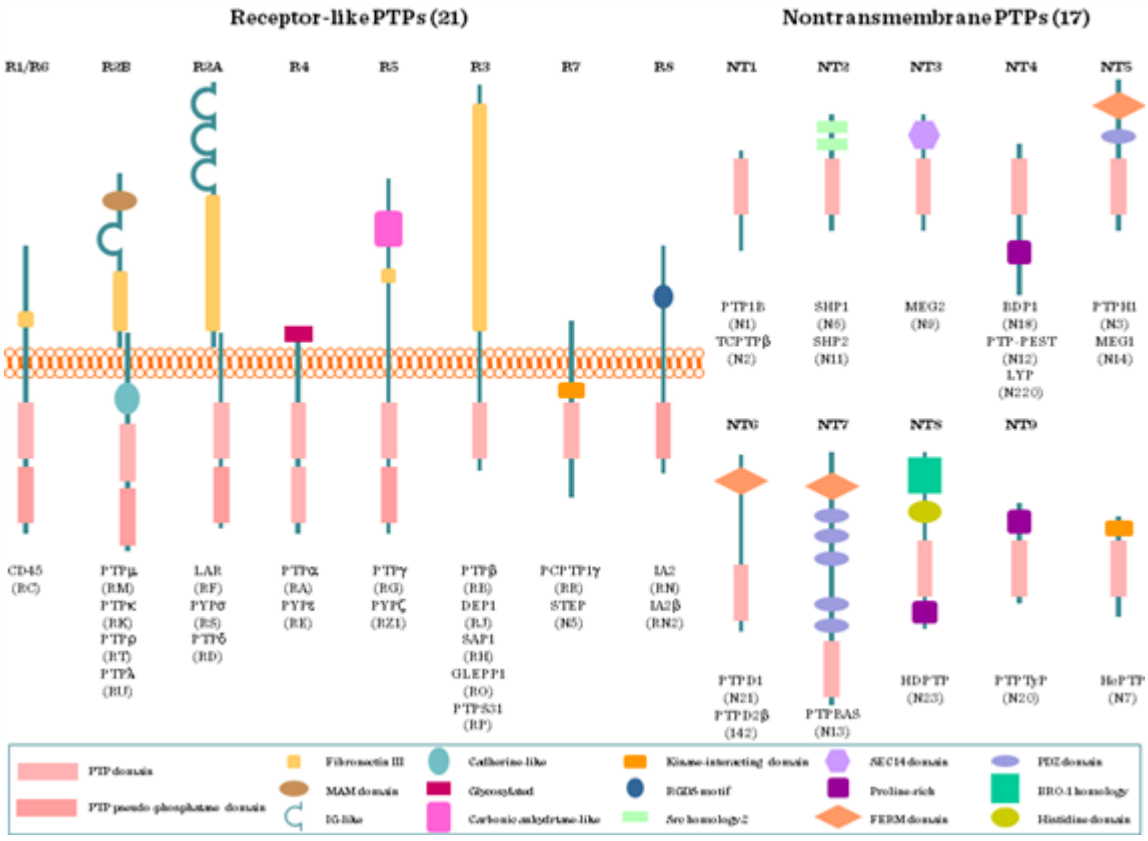


Figure I.2-8: Receptor- and cytoplasmic PTPs

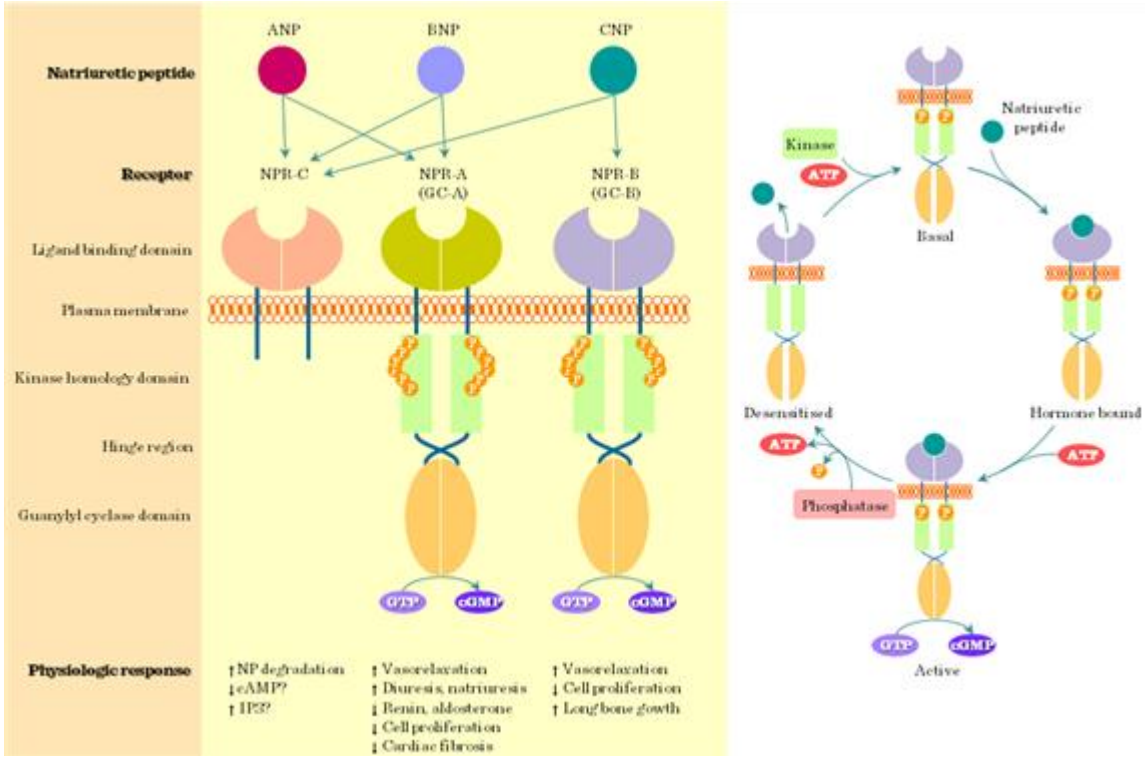


Figure I.2-9: Natriuretic peptide signaling

2.5.1. I.2.3.1 Receptor tyrosine kinases

2.5.1.1. Introduction, definitions

Tyrosine kinases are signaling proteins with catalytic activity to phosphorylate tyrosine residues. Tyrosine-phosphorylation, in turn, is a ubiquitous signaling event in several pathways. There are two major groups of tyrosine kinases:

(1) “Complete” Receptor tyrosine kinases (RTK) are cell surface receptors with intrinsic kinase activity (i.e. own intracellular kinase domain) [e.g. growth factor receptors].

(2) “Incomplete” or Non-receptor tyrosine kinases (nRTK) are cytosolic or membrane-anchored kinases associated with different cell surface receptors and transmit their signal towards the intracellular signaling networks [e.g. Src family kinases, Syk family kinases].

2.5.1.2. Families and structure of receptor tyrosine kinases

There are 90 unique Tyr kinases in the human genome, 58 are RTKs, most of them are growth factor-, cytokine- and hormone receptors (Figure I.2-10). Classes: I– EGFR family (ErbB); II– Insulin rec. family; III– PDGF family; IV– FGF family; V– VEGF family; VI– HGF family (c-Met); VII– Trk family; VIII– Eph family; IX– AXL family; X– LTK family; XI–TIE family; XII– ROR family; XIII– DDR family; XIV– RET family; XV– KLG family; XVI– RYK family; XVII– MuSK family. Some of those are expressed ubiquitously (EGFR), while others are tissue-specific (NGFR).

All receptor tyrosine kinases have extracellular ligand binding domain(s), a trans-membrane domain and intracellular kinase domain(s). The structure of the ligand binding domains is highly variable, they are built from fibronectin III-, cysteine rich-, factor VIII like-, Ig like-, leucin rich-, EGF like-, kringle-, C1r like-, glycine rich-, cadherin or acid box domains, whereas the transmembrane- and kinase domains are similar (Figure I.2-10).

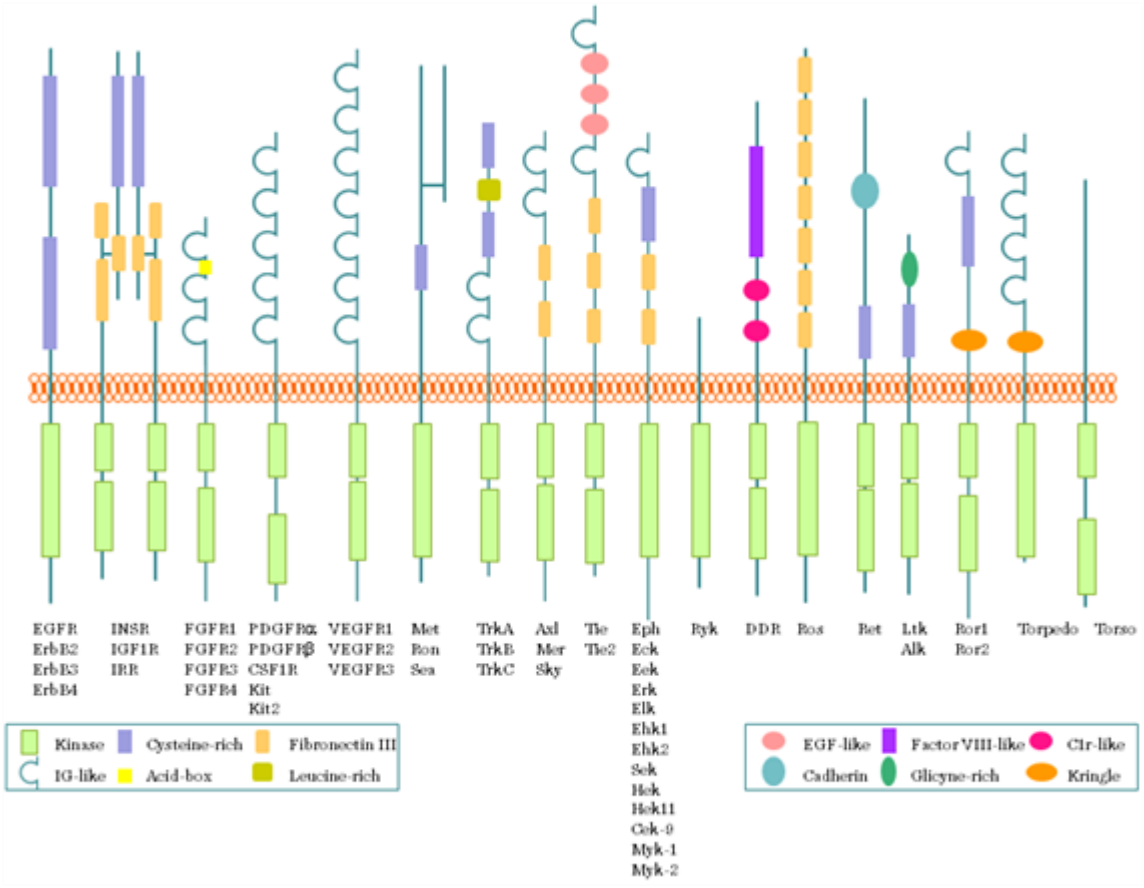


Figure I.2-10: Receptor tyrosine kinase family

2.5.1.3. Signaling through receptor tyrosine kinases

Main steps of RTK signaling (Figure I.2-11, Figure I.2-12 and Figure I.2-13):

(1) Ligand binding

(2) Dimerization (except the insulin receptor, which has a tetrameric structure)

(3) Autophosphorylation

(4) Signal complex (adapter proteins, kinases etc.)

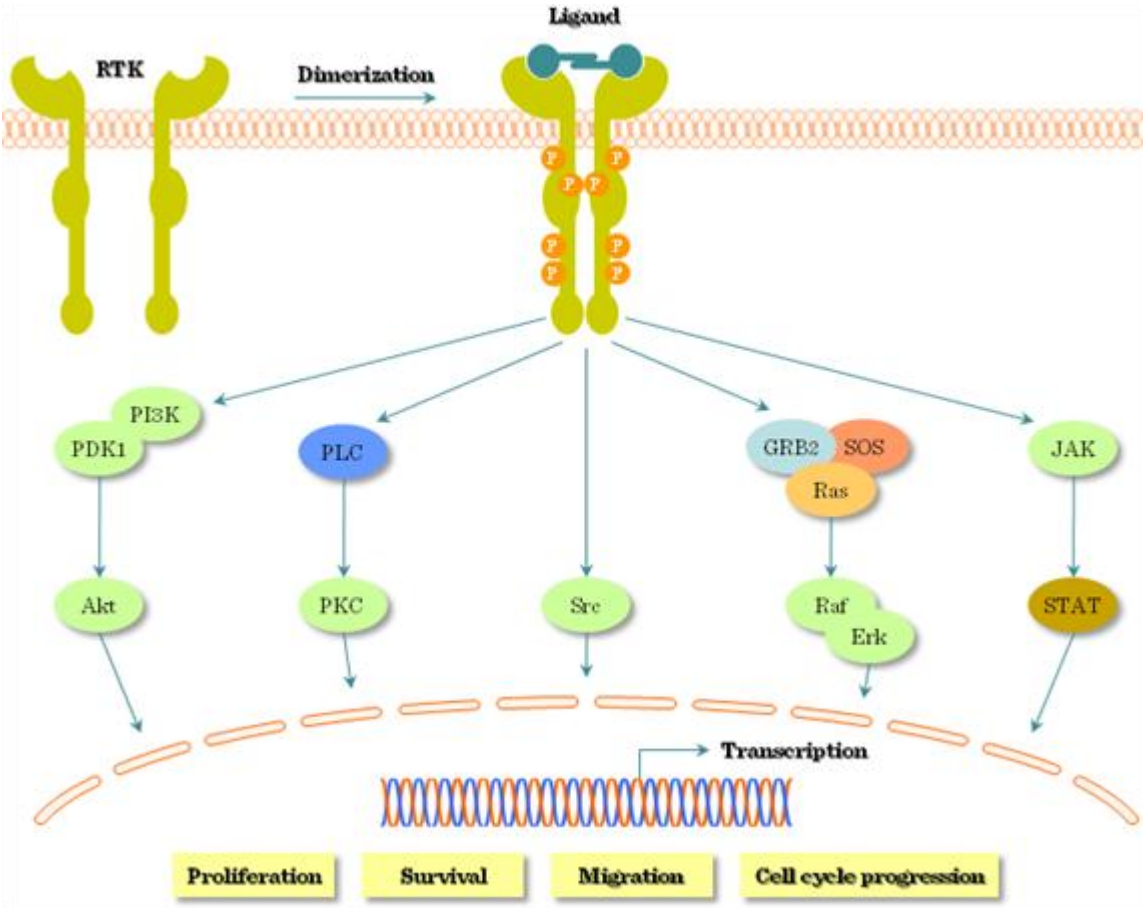


Figure I.2-11: Receptor tyrosine kinase (RTK) signaling

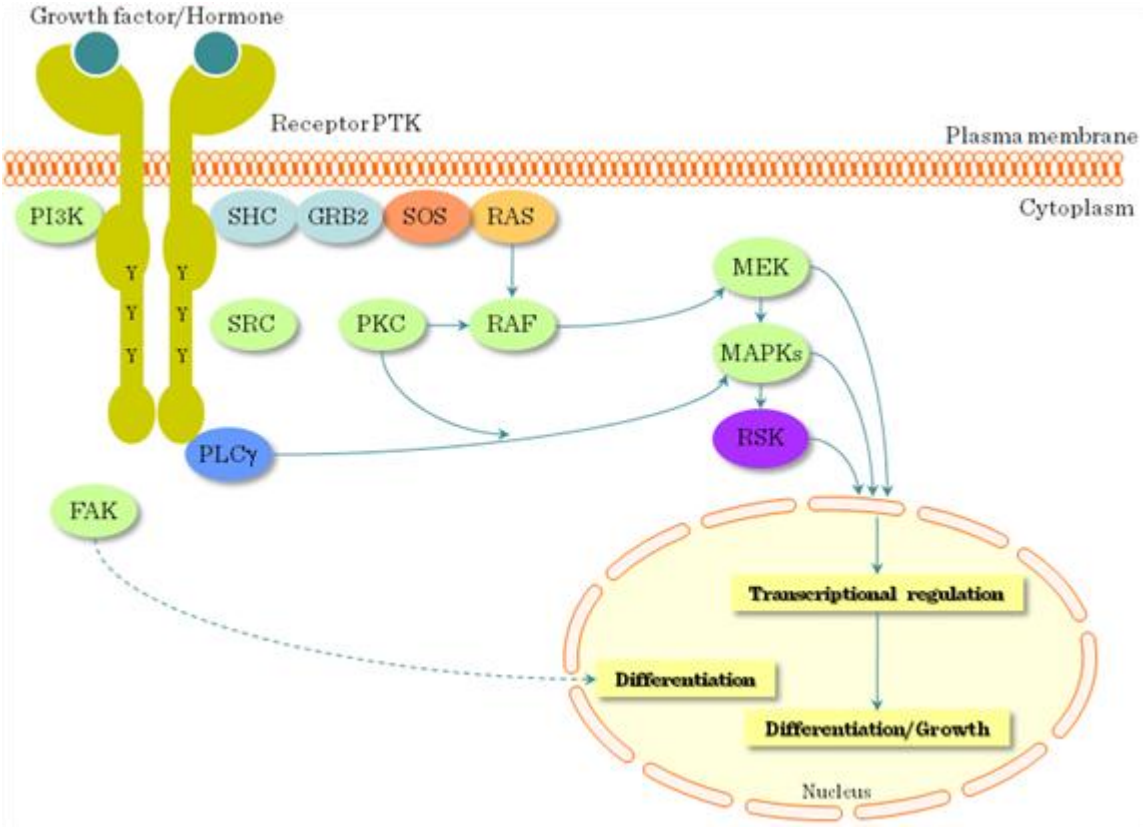


Figure I.2-12: Members of the signaling complex

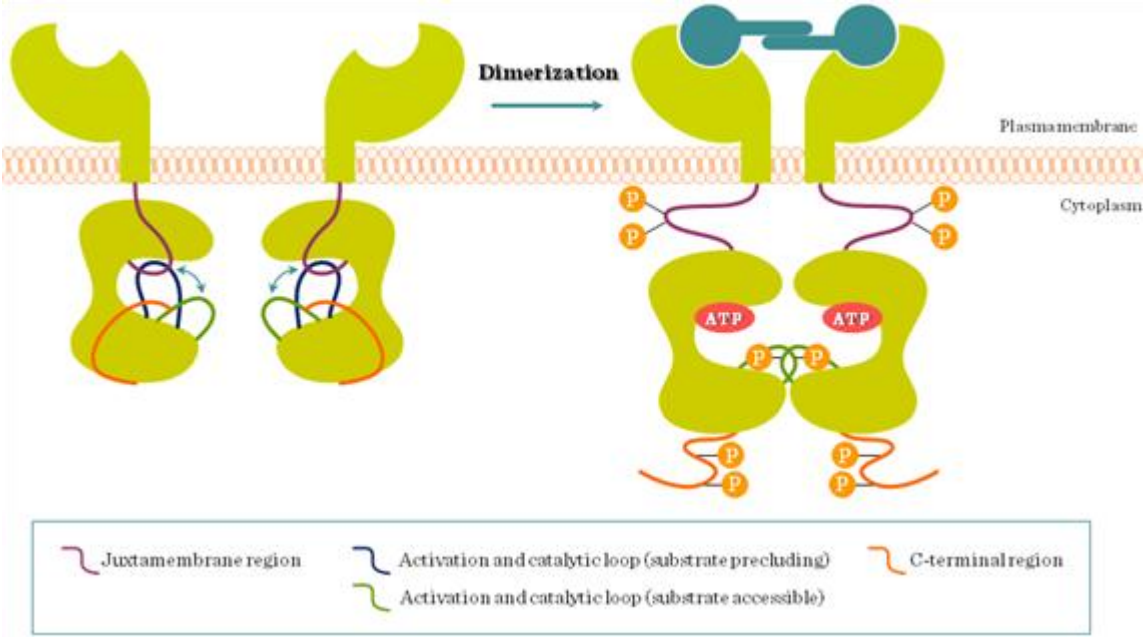


Figure I.2-13: Dimerization of GF receptors

Members of this initial signal complex include (Figure I.2-14):

(1) enzymes/transcription factors e.g. Src/Syk family kinases, SHP-1, PLCg, Sos, Vav, RasGAP, STAT1

(2) adaptors/regulators e.g. Grb2, SLP-76, SOCS1, Nck, Shc, Crk-L, p85

(3) adaptors/docking proteins e.g. FRS2, IRS1, DOK1

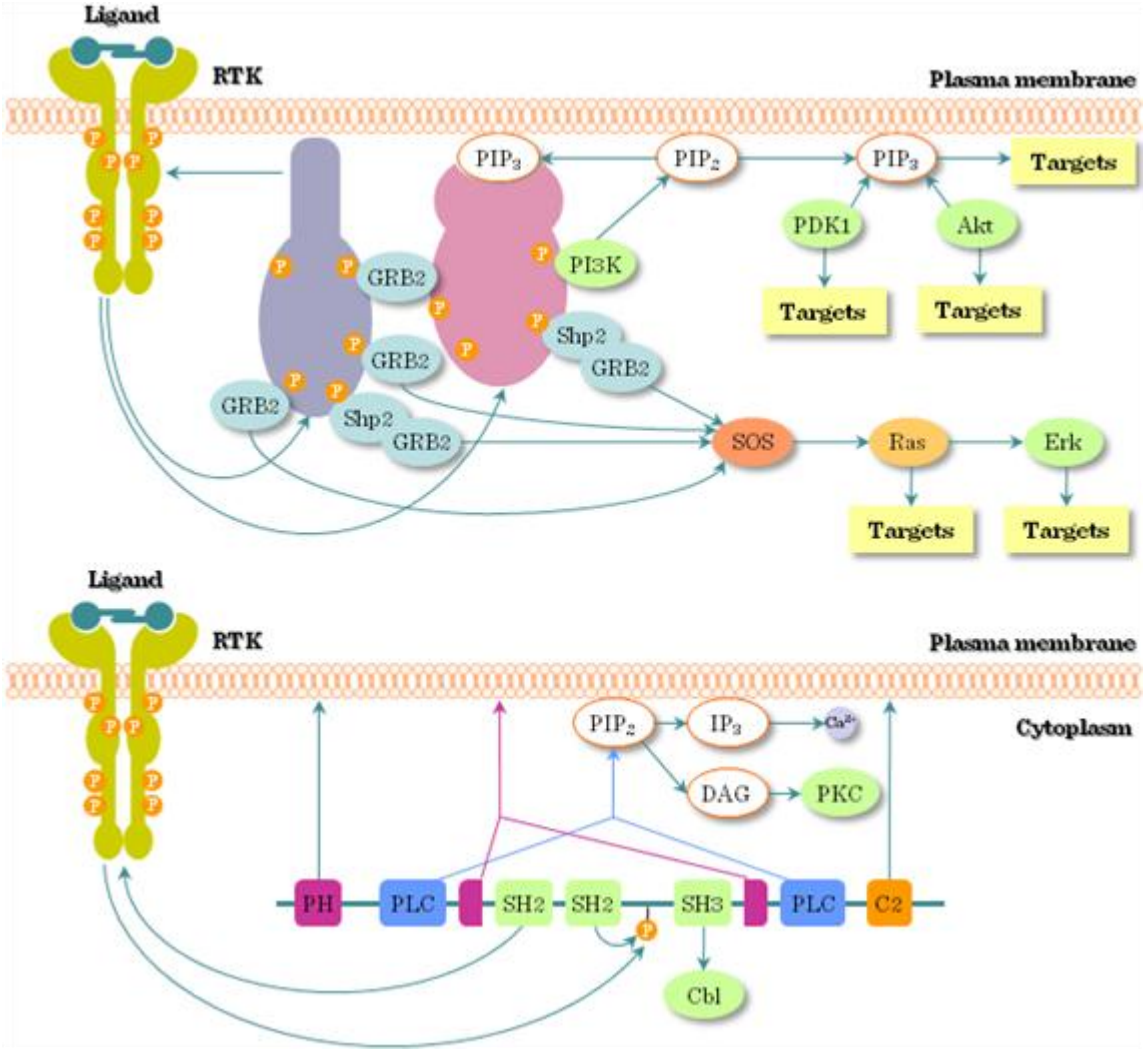


Figure I.2-14: GF receptor signaling pathways

Upon ligand binding dimerization of receptor tyrosine kinases occurs and receptors become autophosphorylated on tyrosine residues of the kinase domain. This leads to the buildup of the initial signal complex with the help of adaptor/docking proteins (Figure I.2-14). These adaptor proteins have Src-homology (SH) domains: SH2 domains associate with the autophosphorylated tyrosine residues of the receptors; while SH3 domains associate with the proline rich domains of further signaling molecules including guanine-nucleotid exchange factors (GEF; e.g. Sos, Vav) which catalyze the GDP-GTP exchange on the monomeric G-protein – Ras – which plays a key role in transducing signal from the growth factor receptors (Figure I.2-14). The GTP-bound Ras is activated and leads to activation of the mitogen-activated protein kinase pathway (MAPK-pathway). Ras proteins have only weak GTPase activity, thus, for their rapid inactivation GAP (GTPase activating protein) is also necessary.

2.5.1.4. Branching of the pathway

After RTK activation more intracellular signaling pathways are activated (Figure I.2-11):

(1) Ras – Raf – MEK – ERK (MAPK pathway)

(2) PLCγ – IP3 – Ca2+ (see I.5.2)

(3) PLCγ – DAG – PKC (see I.5.2)

(4) PI3 kinase (PI3K) – Protein kinase B (PKB) – Glycogen-synthase kinase (GSK)

(5) STAT activation

2.5.1.5. The MAPK pathway

Ras activates Raf, a MAP3K, which is a serine/threonine protein kinase. Raf phosphorylates MEK (MAP2K), a dual specific protein kinase, capable of phosphorylating target proteins both on tyrosine and threonine residues. The substrate of MEK is ERK (MAPK), which is a proline-directed kinase that phosphorylates its target proteins on serine/threonine-proline. ERK has many target proteins and can also translocate into the nucleus thereby regulating the transcription of different genes. MAPK-activated kinases (MK) include:

(1) Cytoplasmic Ribosomal S6 kinases (RSK) [e.g. initiation factors of translation, apoptosis machinery, oestrogen rec., Sos]. In some cases their phosphorylated form can translocate to nucleus [e.g. ATF4, c-Fos, SRF].

(2) Mitogen- and stress-activated kinases (MSK) are found in the nucleus [e.g. CREB, histone H3, HMGN1, ATF1].

(3) MAPK-interacting kinases (MNK) are components of the translation initiation complex.

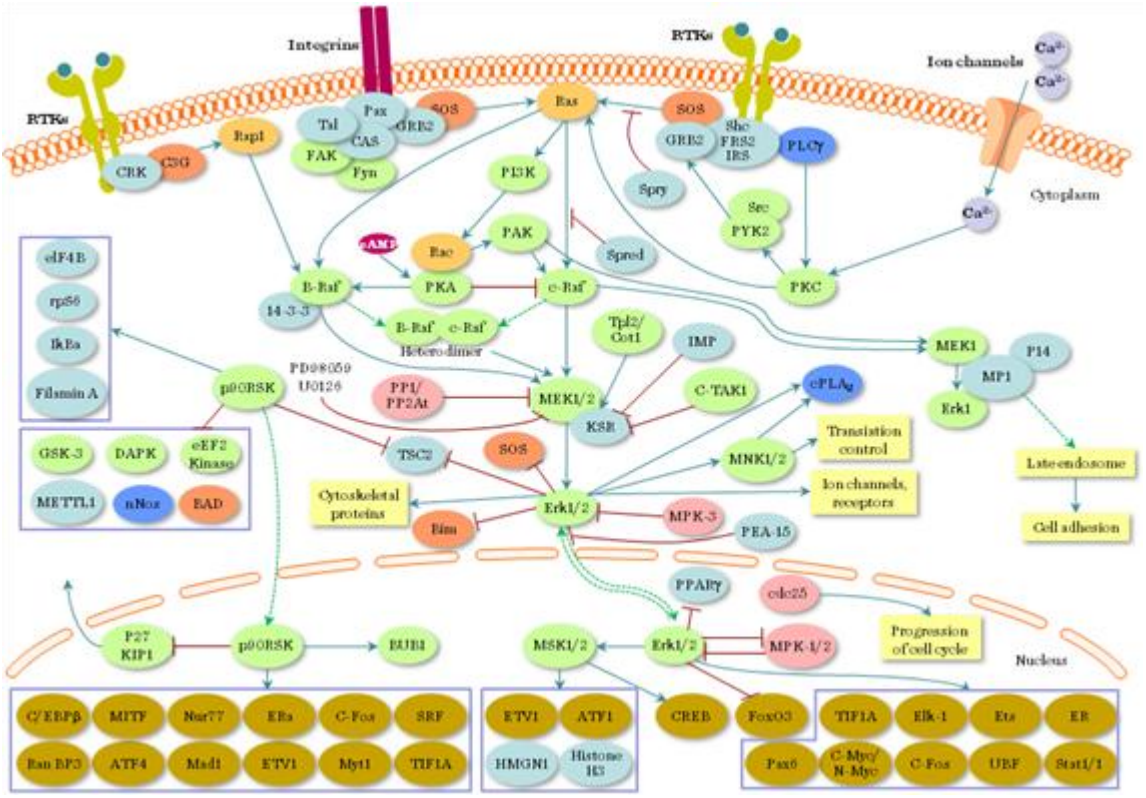


Figure I.2-15: MAPK/ERK in growth and differentation

There are parallel MAPK cascades activated by different signals. The above described “prototypic” MAPK pathway is the ERK-pathway activated by mitogen signals (Figure I.2-15). Cellular stress or cytokines activate the Jnk- or p38-pathways. Members of the MAPK pathways form spatially organized intracellular signaling complexes regulated / held together by scaffold proteins (e.g. IMP, KSR1).

2.5.1.6. Turning-off the pathway

Regulation of the MAPK-pathway is essential to control cell growth and differentiation. Switching-off the activation is done in part by phosphatases (e.g. PTP1B, SHP1/2, DEP1) which dephosphorylate activated members of the pathway. Phosphorylation of GEF (e.g. Sos) decreases their affinity towards the adapter (e.g. Grb2) leading to the dissociation of the initial signaling complex. GAPs inactivate Ras by changing GTP back to GDP. Finally, removal of cell surface receptors by endocytosis also contributes to the stopping of activation.

3. I.3 Intracellular receptors

See chapter II.2.3 Intracellular/nuclear receptor signaling (steroidhormonesandthyroxin).

4. I.4 Intracellular signal transmitting molecules

4.1. I.4.1 G-proteins

4.1.1. Trimeric G-proteins

7-TM receptors associate with G-proteins (=GTP-binding proteins); hence, they are called G-protein-coupled receptors (GPCR), too (Figure I.4-1). G-proteins bind to the intracellular IL2 and IL3 parts of the receptors. Trimeric G-proteins are a complex of α-, β- and γ subunits. In their inactive form, G-protein α subunit binds GDP; upon ligand binding, this GDP is exchanged to GTP resulting in the active form of the Gα, which dissociates from the complex and associates to effector proteins. Finally, GTP is hydrolyzed by the Gα and the inactivated Gα re-associates with the Gβγ-7-TM receptor complex. The Gγ subunit contains C terminal isoprenyl-chains anchoring it into the plasma membrane (Figure I.4-1). Based on their function, Gα subunits have different types:

(1) Gαs: stimulation of adenylyl-cyclase leading to increase of cAMP

(2) Gαi: inhibition of adenylyl-cyclase leading to decrease of cAMP

(3) Gαq: activation of PLC

(4) G12: activation of RhoGEF

Activated Gβγ subunits activate K+- and Ca2+-channels and PI3-kinase isoforms (Figure I.4-2).

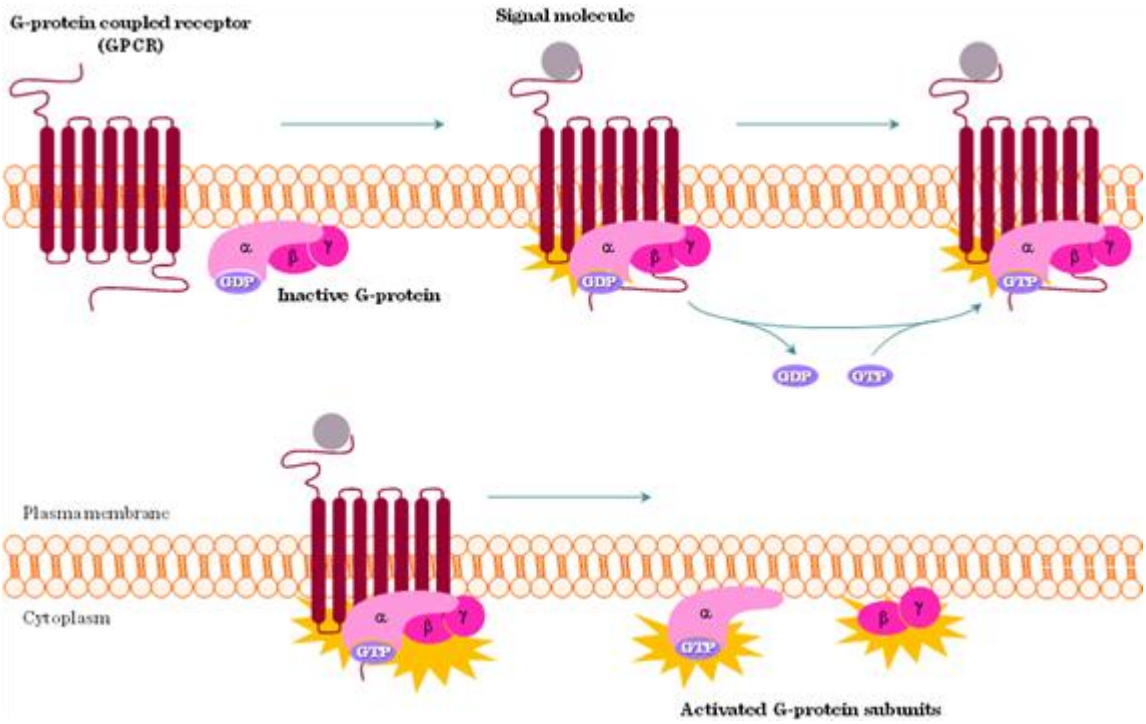


Figure I.4-1: Activation of G-protein-coupled receptors (GPCR)

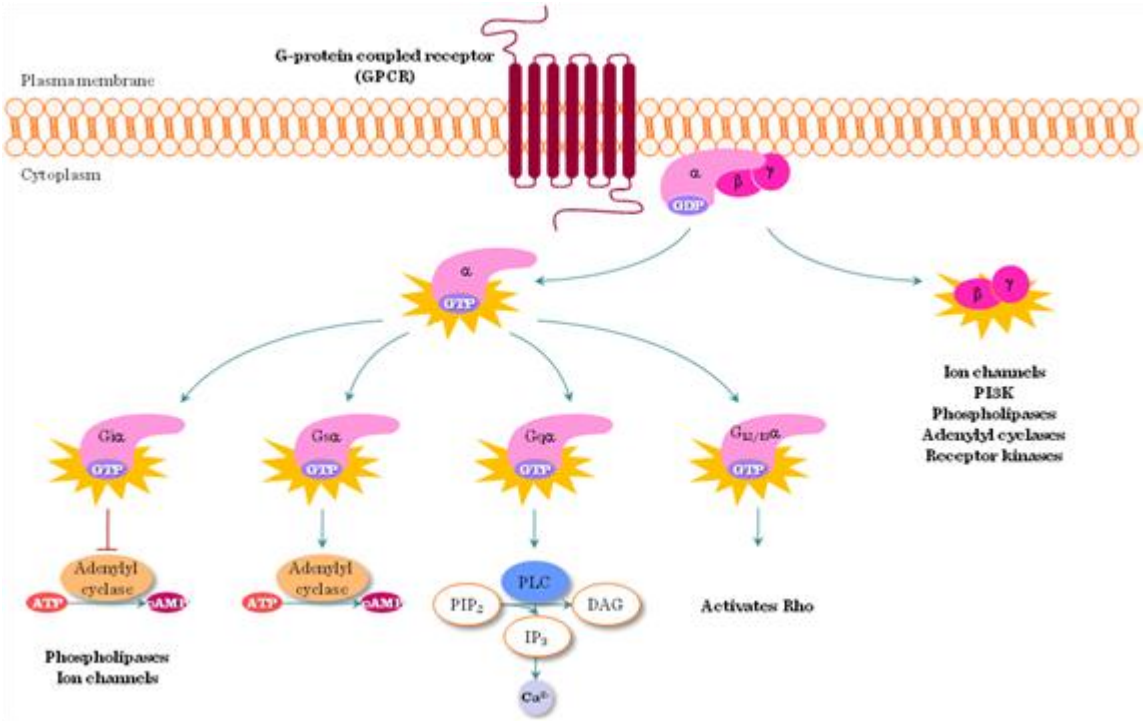


Figure I.4-2: G-proteins

4.1.2. Monomeric G-proteins – Ras

Monomeric G proteins were first discovered as transforming oncogenes in Harvey (H-Ras) and Kirsten (K-Ras) sarcoma viruses; hence their name Ras (=Rat sarcoma). N-Ras was first found in human neuroblastoma. Ras is a 189 amino acid long polypeptide, which is anchored to the membrane through lipid chains. It is of special importance, that mutations in the Ras family (Ras, Rho, Rab, Rap, Rheb) are found in 20-30% of all human tumors. Oncogenic point mutations most frequently affect the GTP-binding region of Ras. Ras is regulated by Guanine-nucleotide exchange factors (GEFs), which catalyse the GDP-GTP exchange of Ras, leading to its activation; and GTPase activating protein (GAP), which enhances the intrinsic GTPase activity of Ras, leading to its inactivation. Ras is involved in signaling through growth factor receptors via the Ras-Raf-MEK-ERK (Mitogen-activated protein kinase=MAPK) cascade (Figure I.2-15). Increased Ras activity (="constitutively active Ras") promotes tumor transformation, for example increased G-nucleotide exchange due to point mutations, or decreased GTPase activity due to point mutations or the lack/inactive form of GAP.

4.2. I.4.2 Second messengers

4.2.1. Definition and types of second messengers

A major question of signal transduction is how the activation of different extracellular receptors by their ligands (hormones, peptides, cytokines etc.), ie. the extracellular signals, are converted, and transduced into the cells. This second layer of signaling is controlled by second messenger molecules, which are diverse in chemical nature: (1)hydrophylic molecules e.g. cAMP, cGMP, IP3, Ca2+; (2)hydrophobic molecules (lipids) e.g. diacylglycerol (DAG), phosphatidyl-inositols; or (3)gases: NO, CO, (H2S).

4.2.2. 3'-5'Cyclic-AMP (cAMP): the "first" second messenger

In the 1950's E. W. Sutherland discovered that the effect of adrenaline on liver cells was mediated through cAMP, for his achievement he was awarded Nobel Prize in Physiology and Medicine in 1971. cAMP is synthesised from ATP by adenylyl-cyclase; and is broken down by cAMP-phosphodyesterase. cAMP activates Protein Kinase A by binding to its regulatory subunits. The targets of PKA include enzymes, structural proteins, and transcription factors like CREB (cAMP-responsive element binding factor) (Figure I.4-3 and Figure I.4-4).

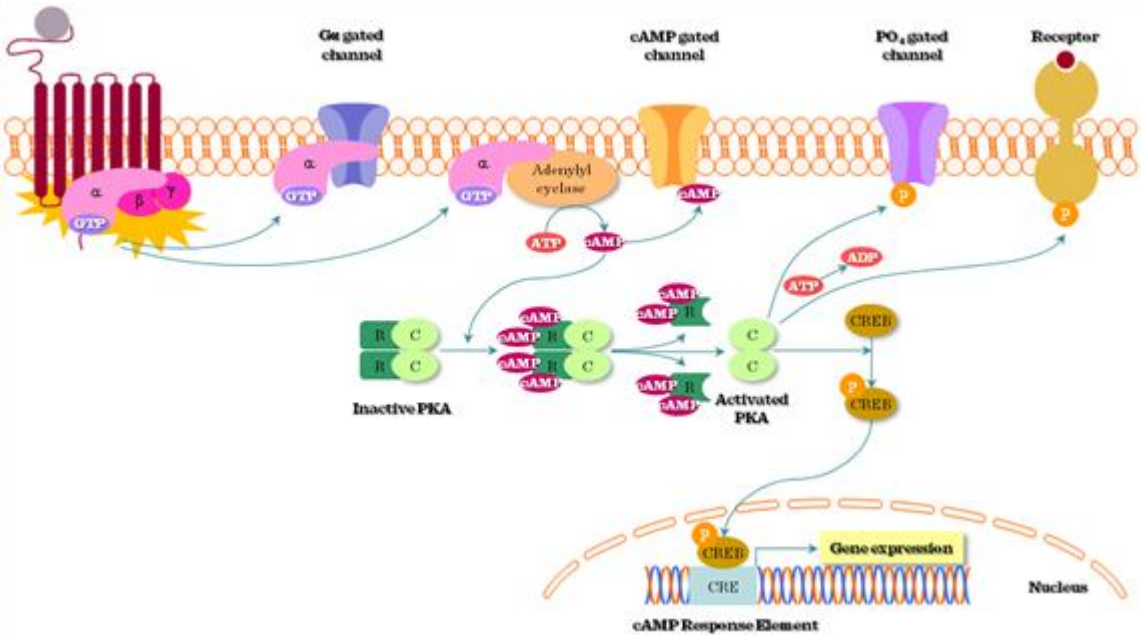


Figure I.4-3: cAMP-PKA pathway

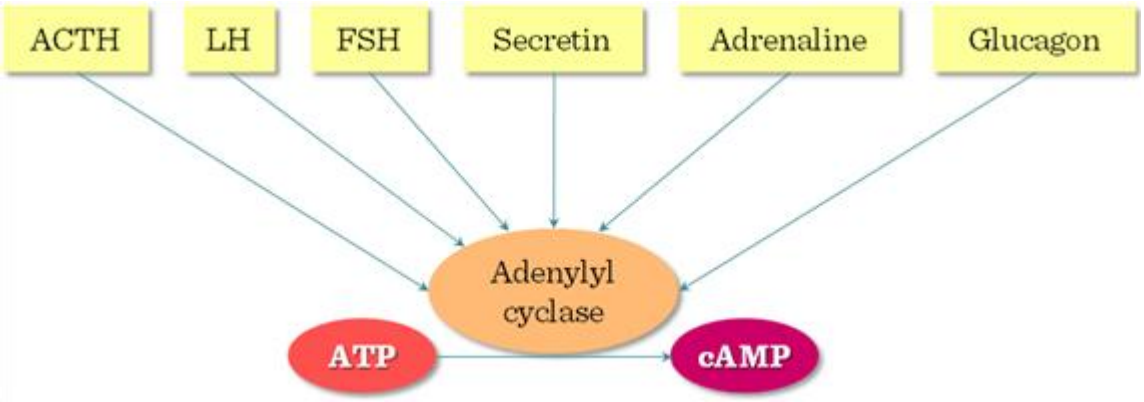


Figure I.4-4: More receptors using the same second messenger system

4.2.3. IP3 and DAG

Phospholipase C cleaves phophatidyl-inositol-4,5-bisphosphate (PIP2) found in the plasma membrane into the soluble inositol-trisphophate (IP3) and the membrane resident diacylglycerol (DAG). IP3 initiates a rise in intracellular Ca2+, DAG activates Protein kinase C (PKC) (Figure I.4-5).

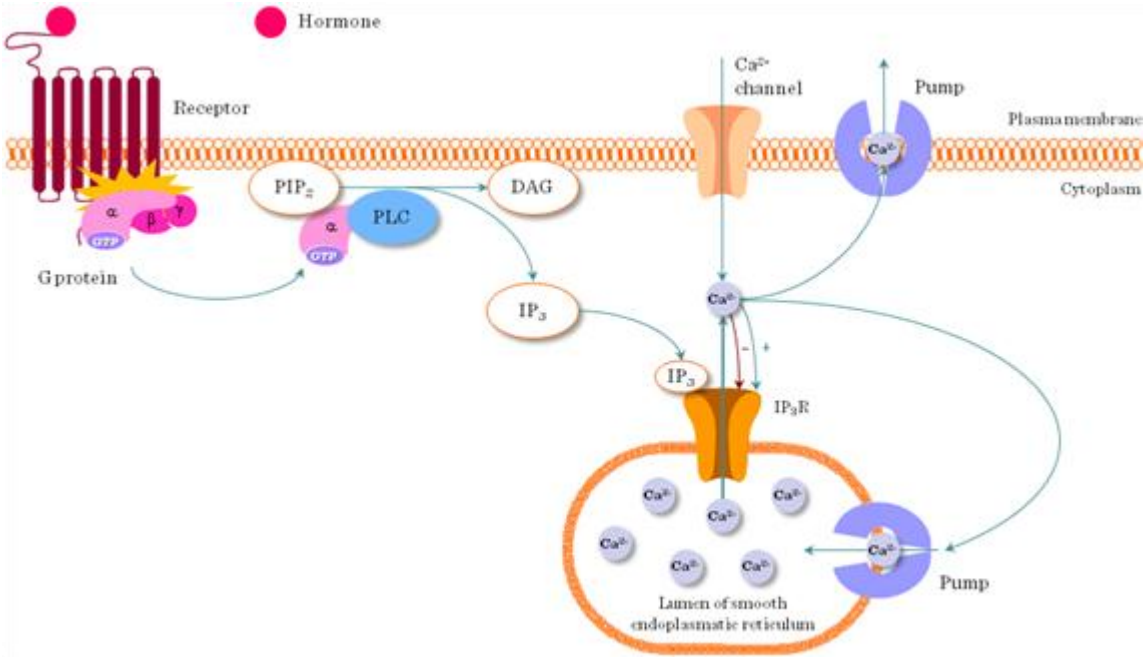


Figure I.4-5: IP3 receptor pathway

4.2.4. Nitric-oxide (NO) and other gases

The “quest” for “Endothelium-derived relaxation factor” (EDRF) was precipitated when multiple research groups found independently that endothelial cells could produce mediator(s) that lead to vasodilatation; this mediator turned out to be NO in 1983. The “star“ of NO rose rapidly: in 1992 NO was named “molecule of the year” by Science magazine; a “Nitric Oxide Society” was established; and in 1998 a shared Nobel Prize was awarded to F. Murad, R.F. Furchgott and L. Ignarro in Physiology or Medicine for the discovery of NO.

NO is synthesized by NO synthase, which has 3 forms: endothelial cells constitutively express eNOS; iNOS is inducible (e.g. in macrophages); and nNOS is neuronal. NOS has 2 major domains: the N-terminal Oxygenase (similar to heme-thiolate proteins), the C-terminal Reductase (similar to NADPH-cytochrome P450 reductase) and a Linker: calmodulin-binding sequence. NO is synthesized from L-arginine, in a chemical reaction where L-Arg is transformed into citrulline coupled by NO production.

NO is a highly active free radical which triggers oxidative stress. However, in smooth muscle cells NO activates guanylyl-cyclase, which produces cGMP from GTP. Protein kinase G is a Ser/Thr protein kinase activated by cGMP. PKG is expressed by vascular smooth muscle cells, platelets, endothelial cells, heart muscle, fibroblasts, renal cells, leukocytes, nervous system and regulates smooth muscle relaxation, platelet function, sperm metabolism, cell division and nucleic acid synthesis.

NO effects are utilized for various medical treatments:

(1) Vascular effects are based on vasodilatation e.g. Nitroglycerin for the treatment of coronary disease (Angina pectoris); for the treatment of erectile dysfunction to induce vasodilatation in the penis (Viagra).

(2) Heart muscle effects include decreased contractility and heart rate.

In the immune system: macrophages produce NO to kill bacteria but in severe systemic infection (sepsis) this can lead to generalized vasodilatation and shock (Septic shock).

4.3. I.4.3 The Ca2+-signal

4.3.1. Physiological role

S. Ringer found that in the presence of Ca2+ isolated frog heart maintained activity for hours, therefore Ca2+ is essential for heart function. Locke described that absence of Ca2+ inhibited neuromuscular transmission. Kamada and Kimoshita discovered in 1943 that introduction of Ca2+ into muscle fibers caused their

contraction. Although Otto Loewi claimed “Ca2+ ist alles.” (=Ca2+ is everything), Ca2+ was identified as second messenger only after cAMP, thus became only the “second” messenger.

Ca2+ is found in 3 forms in the body: free, bound or trapped (hydroxiapatite in calcified tissues e.g. bones, teeth). The plasma Ca2+ level is tightly regulated: hypercalcemia leads to reduced neuromuscular transmission, myocardial dysfunction and lethargy; whereas hypocalcemia leads to increased excitability of membranes, tetany, seizures and death.

The normal range of Ca2+ in plasma or extracellular fluid is 1-2mM; 50-100nM in the intracellular space / cytoplasm; and 30-300mM in the intracellular Ca2+-stores. Cytoplasmic Ca2+ is kept low by Ca2+-ATPases in the plasma membrane and ER (SERCA), and Na+/Ca2+ exchanger in the plasma membrane. Ionophores are lipid-soluble, membrane-permeable ion-carriers e.g. A23187 (524kDa) or ionomycin (709kDa) isolated from Streptomyces.

4.3.2. Measuring intracellular Ca2+

(1) Classically, for the measurement of intracellular Ca2+ concentration changes Ca2+-sensitive photoproteins, for example Aequorin (isolated from the jelly fish Aequoria Victoria) was used, which emits blue light when bound with Ca2+. This was first microinjected into a target cell (e.g. giant squid axon) and then stimulation was applied.

(2) Fluorescent indicators, for example Quin-2, Fura-2 (UV) or Fluo-3, Fluo-4 (visible light) can be used for measuring intracellular Ca2+ level in cell suspensions using flow cytometry or spectrophotometry. Here, the signal represents the summation of individual unsynchronized contributions. Single cell measurement is possible with fluorescent/confocal microscope. On a single cell level the shape of the Ca2+ signal is usually “spike” or “wave”.

(3) Genetically engineered indicators e.g. aequorin-transfected cells or Calmodulin-Myosin light chain Kinase-GFP construct can also be used for Ca2+ measurement.

4.3.3. Phospholipase Cγ (PLCγ) mediated Ca2+ signaling

Signals from cell surface receptors (e.g. GPCR) lead to PLCγ activation. PLCγ is a membrane proximal signaling protein which cleaves phosphatidyl-inositol-bisphosphate (PIP2) into phosphatidyl-inositol-trisphosphate (IP3) and diacyl-glycerol (DAG). IP3 releases Ca2+ from the endoplasmic reticulum, whereas DAG activates Protein kinase C (PKC). This step represents an important branching of the PLCγ pathway (Figure I.4-5). This pathway is activated by a number of different extracellular stimuli through a variety of receptors (Figure I.4-6).

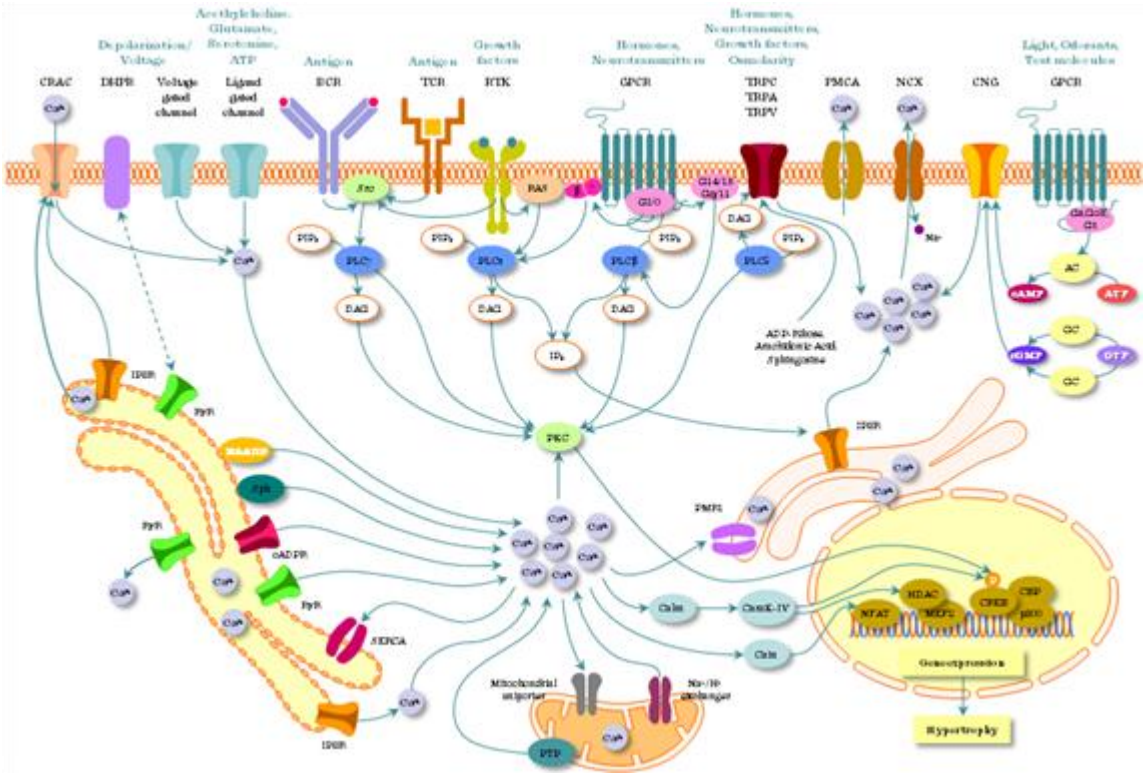


Figure I.4-6: Several pathways use the Ca2+ signal

4.3.4. Ca2+-channels in the ER (Figure I.4-7)

(1) Ryanodine receptor (RyR), expressed in excitable cells (skeletal & cardiac muscle)) has four 560kDa subunits. Its modulators are Ca2+, ATP, calmodulin, FKBP12 (immunophilin).

(2) IP3 receptor (IP3R) has four 310kDa subunits.

Ca2+-induced Ca2+ release (CICR)

When cytoplasmic Ca2+ rises, neighbouring Ca2+ channels are activated progressively. Their opening leads to a Ca2+ “wave”. This is an example of positive feedback.

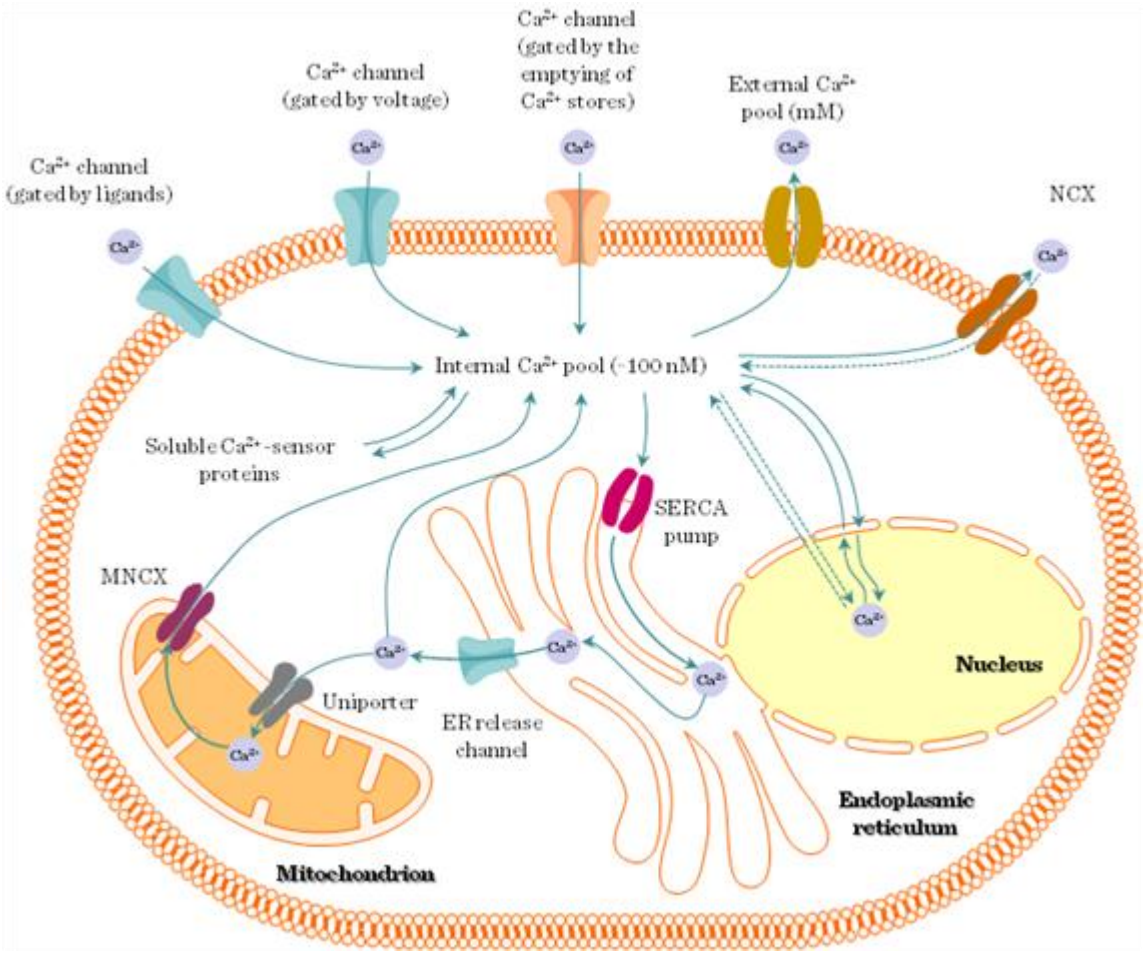


Figure I.4-7: Intra/extracellular compartments of Ca2+-signaling, Ca2+-channels

4.3.5. Besides IP3, “Alternative” Ca2+-releasing 2nd messengers also exist:

(1) Cyclic-ADP-ribose (cADPR) is generated by ADP-ribosyl cyclase (e.g. CD38 ectoenzyme). It participates in panceratic β-cell glucose response and TcR signaling

(2) Nicotinic acid adenine dinucleotide phosphate (NAADP) was first described in sea urchin eggs. It is a mediator of CCK effects on pancreas acinar cells and TcR signaling.

(3) Sphingosine-1-phosphate (S1P) is generated from ceramide by sphingosine-kinases upon activation by FcRs (ε, γ), GFRs (PDGF, VEGF), or cytokine rec. (IL-1, TNFα). S1P transmembrane transport is perfomed by ABCC1, cell surface receptors: S1P1, S1P5.

4.3.6. Ca2+-influx through plasma membrane channels (Figure I.4-7)

(1) Voltage-operated channels (VOCCs) are found on nerve and muscle cells. They open upon depolarization. L, N, P/Q, R and T types

(2) Receptor-operated channels (e.g. Glutamate NMDA rec.).

(3) TRPM2 channels are activated by ADP-ribose or oxidative stress.

4.3.7. Store-operated Ca2+-entry (SOCE)

Also known as “capacitative Ca2+-entry” (1986.). When intracellular Ca2+ stores are depleted plasma membrane Ca2+ channels open and the influx of extracellular Ca2+ follows, mediated by TRP (transient rec. potential) proteins, CRAC (Ca2+ release-activated Ca2+ current) channels e.g. Orai 1 (33kDa) and STIM1 (77kDa Ca2+- sensor transmembrane protein in the ER). Three potential mechanisms of STIM1 action:

(1) Direct interaction between ER and plasma membrane

(2) Movement of STIM1 from the ER to the plasma membrane

(3) The existence of a soluble mediator – CIF (Ca2+-influx factor) (1993.)

4.3.8. Ca2+-regulated target proteins

(1) Calmodulin-dependent (Figure I.4-8): Calmodulin phosphorylates CaM kinases, EF2 kinase, phosphorylase kinase and myosin-light chain kinase (MLCK); dephosphorylates calcineurin, which, in turn activates NFAT (Nuclear Factor of Activated T cells). Calmodulin also regulates Ca2+ transport via plasma membrane Ca2+ ATPases, cyclic nucleotide metabolism through Adenylyl-cyclase and Cyclic Nucleotide Phosphodiesterase, cytoskeleton components (e.g. MAP-2, Tau, fodrin, neuromodulin) and nitric-oxide synthase (NOS).

(2) Calmodulin-independent target proteins include:

a) Neuronal Ca2+ sensors

b) Calpain (Ca2+-activated Cys protease)

c) Synaptotagmin – exocytosis

d) DAG kinase – inactivation of DAG

e) Ras GEFs & GAPs

f) Cytoskeletal proteins a-actinin, gelsolin

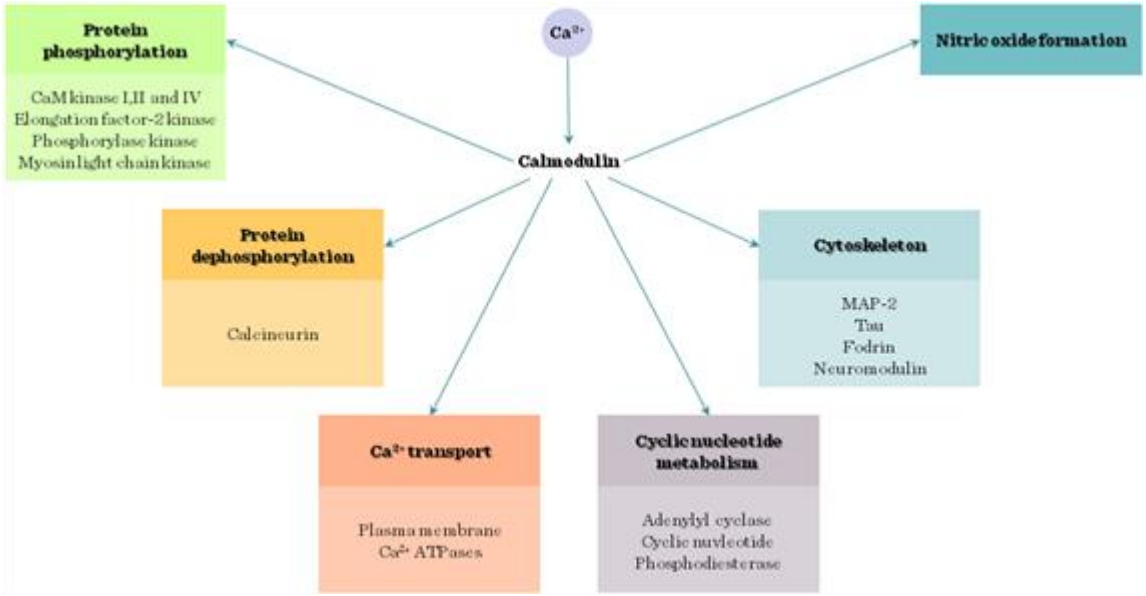


Figure I.4-8: Effector mechanisms of Ca2+-signaling

4.3.9. The structural basis of Ca2+-binding

(1) EF-hand motifs are helix-loop-helix, the loop consists of cca.12AA-s forming the Ca2+-binding site, and they usually form pairs (=unit).

(2) C2 domains contain cca.130 AA-s, forming rigid 8-stranded antiparallel β-sheets.

4.4. I.4.4 Transcription factors

4.4.1. Definition

Transcription factors are sequence-specific DNA-binding factors that control the transmission of genetic information from DNA to mRNA. They act as activators (=promote gene expression) or repressors (=inhibit gene expression) by affecting the recruitment of RNA polymerase to the transcription initiation complex (Figure I.4-9).

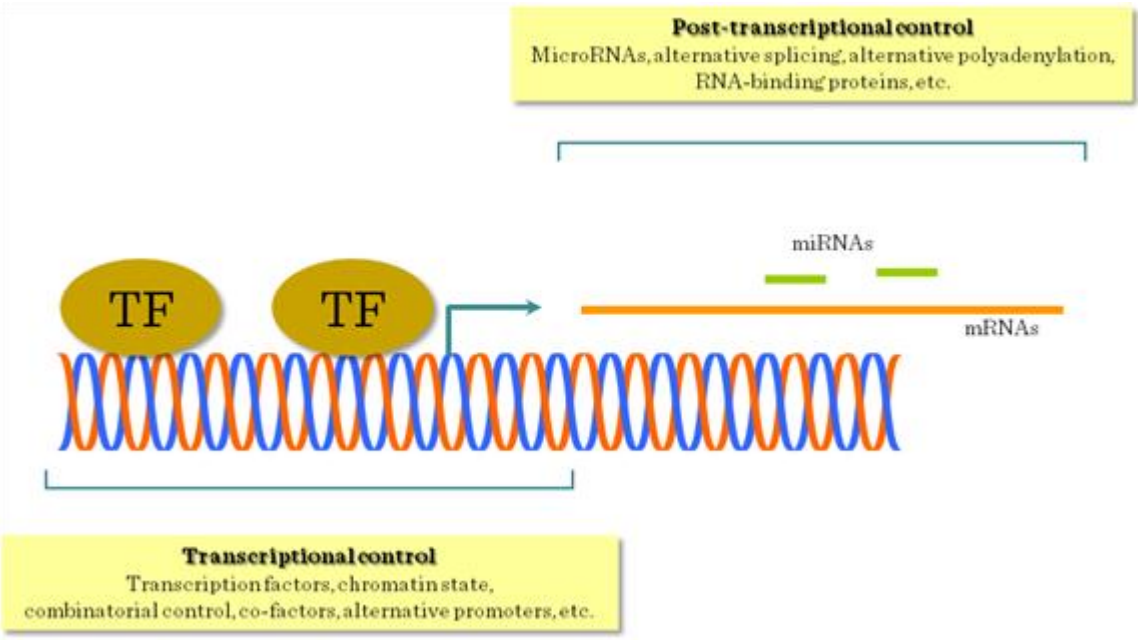


Figure I.4-9: Regulation of transcription

4.4.2. Functional groups

(1) General TFs: constitutively active, present in all cells at all times, bind TATA box, form pre-initiation complex e.g. TFIIA-H.

(2) Specific transcription factors/upstream transcription factors are conditionally active (Table I.4-1).

A) Developmental (cell specific) e.g. GATA, MyoD, Hox, Winged helix

B) Signal-dependent

a) extracellular ligand (e.g. nuclear receptors)

b) intracellular ligand (e.g. SREBP, p53)

c) cell-membrane rec. dependent

d) resident nuclear CREB, AP-1, Mef-2

e) latent cytoplasmic STAT, NFAT, NFkB, Notch

Table I.4-1: Some important transcription factors

Family	Representative transcription factors	Functions
Homeodomain		
Hox	Hoxa-1, Hoxb-2, etc.	Axis formation
POU	Pit-1, Unc-86, Oct-2	Pituitary development; neural fate
LIM	Lim-1, Forkhead	Head development
Pax	Pax1, 2, 3, etc.	Neural specification; eye development
Basic helix-loop-helix (bHLH)	MyoD, achaete, daughterless	Muscle and nerve specification; Drosophila sex determination
Basic leucine zipper (bZip)	C/EBP, AP1	Liver differentiation; fat cell specification
Zinc finger		
Standard	WT1, Krüppel, Engrailed	Kidney, gonad, and macrophage development; Drosophila segmentation
Nuclear hormone receptors	Glucocorticoid receptor, estrogen receptor, testosterone receptor, retinoic acid receptors	Secondary sex determination; craniofacial development; limb development
Sry-Sox	Sry, SoxD, Sox2	Bend DNA; mammalian primary sex determination; ectoderm differentiation

Gene transcription is regulated in a complex manner: the basic transcription machinery (general transcription factors and the RNA polymerase) interacts with numerous co-regulators (specific transcription factors). Activators bind to enhancer elements, repressors bind to silencer elements of the DNA upstream from the TATA box. However, the exact positions of such regulatory DNA elements are highly variable.

4.4.3. Structure

Generally, transcription factors contain (1) a DNA-binding domain (DBD) responsible for the direct interaction with DNA response elements; (2) a signal-sensing domain (SSD) responsible for the detection of extracellular signals e.g. ligand-binding; and (3) a transactivation domain (TAD) interacting with transcription co-regulators (Figure I.4-10). Most transcription factors contain helix-loop-helix, zinc-finger or leucin-zipper motifs and bind to the DNA as dimers (Figure I.4-11).

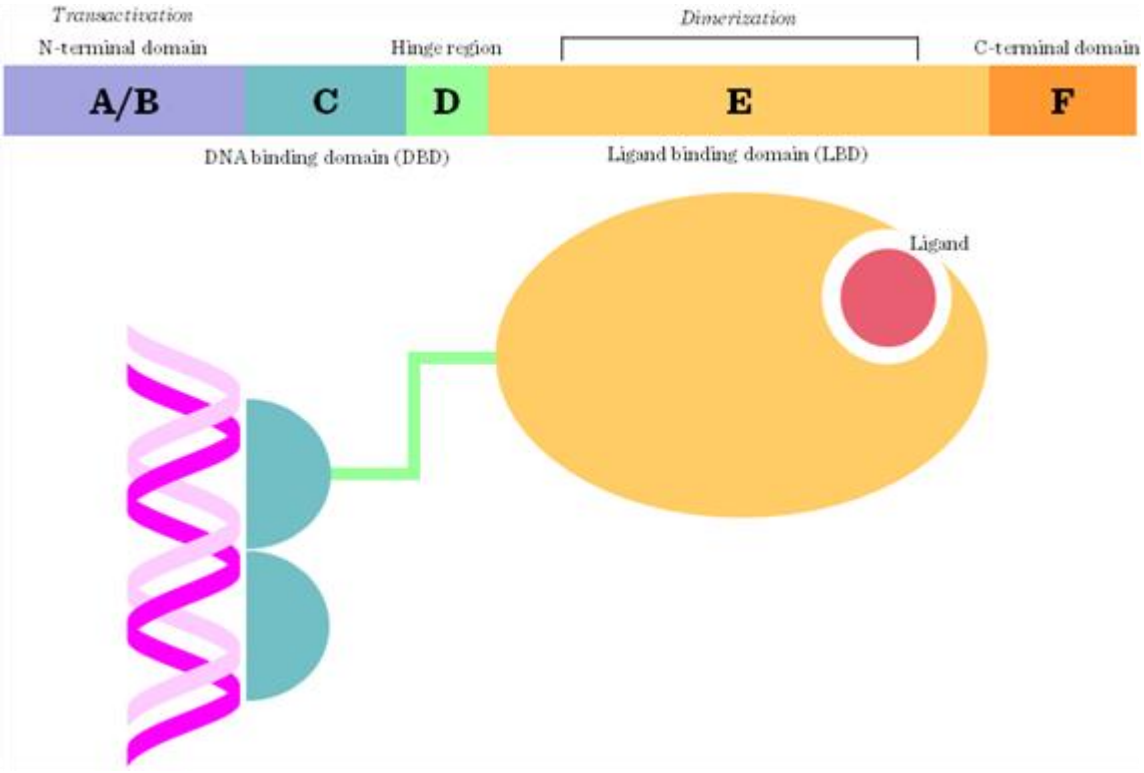


Figure I.4-10: Functional domains of transcription factors

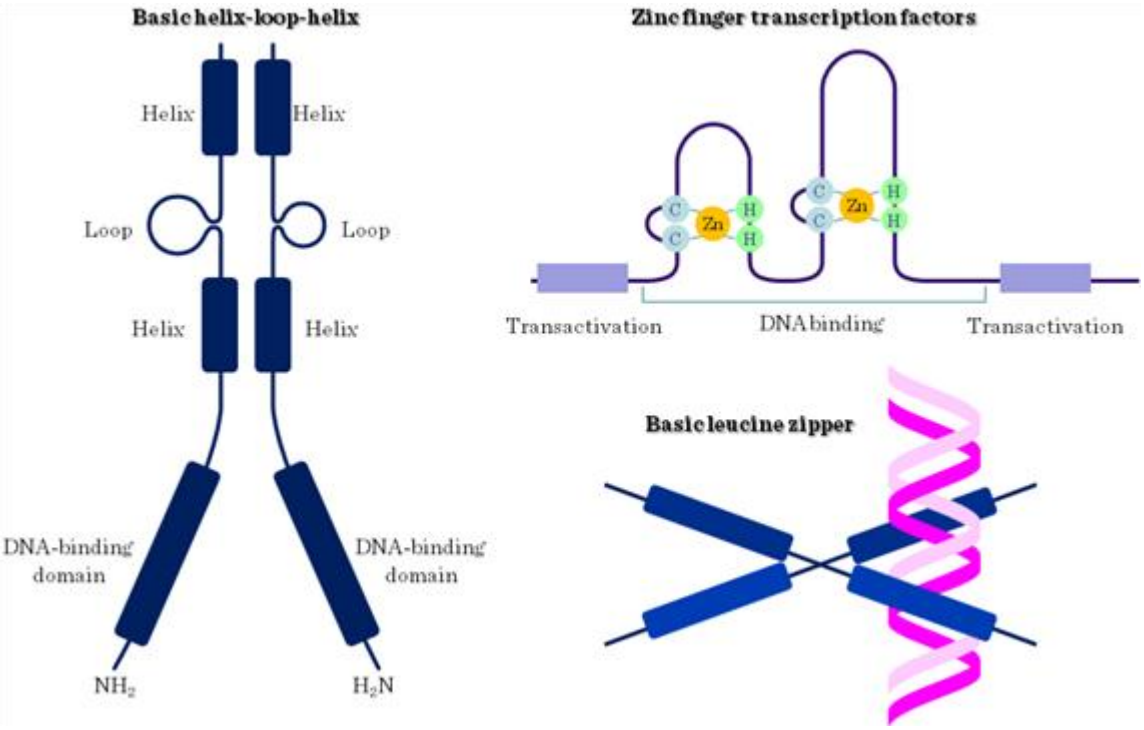


Figure I.4-11: Structural groups of transcription factors

Structural groups of transcription factors (Superclasses):

(1) Helix-loop-helix e.g. MyoD, c-Myc

(2) Leucin zippers e.g. AP-1, CREB

(3) Zinc-coordinating DNA-binding domains e.g. Zinc fingers: nuclear receptors for steroids, thyroid hormone; GATA factors

(4) Helix-turn-helix e.g. Homeobox; Forkhead / winged helix

(5) Beta-scaffold factors with minor groove contacts e.g. NFkB. NFAT, STAT, p53

(6) Others

4.4.4. Transcription factors controlling T cell differentiation

T lymphocytes are central players of the adaptive immune mechanisms. They derive from the bone marrow common lymphoid precursor, then, the early progenitors migrate to the thymus where they undergo a series of central differentiation steps, which are tightly controlled by specific transcription factors (Figure I.4-12). Finally, T cells leave the thymus as helper (CD4+) or cytotoxic (CD8+); this lineage decision is also under the control of transcription factors (Figure I.4-13). In the peripheral lymphatic organs, naïve CD4+ T cells reach their final differentiation stages: Th1, Th2, Th17 and Treg subpopulations, controlled by T-bet, GATA-3, RORγ and FoxP3 transcription factors, respectively (Figure I.4-14).

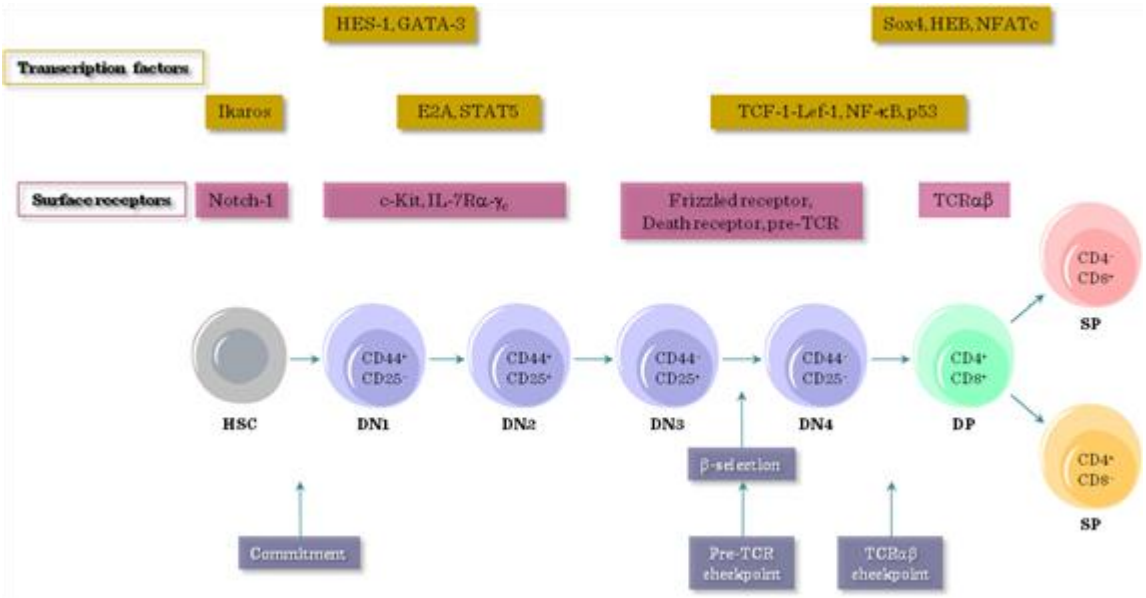


Figure I.4-12: Role of transcription factors in thymocyte development

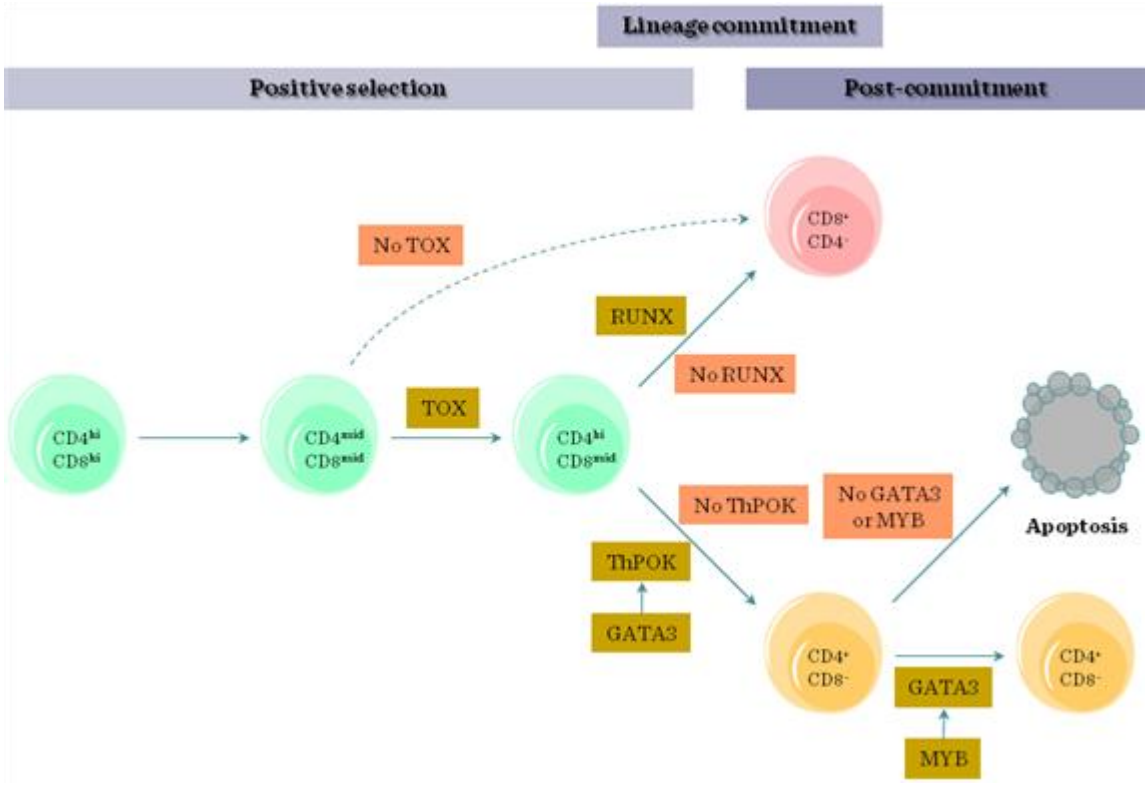


Figure I.4-13: Th - Tc cell decision

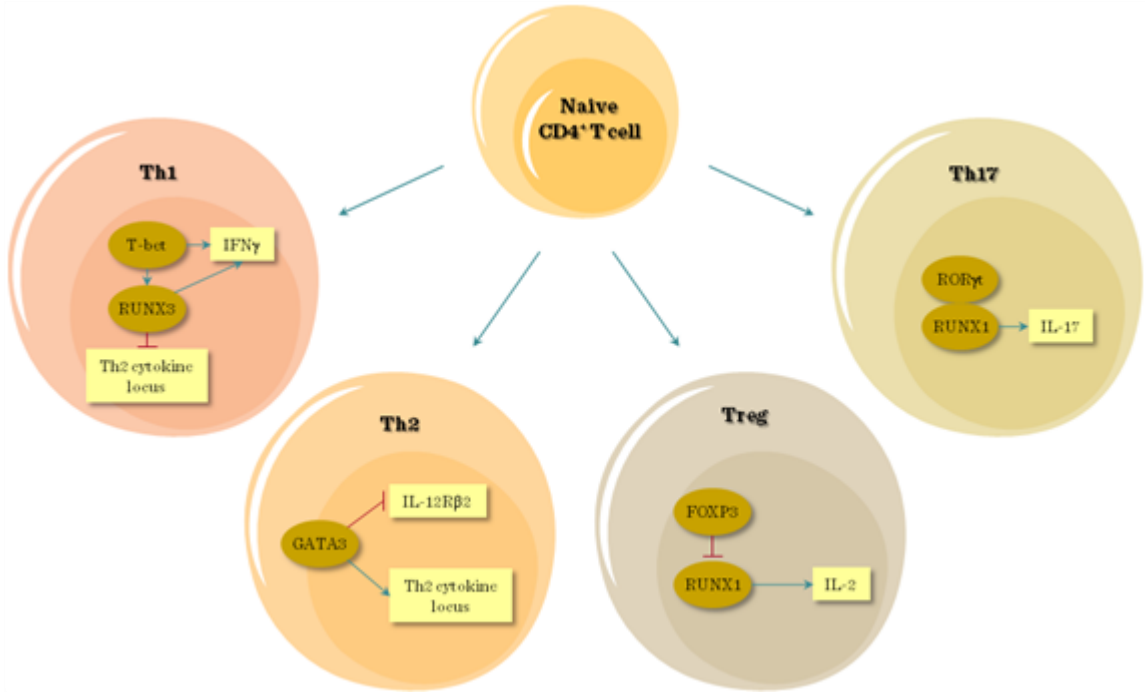


Figure I.4-14: Th differentiation

4.4.5. Transcription factors in diseases

Some transcription factors are directly involved in diseases, for example:

(1) IPEX syndrome (Immuno-dysregulation Poly-endocrinopathy Enteritis X-linked), “Scurfy” phenotype in mouse – FoxP3

(2) Rett-syndrome – MECP2

(3) Li-Fraumeni-syndrome – p53

4.4.6. Studying transcription factors

(1) Transcription factor activity might be tested by

a) Luciferase reporter assay: transfection of the target cells with a plasmid containing the luciferase gene under the control of the promoter to be studied. Upon transcription factor-binding light is emitted.

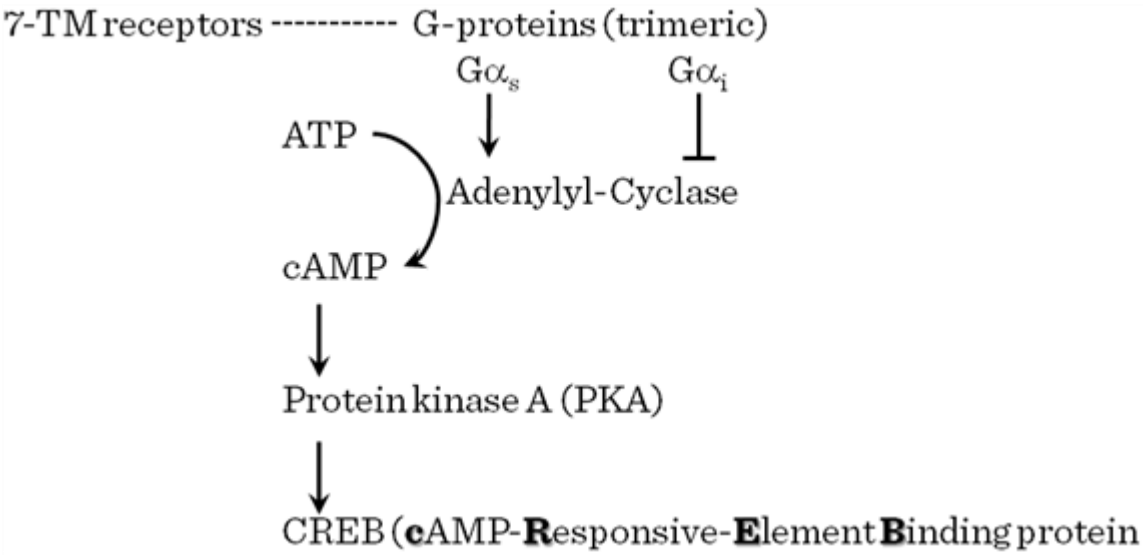
b) Chromatin immunoprecipitation (ChIP): after the biological treatment the activated transcription factors are fixed to the DNA by formaldehyde, then the genomic DNA is extracted and fragmented. The fragments are precipitated by transcription factor specific antibody(s). The precipitate is disrupted and gene-specific PCR or sequencing (ChIP-Seq) can be performed on the purified DNA. ChIP can be combined with microarray (ChIP-on-chip). Thus, high throughput screening of gene networks controlled by a transcription factor becomes possible.

c) Electrophoretic Mobility Shift Assay (EMSA) is based on the alteration in the migration speed of DNA in complex with transcription factor(s).

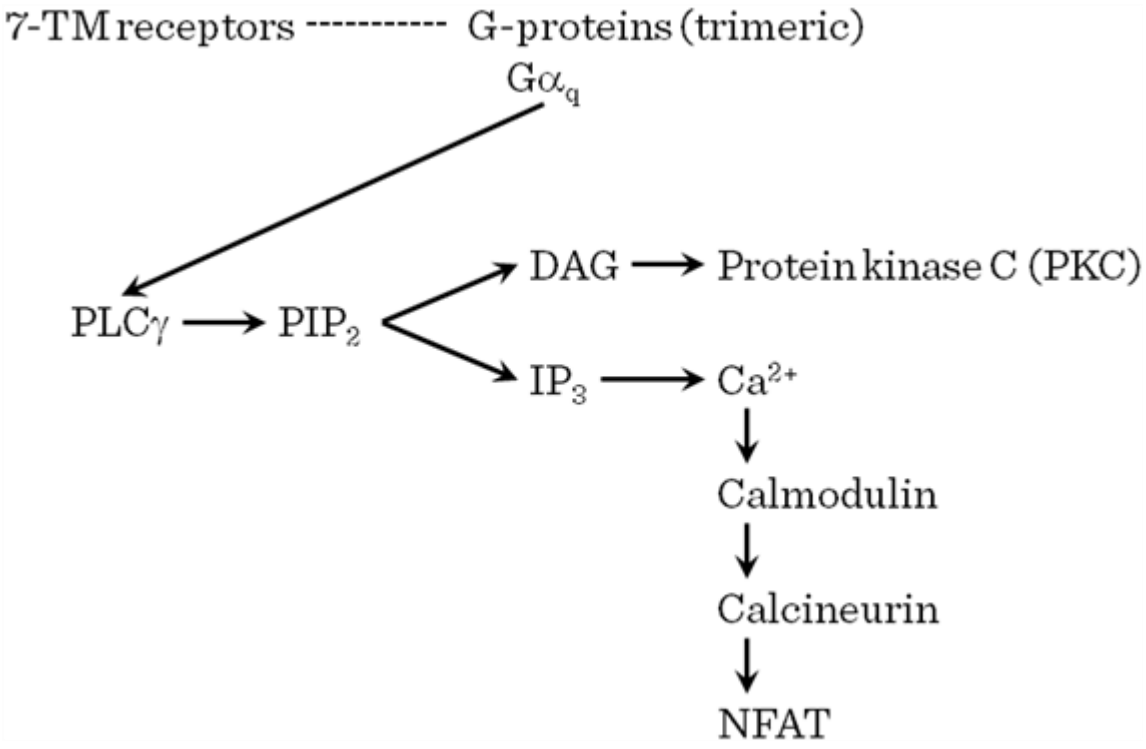
(2) Transcription factor interactions (physical association) can be assessed by co-immunoprecipitation.

5. I.5Overview of major signaling pathways

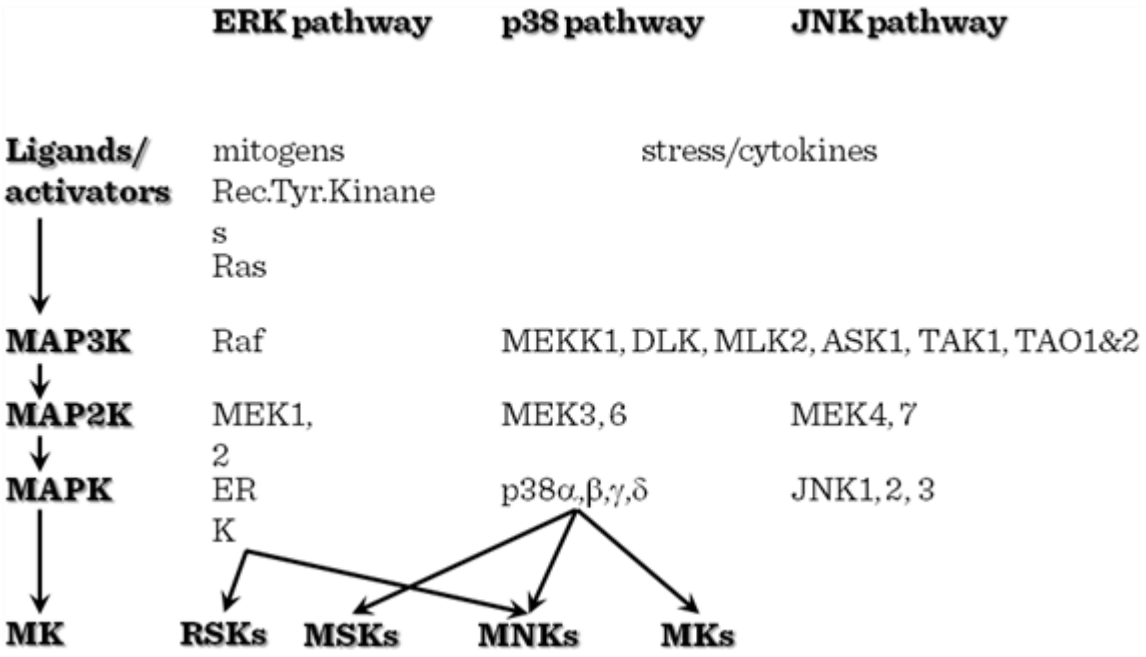
5.1. I.5.1 cAMP-PKA pathway



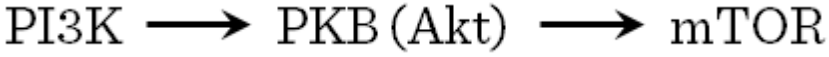
5.2. I.5.2 PLCγ-DAG-PKC



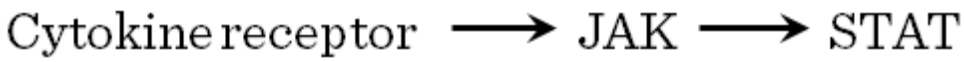
5.3. I.5.3 MAPK-pathway



5.4. I.5.4 PI3-kinase-PKB (Akt)



5.5. I.5.5 JAK-STAT



Chapter 3. II Detailed (systematic) signal transduction

1. II.1 Signaling in the immune system

The immune system functions as a finely regulated network of innate and adaptive mechanisms, with the ability to recognize and distinguish between self and non-self structures. Immunological steady state is continuously maintained on one hand by attacking and eliminating foreign invaders and tumor cells, and providing tolerance of important self-antigens on the other hand. Immunological recognition molecules are cell surface receptors (T cell receptor, B cell receptor, Fc receptors, Complement receptors, Toll-like receptors etc.), which, in most cases, have no intrinsic enzymatic activity, hence they use cytoplasmic non-receptor tyrosine kinases and adaptor molecules for signaling. Tyrosine-phosphorylation is a common event during immunological signaling; thus specialized tyrosine containing signal sequence motifs have evolved: ITAM (Immunoreceptor Tyrosine-based Activation Motif) and ITIM (Immunoreceptor Tyrosine-based Inhibition Motif). ITAM is a specific sequence of amino acids (YXXL) occurring twice in close succession in the intracellular tail of a receptor, whereas ITIM sequence is as follows: I/VXXYXXL. Signals through these receptors are converted into a plethora of complex biological responses: proliferation, differentiation, phagocytosis, apoptosis or anergy.

1.1. II.1.1 Signaling in the specific immune system 1: Bcellsignaling

1.1.1. The B cell receptor (BcR) complex

B-lymphocytes are part of the adaptive immune system, their antigen recognition molecule is the B cell receptor (BcR), which is structurally a cell surface-bound monomeric immunoglobulin molecule. Having only a short transmembrane and intracellular part, the BcR associates with the Igα/β chains, which contain ITAM motifs. The BcR complex contains other co-stimulatory molecules as well: CD19, CD20, CD21, CD23 and CD45.

1.1.2. Activation of the BcR and signaling pathways

The BcR can be activated through cross-linking by the antigen molecule (Figure II.1-1 and Figure II.1-2). Protein antigens (“T cell dependent antigens”) with variable epitopes can cross link only a limited number of BcRs, which alone leads to incomplete B cell activation. In this case a second simultaneous activating cytokine signal deriving from helper T cells is indispensible. Polysaccharide and lipid antigens, on the other hand, possess repeating epitopes in large number, thus, cross linking several BcRs and leading to complete B cell activation without T cells (“T cell independent antigens”).

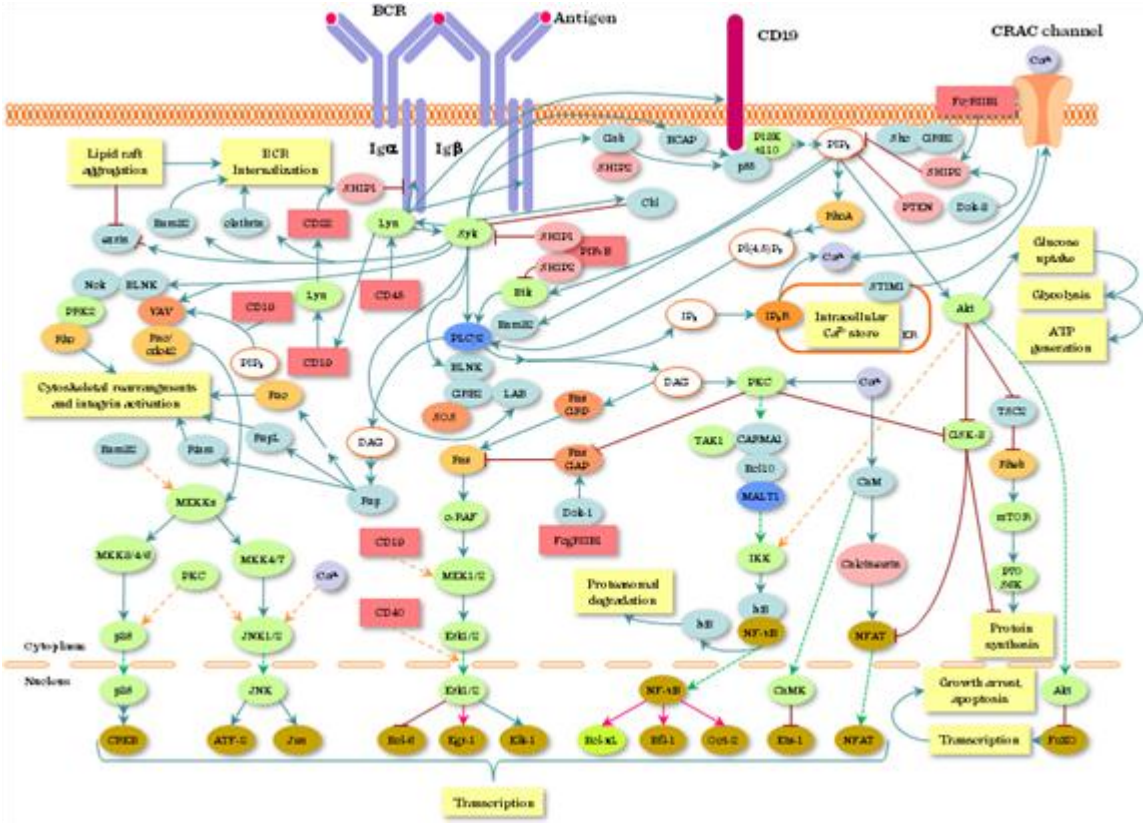


Figure II.1-1: Overview of BcR signaling

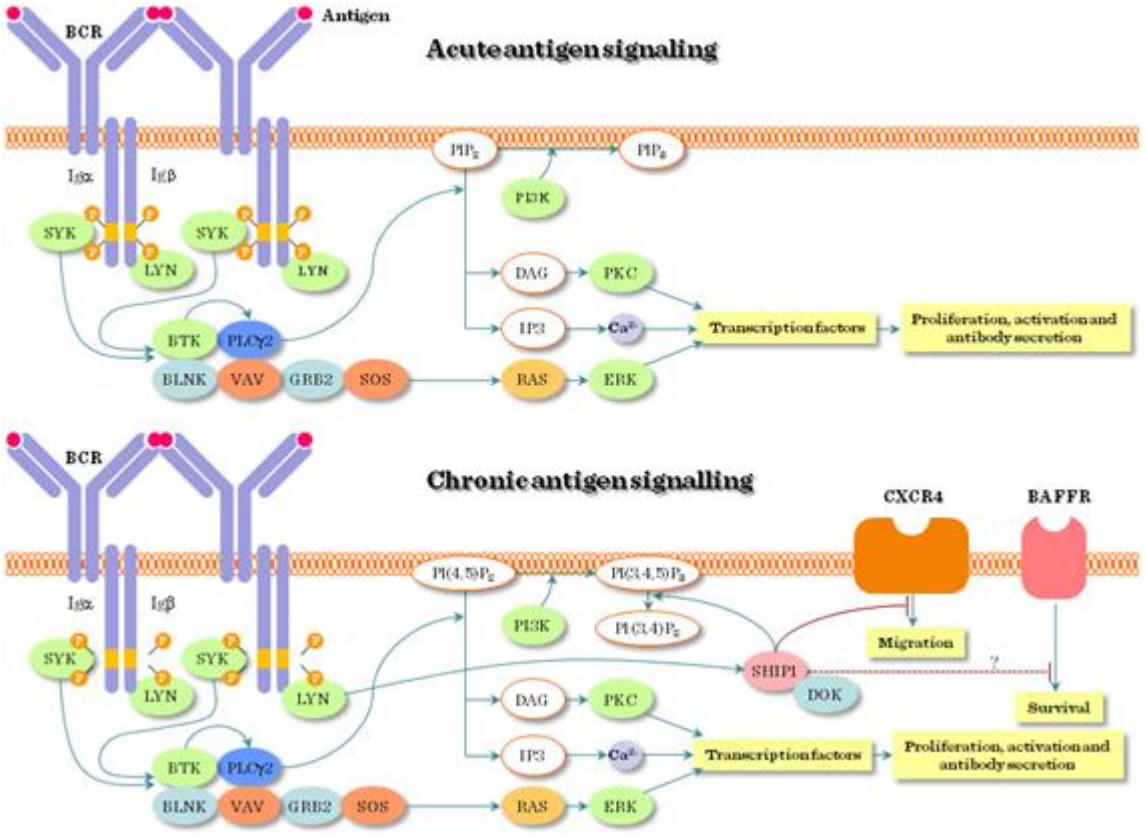


Figure II.1-2: Short/long term BcR stimulation

In either case, antigen cross-linking leads to the activation of Fyn and Lyn, two Src family kinases, which phosphorylate the ITAMs of the Igα/β chains. These phosphorylated ITAMs provide docking site for the SH2 domains of Syk, which is a central non-receptor tyrosine kinase in BcR signaling. Syk activates Grb2 and PLCγ, which initiates the DAG and IP3 pathways, leading to PKC activation and the rise of the intracellular Ca2+ level, respectively. Calmodulin activates calcineurin leading to NFAT activation. Other pathways include the MAPK pathways, NFκB activation and the PI3K-Akt pathway (regulated by the CD19 co-stimulatory molecule). The non-canonical NFκB pathway is activated by BAFFR (a member of the TNF receptor family) leading to B cell survival (Figure II.1-3). Finally, on the transcription factor level, NFAT, AP-1, NFκB and ERK are activated leading to gene expression changes. The most important biological effects of the BcR signaling are the clonal proliferation and peripheral differentiation (into plasma- or memory cells) of B cells.

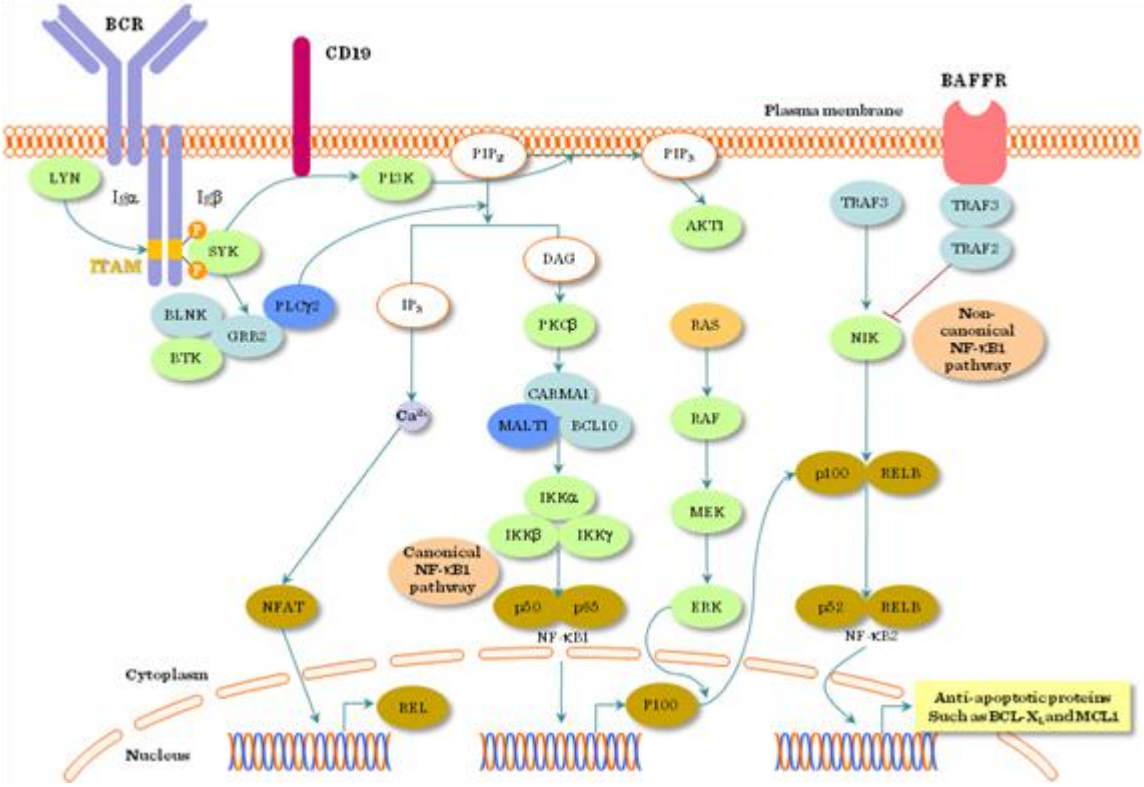


Figure II.1-3: Co-stimularory pathways of BcR signaling

1.2. II.1.2 Signaling in the specific immune system 2: Tcellactivationandsignaling

1.2.1. The T cell receptor (TcR) complex

T lymphocytes perform a wide range of functions in the adaptive immune system: from the regulation of central phase of the immune response through cytokines to cytotoxic effector functions. Their antigen recognition molecule is the T cell receptor (TcR), which is a heterodimeric molecule made up from either α/β or γ/δ chains. The TcR is complexed by the multichain signaling complex CD3 from which ζ chains contain ITAM sequences (Figure II.1-4). The TcR/CD3 complex is completed by accessory (e.g. CD4, CD8, CD45 etc.) and co-stimulatory (e.g. CD28, CTLA-4, PD-1L, ICOS etc.) molecules on the cell surface.

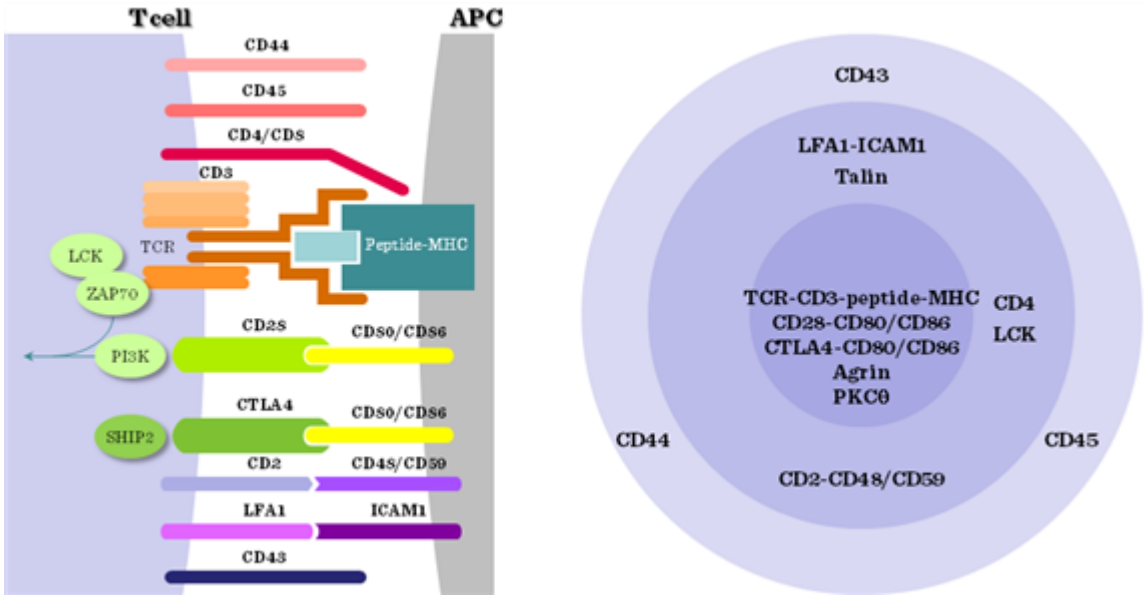


Figure II.1-4: Molecules of the "immunological synapse"

1.2.2. Activation and signaling through the TcR

Contrasting to B cells, T cells can only be activated by processed antigen fragments (8-20 amino acid peptides) bound to MHC I or II molecules on the surface of antigen presenting cells ("MHC-restriction"). Upon close binding between the peptide-MHC complex and the TcR a sequence of signaling events follow (Figure II.1-5).

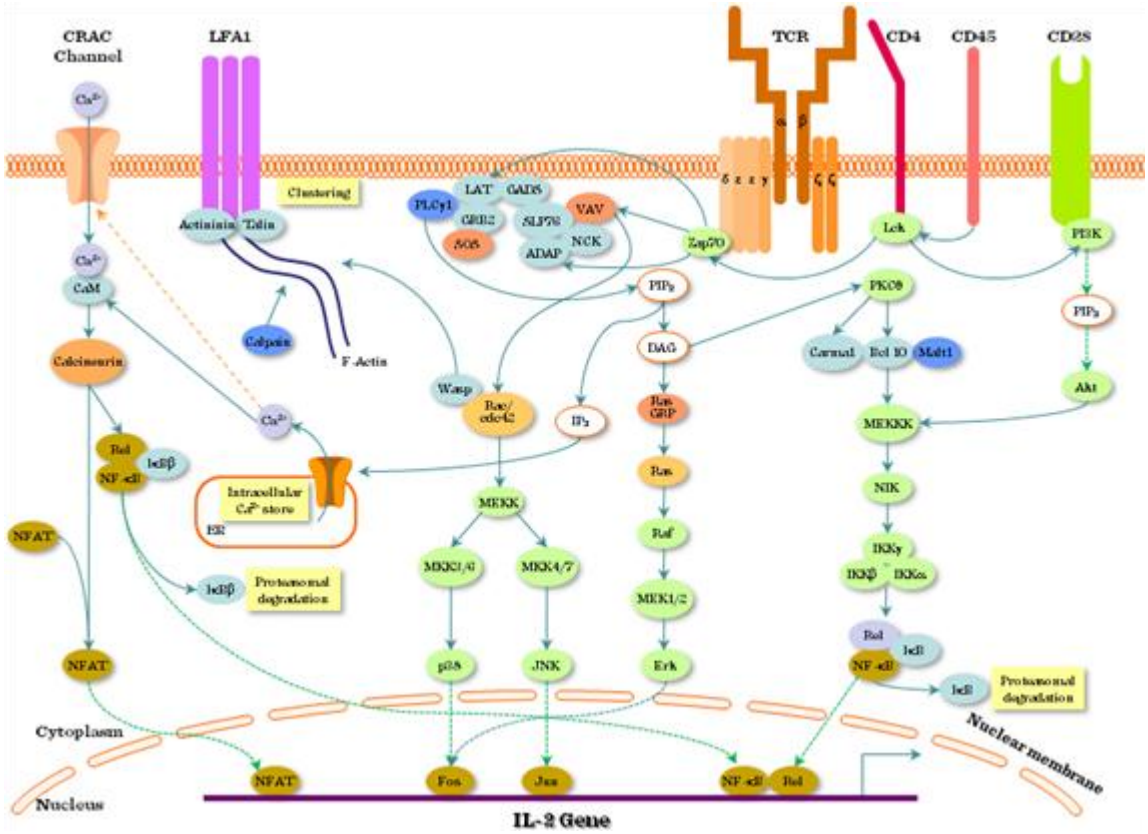


Figure II.1-5: Overview of TcR/CD3 signaling pathway

First, the CD45 phoshatase removes an inhibitory phosphate group from the Src family kinase Lck, which, in turn, phosphorylates the ζ chain ITAMs of the CD3. Next, ZAP-70 (70kDa Zeta-chain Associated Protein

kinase), homologous to Syk in B- and mast cells, docks to the phosphorylated ITAMs on the CD3 ζ chains and gets phosphorylated by Lck and itself (autophosphorylation). The activated ZAP-70 becomes a key organizer of downstream TcR signaling steps. Two important target molecules of the ZAP-70 are the adapter proteins LAT and SLP-76. Phosphorylation of these molecules leads to the formation of a multimolecular complex involving GRB2, Itk, GADS and Vav that results in activation of PLCγ1. PLCγ1, in turn, cleaves PIP2 producing two second messengers: IP3 and DAG. DAG initiates two major pathways: the Ras and PKCθ signaling. Ras triggers the MAP-kinase cascade that results in the activation of transcription factors (e.g. AP-1), while activation of PKCθ activates the NFκB pathway leading also to transcriptional regulation.

IP3 releases Ca2+ from the endoplasmic reticulum (intracellular Ca2+-store) that is followed by the opening of plasma membrane Ca2+ channels as well (capacitative influx). Elevated intracellular Ca2+ level then activates calcineurin, calmodulin and finally the transcription factor NFAT. As a consequence of all above mentioned signaling cascades a number of transcription factors are activated (AP-1, NFAT, NFκB) leading to complex gene expression changes in activated T cells (Figure II.1-6).

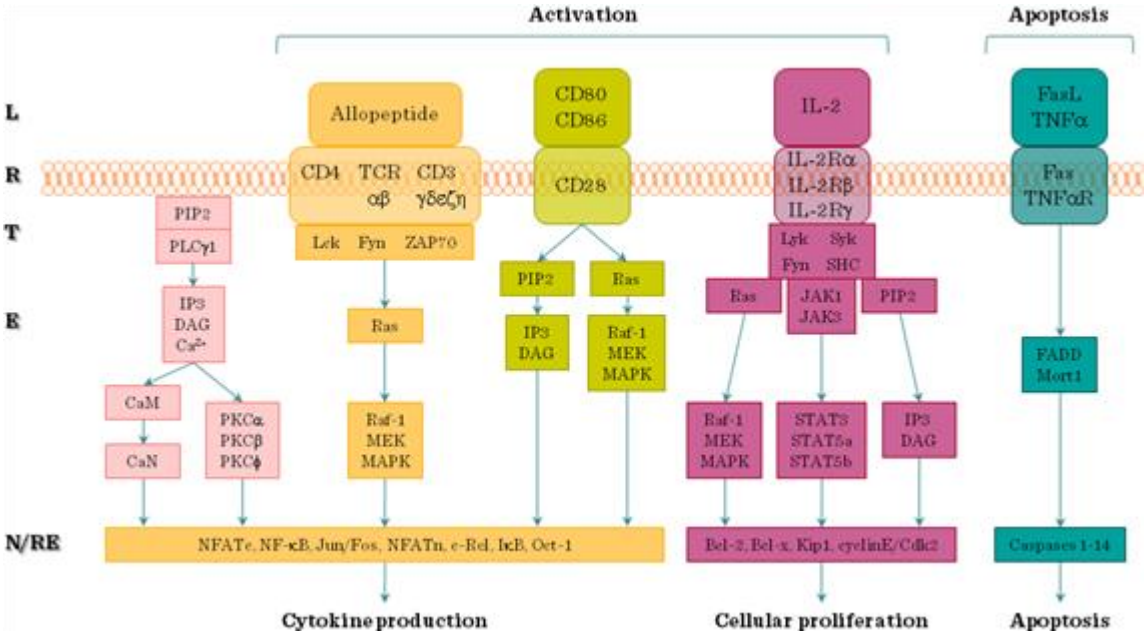


Figure II.1-6: T cell activation pathways

1.2.3. Lipid rafts and the immunological synapse

Recent advances in membrane cell biology have shown that the plasma membrane is not a vast “ocean” of uniform freely diffusing lipid molecules but contains important structural asymmetries. Cholesterol and sphingolipid-rich microdomains of the plasma membrane, also known as “lipid rafts” are responsible for the precise organization of the above-described signaling events. These rafts provide a platform for the molecules of the TcR signaling complex and regulate their fine molecular interactions. Importantly, lipid rafts are in close connection with the cytoskeleton network.

For a successful T cell activation the TcR signal alone is insufficient, a second, co-stimulatory signaling is also necessary (Figure II.1-7). The immunological synapse (A.Kupfer and M. Dustin) is the attachment surface between the T cell and the APC; a Supramolecular Activation Complex (SMAC) consisting of a central (c) region containing the TcR complex, CD4, CD28 and a peripheral (p) region containing adhesion molecules e.g. LFA-1 (Figure II.1-4). Besides the binding of important ligand-receptor pairs inside the synapse, the exclusion of the CD45 phophatase is also an important factor in T cell activation. The absence or presence of the CD28 co-stimulatory signal determines whether the TcR signal causes activation or anergy (functional inactivation) of the T cell (Figure II.1-7).

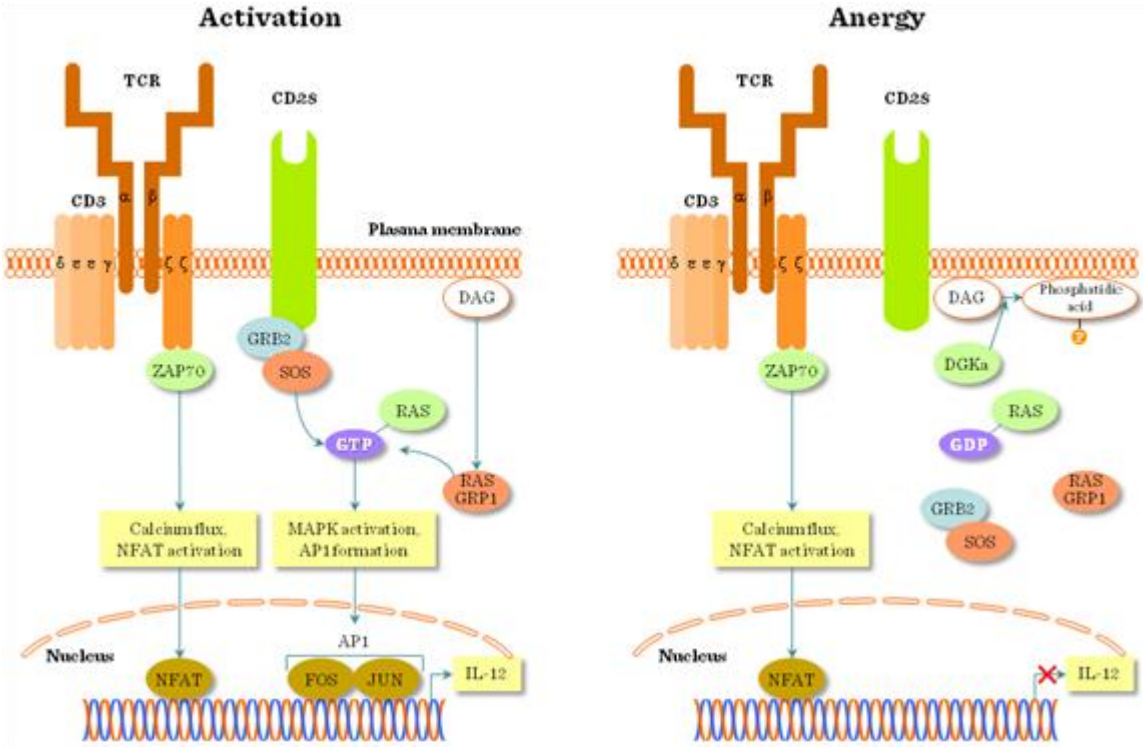


Figure II.1-7: Co-stimulatory pathways regulate the TcR signal

1.3. II.1.3 Fcg Receptor signaling

1.3.1. Introduction

Fc-receptors (FcR) bind the constant Fc region of the immunoglobulin molecules. Based on their immunoglobulin isotype-specificity the following FcR groups can be distinguished: FcαRI (IgA), Fcα/μR, Fcγ Receptors (I, IIa/b/c, IIIa/b) (IgG), FcεRI/II (IgE), FcRH1-6, FcRX and FcRY.

1.3.2. Role and expression of Fcg receptors

Leukocyte Fc receptors promote the phagocytosis and killing of opsonized particles and deliver signals that stimulate the microbicidal activity of leukocytes. The most important Fc receptors of phagocytes are the Fcg receptors, which bind IgG immuncomplexes (Figure II.1-8). Their activity stimulates phagocytic or cytotoxic cells to destroy microbes, or infected cells by antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC).

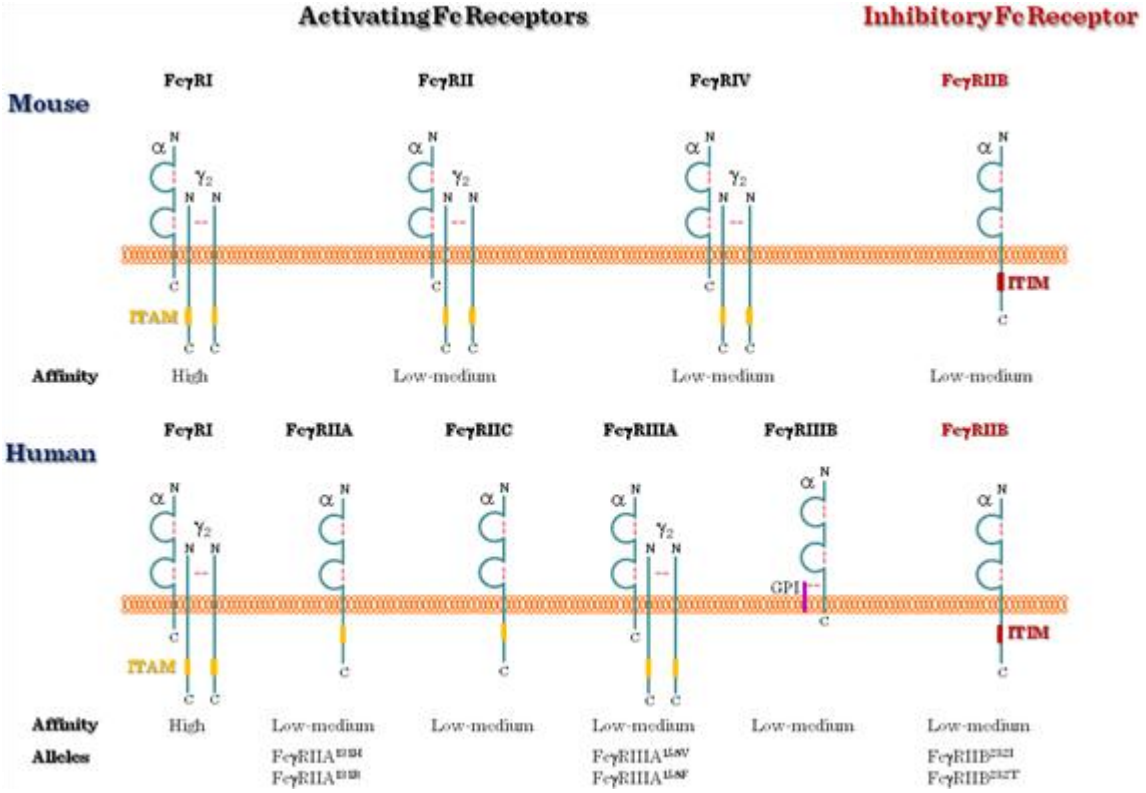


Figure II.1-8: Types of Fcgamma receptors

1.3.3. Fcg receptor ITAM/ITIM

Similarly to the BcR and the TcR, Fcg receptors generate signals through ITAMs, too (Figure II.1-9). For example, the intracellular tail of FcγRIIA and C, and in the g chains of FcγRI and FcγRIIIA contain ITAMs. After ligand binding, tyrosine (Y) residue of the ITAM is phosphorylated by tyrosine kinases, and a signaling cascade is generated within the cell.

FcγRIIB1 and FcγRIIB2, on the other hand, have ITIM sequences and, thus, are inhibitory Fc receptors: they do not induce phagocytosis, instead, they counteract the antigen-induced BcR signal on B cells and shut off B cell activation. This inhibitory signal is controlled by SHP-1 and SHIP-phosphatases.

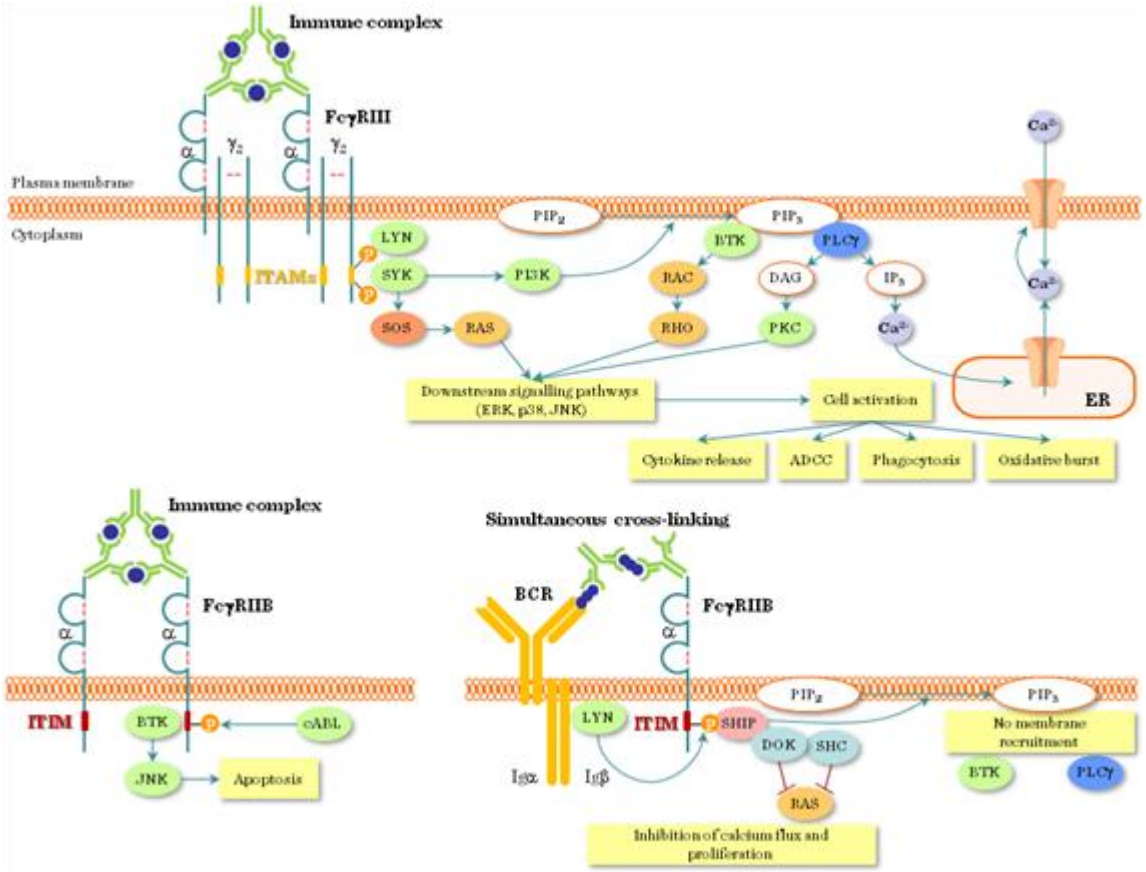


Figure II.1-9: Activator and inhibitory Fcγ receptor signaling

1.3.4. Fcg receptor signal transduction pathway

The clustering of these Fcg receptors with IgG1 or IgG3-coated particles or cells delivers an activation signal to phagocytes (Figure II.1-10). Activation signal requires cross-linking of the FcR a chains by several linked Ig molecules (e.g. Ig coated microbes, immuncomplexes). The signal transduction starts with Src kinase-mediated tyrosine phosphorylation of the ITAMs followed by SH2 domain-mediated recruitment of Syk family kinases to ITAMs , activation of PI-3 kinase, recruitment of adapter molecules like SLP-76 and BLNK, and the activation of enzymes like phospholipase Cg and Tec family kinases. Consequently, IP3 and DAG is generated and intracellular free Ca2+ is mobilized. These signal pathways in leukocytes induce gene transcription of cytokines, inflammatory mediators, microbicidal enzymes, activation of the cytoskeleton, alltogether leading to phagocytosis, cell migration and degranulation.

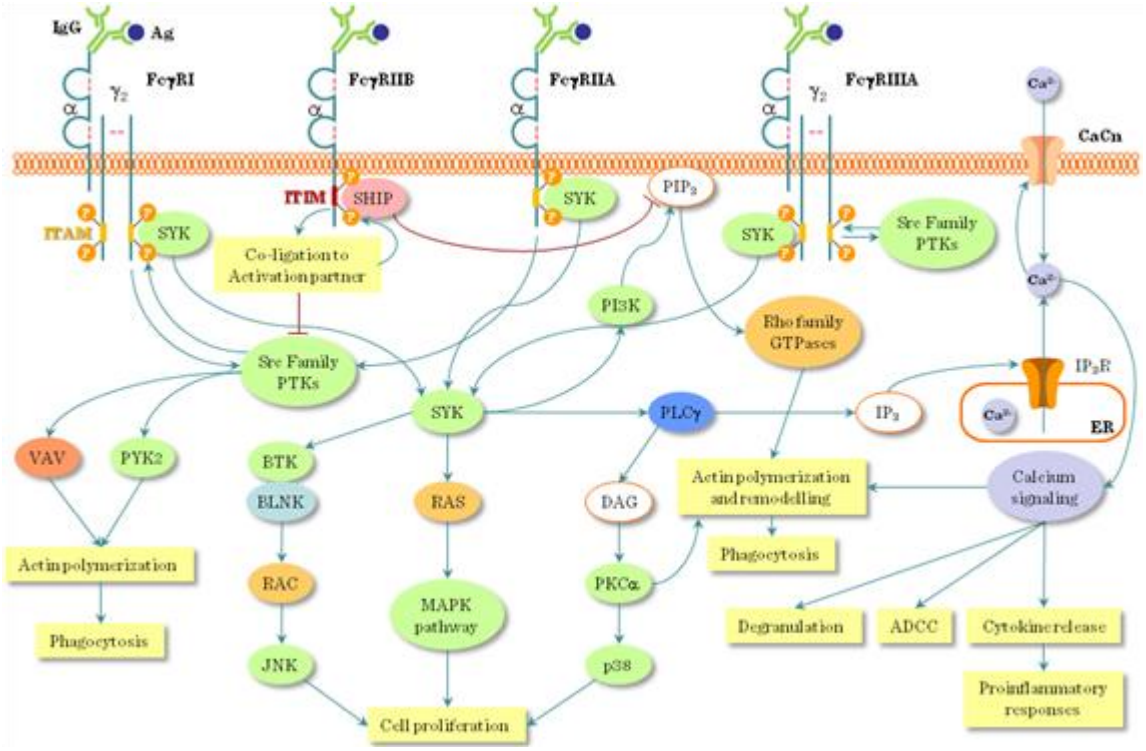


Figure II.1-10: Overview of Fcγ receptor signaling

1.4. II.1.4 Fce Receptor signaling

FcεRs have the ability to bind IgE. Although a lot is known about FcεRI function, the exact role of FcεRII still needs further studies.

1.4.1. The structure and expression of FcεRs

FcεRI (high-affinity IgE receptor) consists of α, β and γ chains (Figure II.1-11). It is expressed as an αβγ2 tetramer on mast cells and basophils, and as an αγ2 trimer on human antigen-presenting cells, monocytes, eosinophils, platelets and smooth-muscle cells. The α chain has 2 extracellular domains which are responsible for IgE binding. The intracellular parts of the β and γ chain contain ITAMs (immunoreceptor tyrosine-based activation motifs) being important in signal transduction.

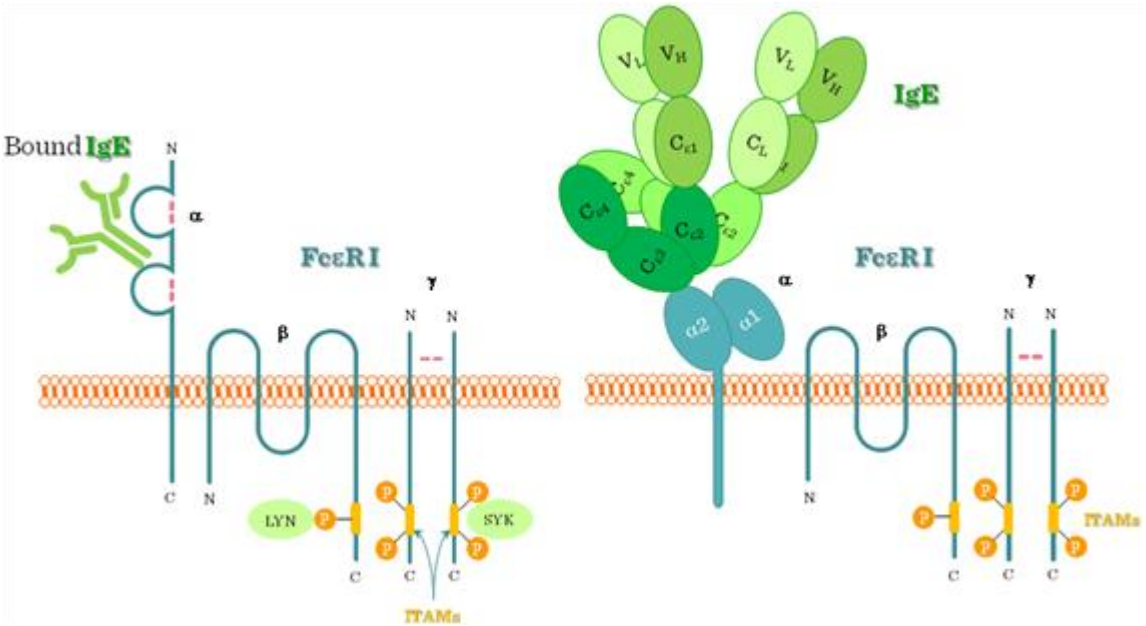


Figure II.1-11: IgE bound FcεR I

FcεRII (CD23, the low affinity receptor) is structurally different from all immunoglobulin-binding receptors because it belongs to the C-type lectin superfamily (Figure II.1-12). It consists of 3 C-type lectin domain heads, C terminal 'tails', an extracellular trimeric coiled coil 'stalk' and a short N-terminal intracellular sequence that exists in two splice variants. The coiled coil 'stalk' can undergo proteolysis resulting in soluble forms of CD23. CD23 does not only bind IgE but also CD21 (expressed by B cells, follicular dendritic cells, activated T cells and basophils) that might be important in allergic processes and in the regulation of IgE through the complement system.

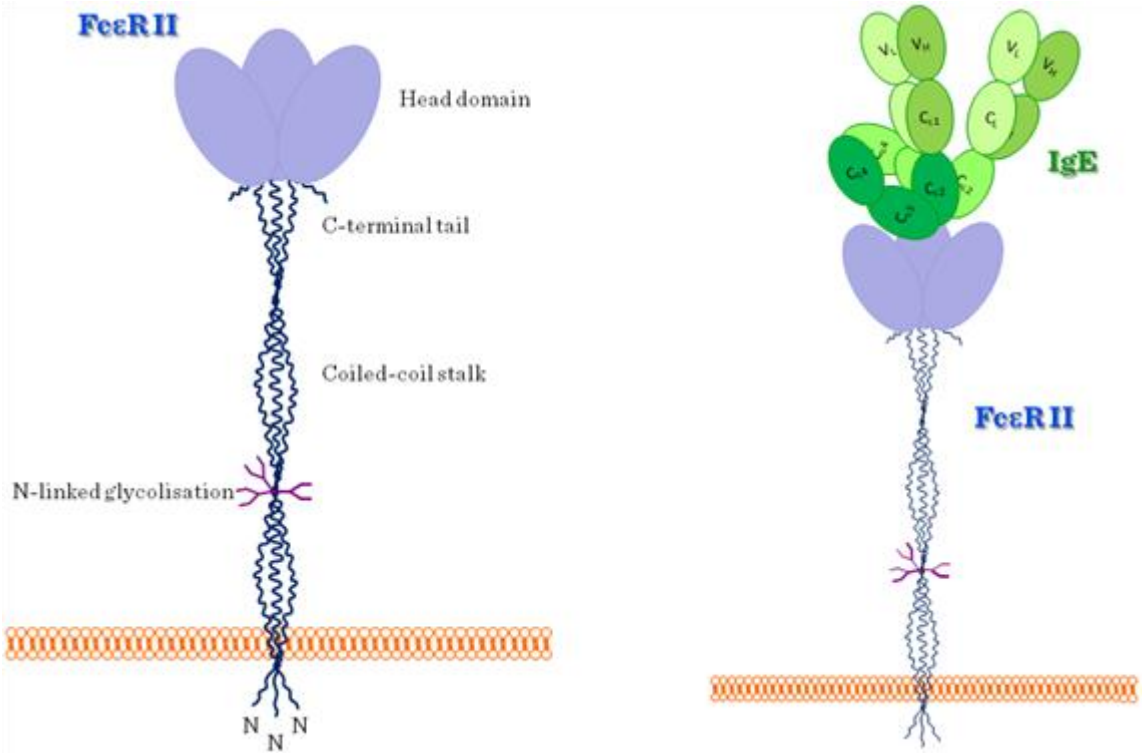


Figure II.1-12: IgE bound FceR II

1.4.2. FcεRI mediated signaling

Type I hypersensitivity reactions, for example anaphylaxis, hay fever, food allergies, other allergic diseases or asthma, are the most important pathological conditions mediated by FcεRI. Allergens like plant pollens, insect venoms are recognized by the immune system and IgE type antibody is produced against them. IgE then binds to the FcεRI receptors of mast cells, called sensibilisation. When the body meets the same allergen for the second time, crosslinking of the FcεRI-bound IgE molecules leads to activation of the cells (Figure II.1-13 and Figure II.1-14). Following FcεRI aggregation, ITAMs of the FcεRI become phosphorylated and protein tyrosine kinases Fyn and Lyn become activated, resulting in tyrosine phosphorylation of Syk non-receptor tyrosine kinase and Gab2 (Grb2-associated binding protein). These initial steps of FcεRI signaling share close homology with those of the TcR (Figure II.1-15). Gab2 binds to phosphatidylinositol 3-kinase (PI3K) and PI3K activation leads to Btk (Bruton's tyrosine kinase)-dependent phosphorylation of phospholipase C, that results in Ca2+ mobilization. PI3K might also enhance Ca2+ mobilization through phospholipase D mediated sphingosine-kinase activation. Parallel to PI3K, the MAP-kinase cascade is activated as well. Increased cytoplasmic Ca2+ leads to degranulation of the mast cells resulting in exocytosis of vasoactive amins (e.g. histamine) and proteases. The activation of the MAP-kinase cascade together with the increased Ca2+ signal enhances transcriptional activation of different genes leading to the synthesis of inflammatory cytokines, for example TNFα. Enzymatic modification of arachidonic acid results in the production of lipid mediators like prostaglandins or leukotrienes. The above mentioned mediator substances are responsible for the symptoms of hypersensity reactions, for example vascular leak, broncho-constriction, gastrointestinal hypermotility, inflammation and tissue damage.

Besides its pathogenic role, FcεRI has important physiological function in the immune response against parasites. Helminthes recognized by the immune system induce the production of IgE. IgE binds on the appropriate epitope of the parasite, thus, eosinophils can attack it through binding with their high-affinity FcεRI. During the exocytosis of eosinophilic granules cationic granule proteins, like major basic proteins, eosinophil basic proteins, and enzymes, like eosinophil peroxidase, are released leading to the killing of the parasite.

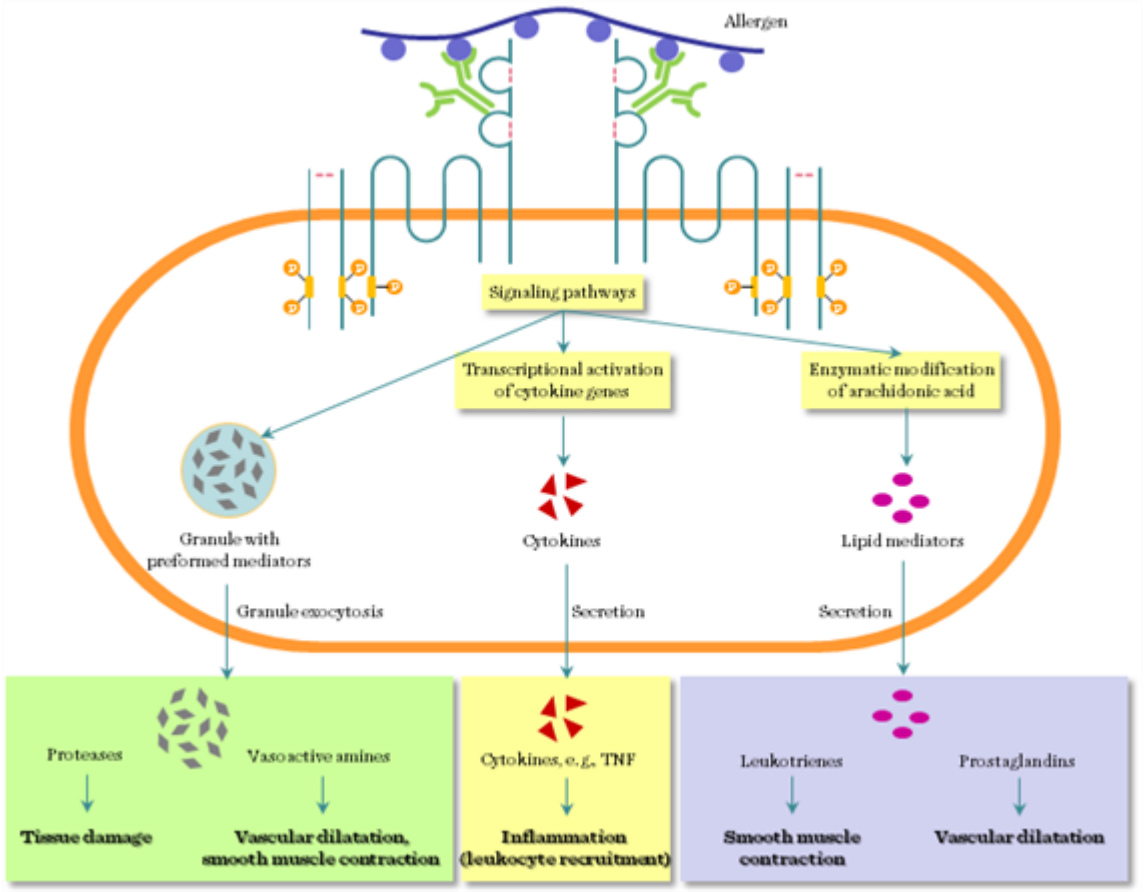


Figure II.1-13: Biological effects of FcεR signaling

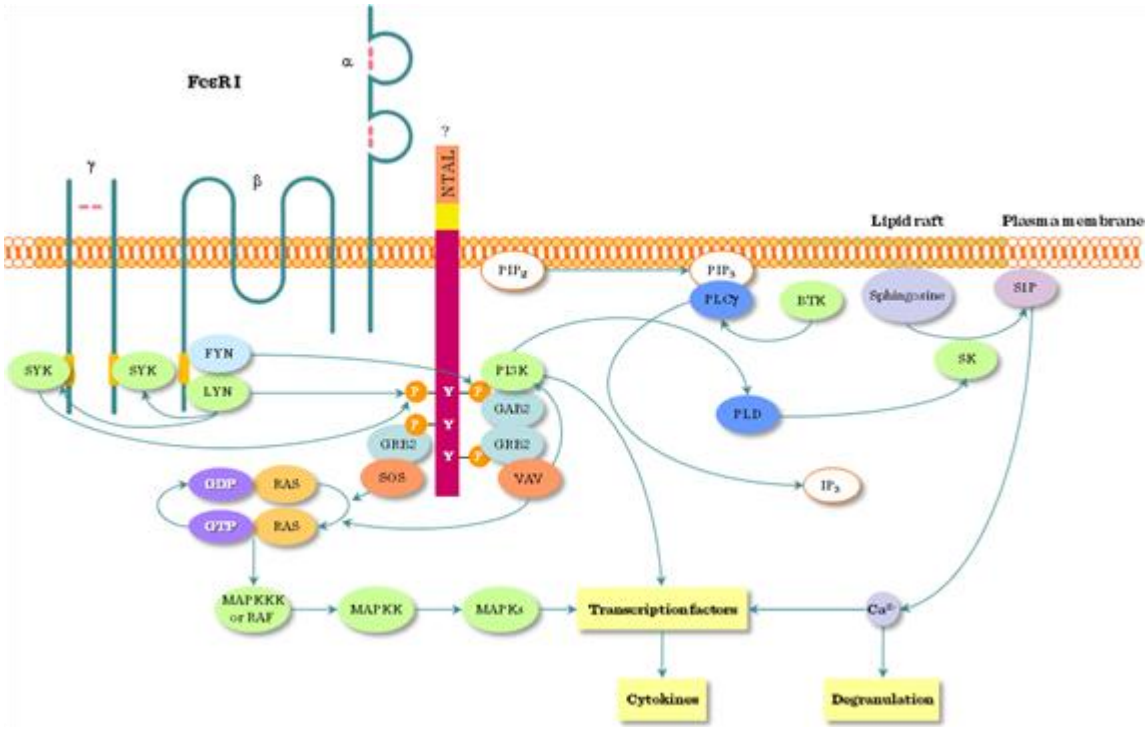


Figure II.1-14: FceRI mediated signaling

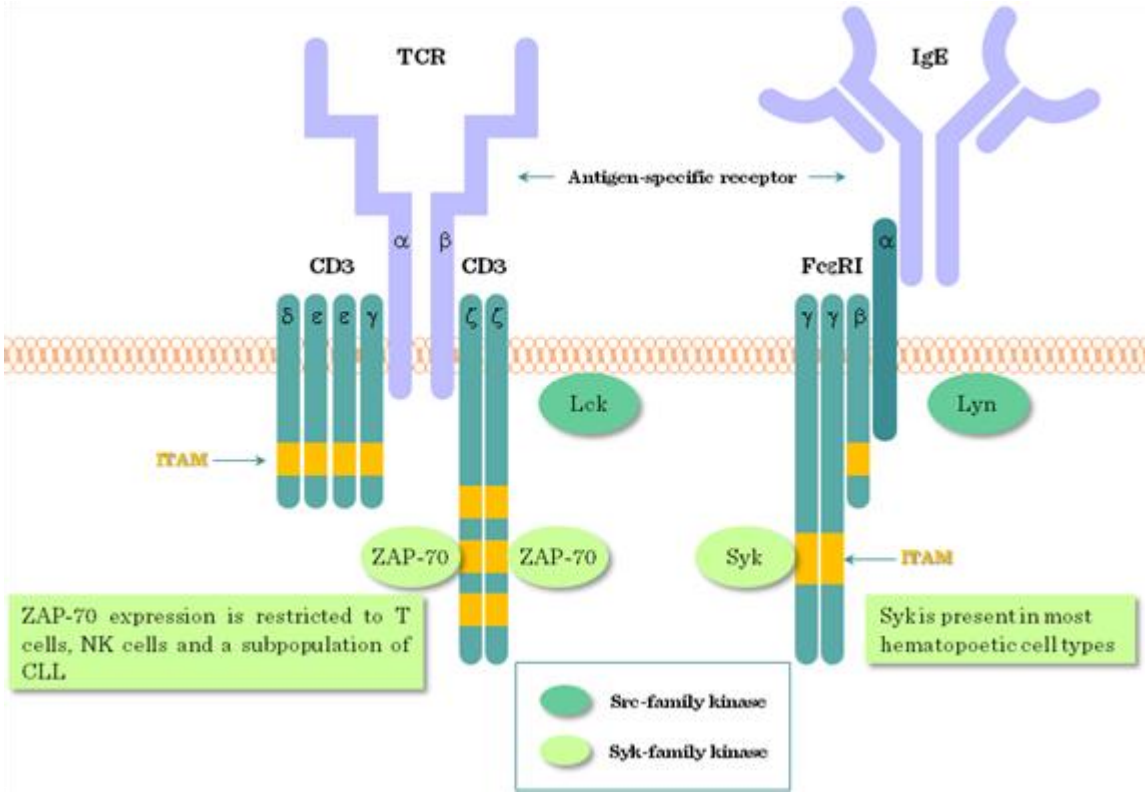


Figure II.1-15: Similarities in TcR and FcεR signaling

1.4.3. FcεRII (CD23) mediated processes

CD23 might mediate positive or negative regulation of IgE synthesis according to different models. Negative regulation of IgE synthesis occurs when membrane bound CD23 and IgE are co-ligated by allergen–IgE complexes. CD23 might also be important in the pathogenesis of food allergic diseases by transporting the

allergen from the gut lumen to the mucosa. The inhibitory function of CD23 suggests that it could serve as a basis for anti-allergic drug development in the future.

1.5. II.1.5 Cytokine signaling

1.5.1. Definition

Cytokines are small molecular weight glycoproteins that act at low concentrations on high affinity, specific cell surface receptors. In most cases they act on the cell(s) that are in the close vicinity of the producing cell (paracrine action), but some of them has autocrine (target cell = producing cell) or endocrine effects (via the circulation), too.

1.5.2. Division, groups

From a structural point of view, 3 groups were defined: (1) 4 α-helix bundle family (comprising from the IL-2-, IFN- and IL-10 subfamilies); (2) IL-1 family; and (3) IL-17 family.

From a functional point of view, we distinguish (1) haematopoetic cytokines (e.g. GM-CSF, G-CSF, M-CSF, erythropoetin, thrombopoetin); (2) cytokines that regulate lymphocyte activation and differentiation (immunoregulatory cytokines); and (3) inflammatory cytokines (IL-1, IL-6, TNFα). Immunoregulatory cytokines can be further classified based on the helper T cell subset that produces them:

a) Th1 cytokines: IL-2, TNFα, IFNγ;

b) Th2 cytokines: IL-4, 5, 13

c) Th17: IL-17A-F

d) Treg: TGFβ, IL-10

1.5.3. Receptors

The following cytokine receptor classes can be distinguished: class I (hematopoetin family), class II (IFN, IL-10), and TNF-receptor family (Figure II.1-16). Class I receptors are heterodimer/trimer molecules that can be further divided into subgroups:

(1) receptors for erythropoetin, growth hormone and IL-13

(2) receptors with common β chain (IL-3, IL-5, GM-CSF);

(3) receptors sharing a common γ chain (IL-2, IL-4, IL-7, IL-9, IL-15);

(4) receptors sharing a common gp130 subunit (IL-6 receptor subfamily) (Figure II.1-17).

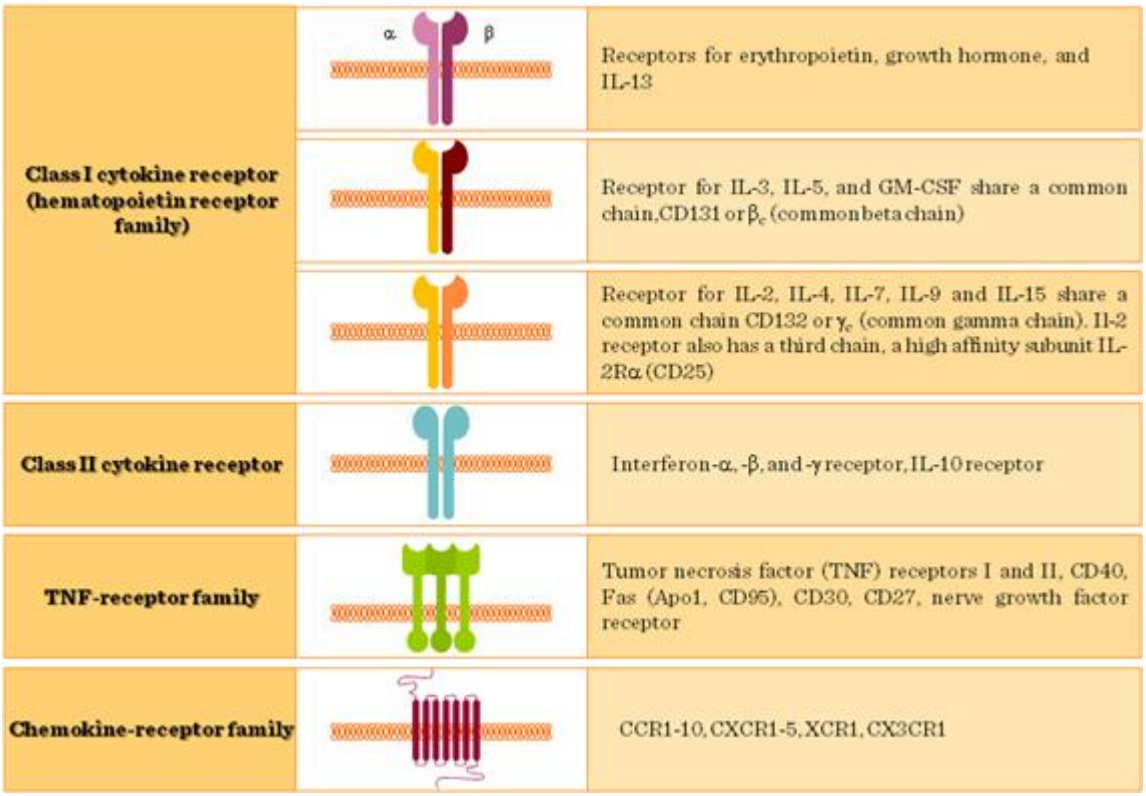


Figure II.1-16: Cytokine receptors

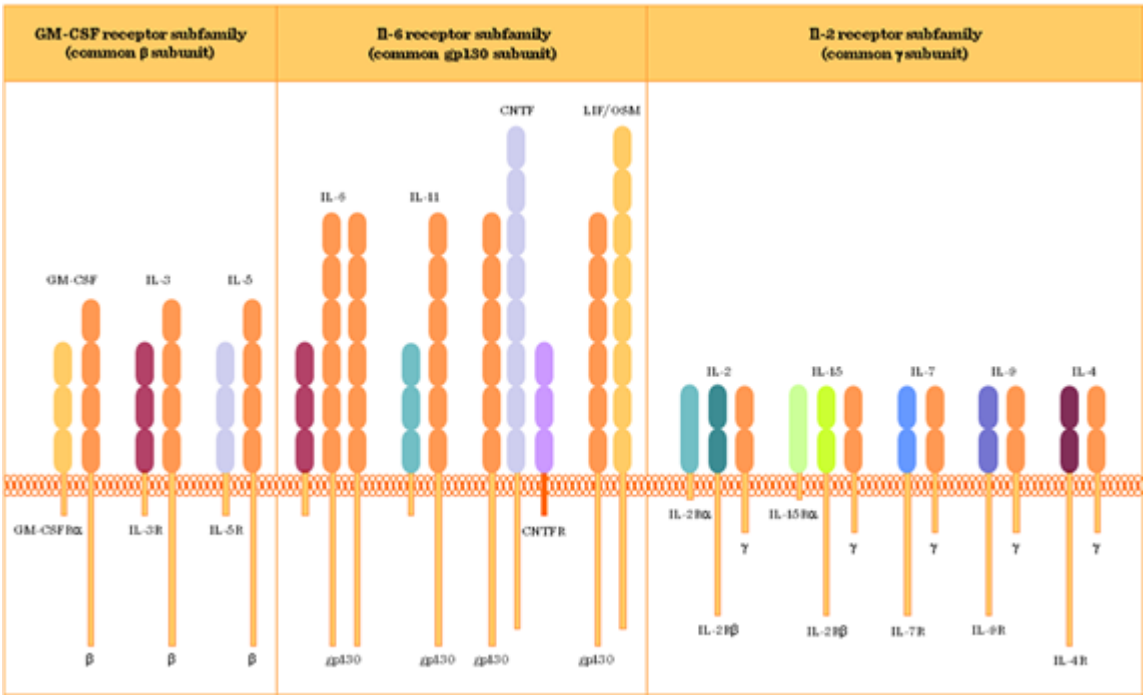


Figure II.1-17: Characteristics of multichain cytokine receptors

1.5.4. Janus kinases (JAKs)

JAKs (JAK1, 2, 3 and TYK2) (120-140kDa) associate to the cytoplasmic part of cytokine receptors. They were first named “Just Another Kinase”, later „Janus (Roman god of doorways with two faces heading opposite directions) kinases”. This latter name has a structural basis: all JAKs have adjacent “Kinase” and “Pseudokinase” domains. The basic structural elements of JAKs are the “JH” (Janus homology domains). The

JH1: kinase, JH2: pseudokinase, JH3: SH2, JH4-7: FERM (=band 4.1, ezrin, radixin and moesin) domain (Figure II.1-18). The FERM domain binds to the proline-rich membrane proximal part of cytokine receptors. Phosphorylation of two neighboring tyrosine residues in the kinase domain is critical in the activation of the molecule. Class I.1. and I.2. (see above) receptors associate with JAK2; Class I.3. receptors associate with JAK1 and JAK3; Class I.4. and Class II receptors associate with JAK1, JAK2 and TYK2.

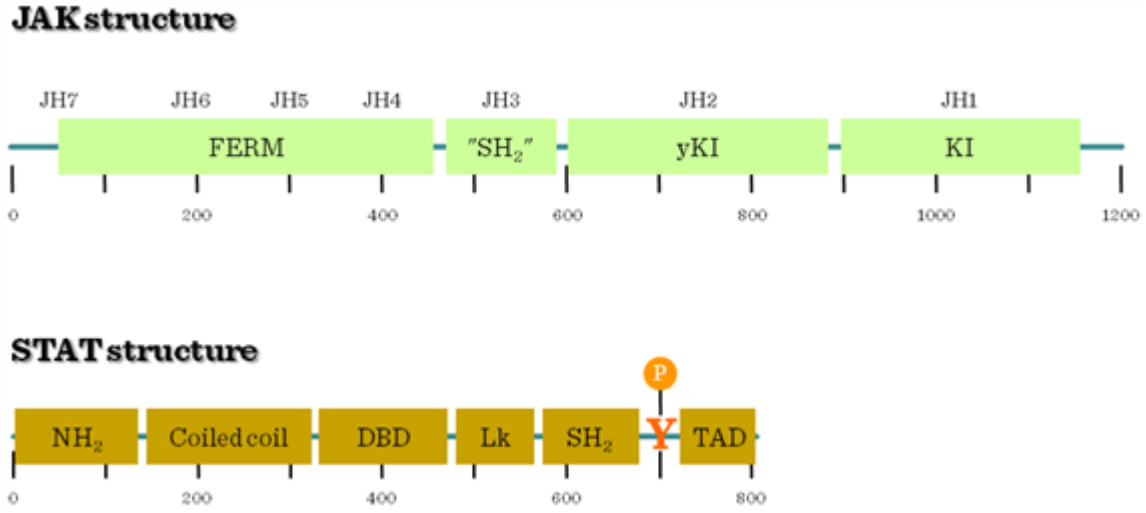


Figure II.1-18: The structure of JAK and STAT proteins

1.5.5. Signal Transducer and Activator of Transcription (STATs)

STATs are the main target molecules of JAKs (Figure II.1-18). They contain an NH2 domain (Dimerization, DNA-binding and Nuclear transport), a coiled-coil (binding of regulator proteins), a DNA-binding domain (DBD), a linker (Lk), an SH2 (receptor recruitment and dimerization) and a transcriptional activation domain (TAD). Phosphorylation of the tyrosine residue between the SH2 and TAD domains is critical in the activation of the molecule. STAT1 and 2 are involved in IFN signaling; STAT3 mediates IL-6 & IL-10 family, IL-21 and IL-27 signaling controlling Th17 differentiation; STAT-4 mediates IL-12 and IL-23 signaling controlling Th1 differentiation; STAT5a & b mediate IL-3, IL-5 and GM-CSF signaling; and STAT-6 is involved in IL-4, IL-13 signaling driving Th2 differentiation and allergic immune responses.

1.5.6. Cytokine signaling

Upon ligand binding the receptor chains dimerize which leads to the activation of the associated JAKs (Figure II.1-19). Activated JAKs phosphorylate each other and the receptor chains. STATs bind to the phosphorylated receptors and, in turn, they are phosphorylated by JAKs. Activated STATs form dimers and translocate to the nucleus where they act as transcription factors. For example, type I IFNs (IFNα and IFNβ) activate STAT1/2 heterodimers which bind to ISRE (=IFN-sensitive response elements) sequences, whereas type II IFN (IFNγ) signaling activates STAT1 homodimers which bind to GAS (=IFNγ-activated site) sequences.

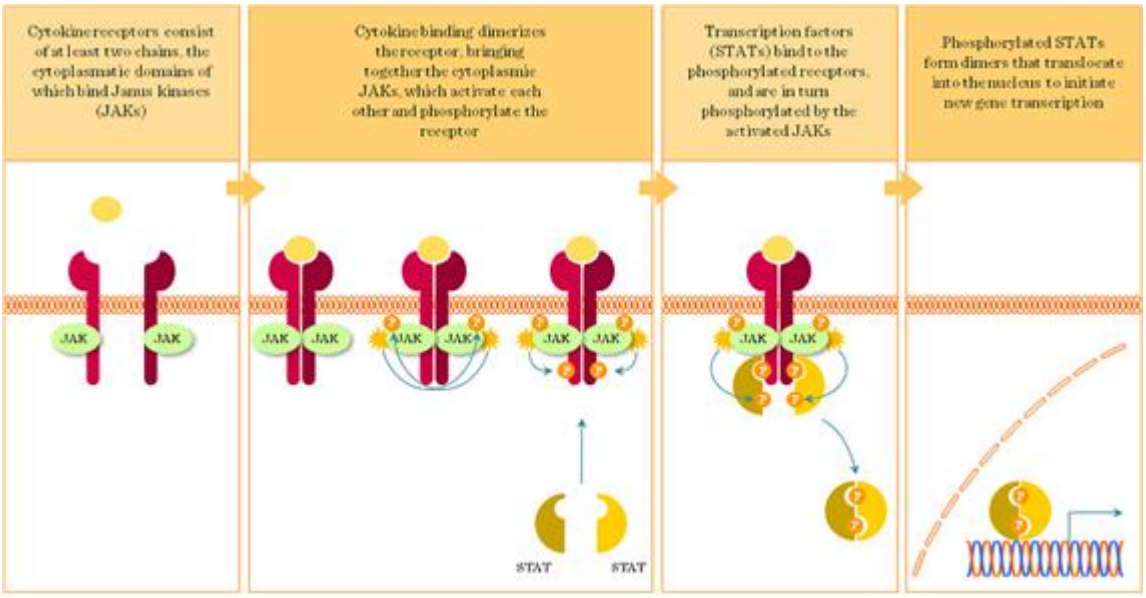


Figure II.1-19: Overview of cytokine signalling

1.5.7. Regulation of JAK/STAT signaling

The JAK/STAT pathway is controlled by four major mechanisms.

(1) Phosphatases SHP-1/2 and CD45 dephosphorylate JAK, whereas SHP-2, PTP1B, TC-PTP and PTP-BL dephosphorylate STAT proteins.

(2) Control of nuclear export/import by NES (nuclear export sequence) or NLS (nuclear localization sequence).

(3) SOCS (suppressors of cytokine signaling) e.g. PIAS=Protein Inhibitor of Activated STATs

(4) Serine-phosphorylation, acetylation or O-glycosylation of TAD.

1.5.8. Clinical implications: JAK inhibitors

JAK inhibitors (e.g. Lestaurtinib; Tofacitinib; Ruxolitinib) are being tested in the treatment of hematological diseases e.g. polycythemia vera, thrombocytemia, myeloid metaplasia, myelofibrosis; and autoimmune diseases like psoriasis and RA.

1.5.9. TNF receptor signaling

Upon ligand binding, TNF receptor chains form trimers, which leads to conformational change and the subsequent dissociation of the inhibitor SODD (=silencer of death domains) from the intracellular “death domain”. The adaptor protein TRADD (=tumor necrosis factor receptor type 1-associated DEATH domain protein) binds to the death domains and serves as a platform for further protein association (Figure II.1-20 and Figure II.1-21).

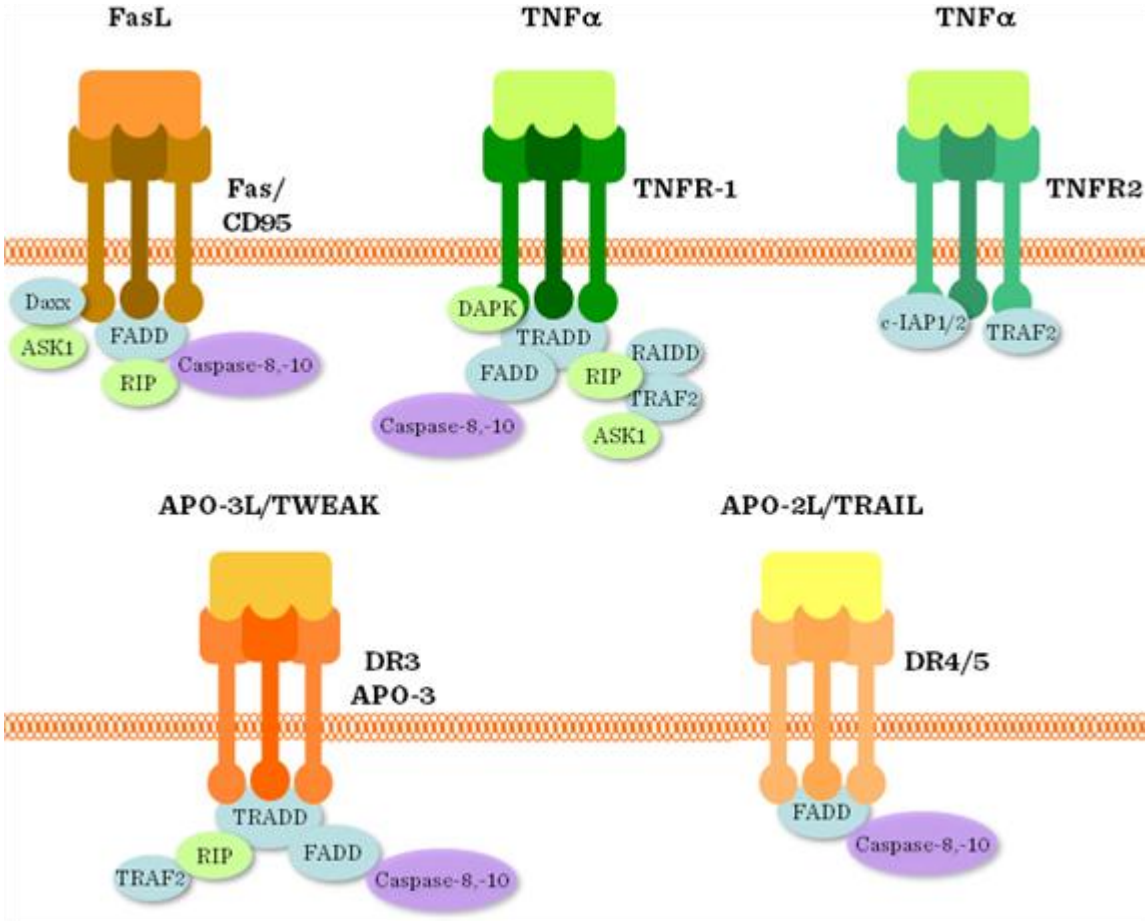


Figure II.1-20: TNF receptor mediated apoptosis I

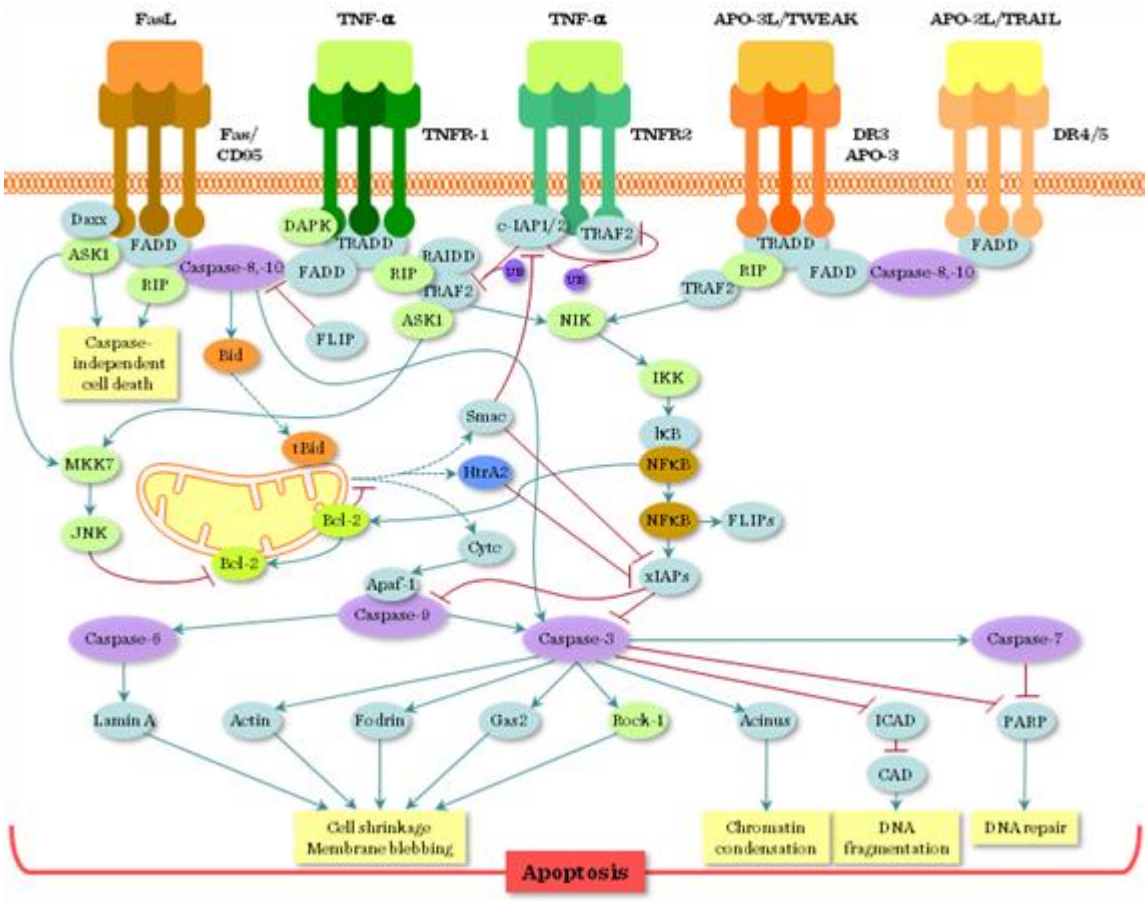


Figure II.1-21: TNF receptor mediated apoptosis II

Three major signaling pathways are activated:

(1) NF-κB pathway: TRAF2 (=TNF receptor-associated factor 2) and RIP (=receptor interacting protein) are recruited to TRADD. RIP, a Ser/Thr kinase, activates IKK (IκB kinase), which, in turn, activates NF-κB. The activated NF-κB translocates to the nucleus and controls the transcription of cell survival, proliferation, inflammation and apoptosis genes (generally anti-apoptotic).

(2) Activation of MAPK pathways: From the three major MAPK pathways, strong activation of the stress response JNK pathway, moderate activation of p38 and minimal activation of the ERK pathway occurs after TNF receptor activation. TRAF2 recruits MEKK1 (=Mitogen-activated protein kinase kinase kinase 1) and ASK1 (=Apoptosis signal-regulating kinase 1), which phosphorylate MKK7 (=Mitogen-activated protein kinase kinase 7). MKK7 phosphorylates JNK (=c-Jun N-terminal kinase), which in turn, translocates to the nucleus and activates the transcription factors c-Jun and ATF2 (=Activating transcription factor 2). This pathway controls genes of cell differentiation, proliferation and apoptosis (generally pro-apoptotic).

(3) Death signaling ("Extrinsic apoptosis pathway ", see Chapter II.4 for details): TNFR1 does not induce this pathway as strong as for example the Fas molecule. TRADD binds FADD, which recruits pro-Caspase-8. Autocatalytic cleavage activates Caspase-8, which initiates the downstream events of the apoptotic cascade: Caspase-3 and Bid (=BH3 interacting domain death agonist), a pro apoptotic member of the Bcl-2 family leading to Cytochrome C release from the mitochondria.

1.6. II.1.6 Chemokine signaling

1.6.1. Definition

Chemokines are 90-130 amino-acid polypeptides, which control the chemotaxis of different leukocytes. They regulate normal leukocyte traffic as well as the recruitment of cells to inflammatory sites. Chemokines enhance

cell adhesion, activate effector leukocytes, contribute to the development of the inflammatory reaction and the development of lymphoid tissues.

1.6.2. Nomenclature, groups and receptors

Classiffication of chemokines is based on the spacing of their structurally conserved first cysteine (C) residues. In CXC (α chemokines) the cysteins are separated by a single amino acid; in CC (β chemokines) the cysteins are adjacent to each other, C (γ chemokines) have only two cysteine residues: one N terminal and one downstream; whereas in CX3C (δ chemokines) the cysteins are sparated by 3 other amino acids. Their receptors are named CXCR, CCR etc. indicating the type of chemokine that is bound to them.

1.6.3. Signaling

Chemokine receptors belong to the 7-transmembrane spanning (7-TM) / G-protein coupled receptor family. They activate the PLC > PIP2 > IP3 > Ca2+-signal, PLC > PIP2 > DAG > PKC and MAPK pathways (Figure II.1-22 and Figure II.1-23). These pathways promote actin polymerization, cytoskeleton rearrangement, and expression of adhesion molecules, which, together lead to chemotaxis.

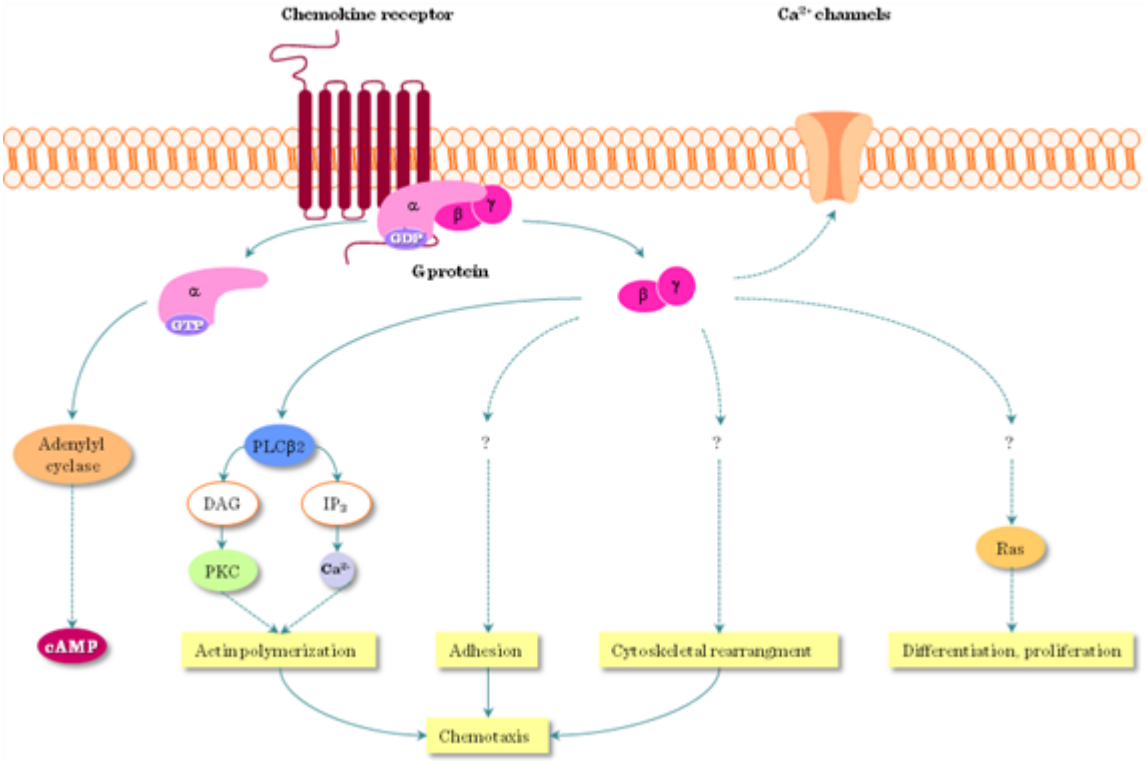


Figure II.1-22: Chemokine signal through receptors coupled with G-proteins

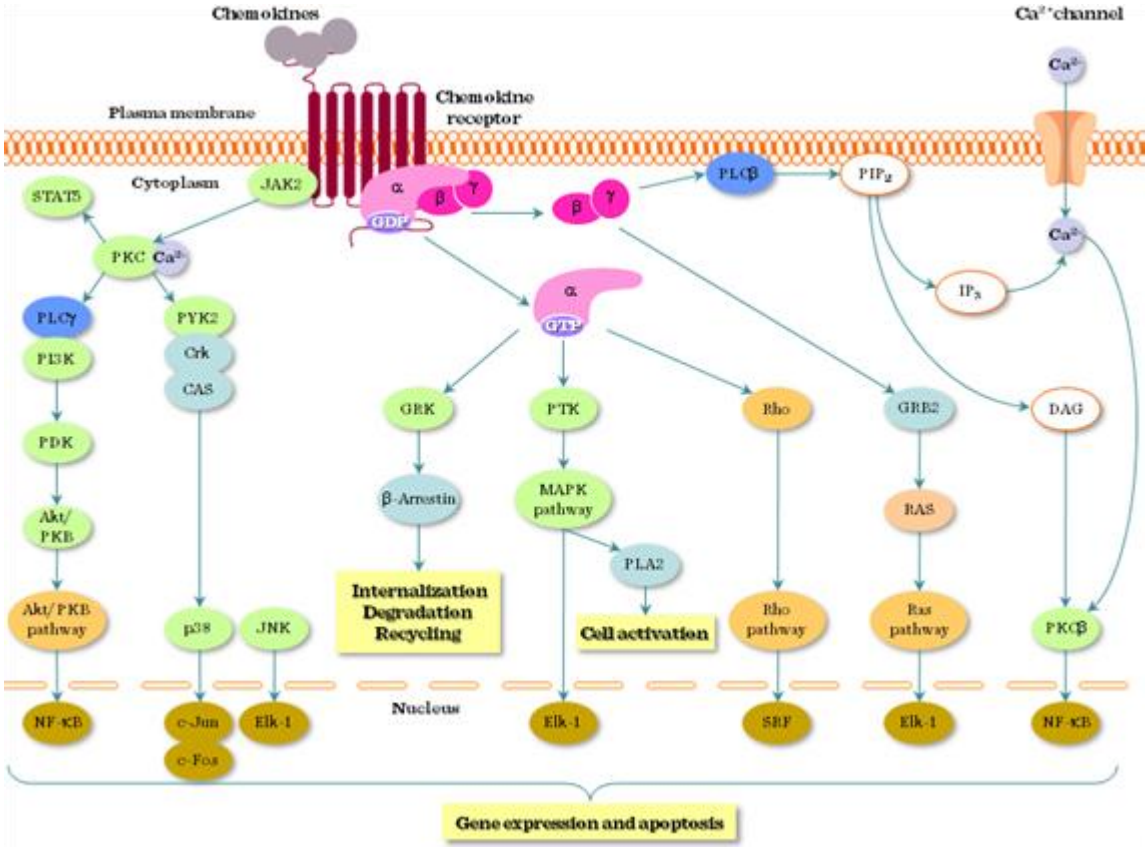


Figure II.1-23: Chemokine signaling pathways

1.6.4. Implications in diseases

Constitutively expressed chemokine receptors (e.g. CCR6, 8, 10, 11; CXCR4, 5, 6) regulate basal homing and trafficking of leukocytes, while inducible chemokine receptors (e.g. CCR1-5, 7; CXCR1-3) control inflammation. CCR1, 2 and 5 might play a role in multiple sclerosis, rheumatoid arthritis, asthma and nephritis; CCR5 and CXCR4 in AIDS; CXCR1 and 2 in sepsis and atherosclerosis.

1.7. II.1.7 Signaling in the innate immune system, PRR signaling

The innate immune system recognizes common microbial molecules, called pathogen-associated molecular patterns (PAMPs), which are essential for the survival of those organisms and are not found in mammals. Most phagocytes have pattern-recognition receptors (PRR-s) for these common PAMPs but they can also be recognized by a series of soluble PRRs in the blood that function as opsonins and initiate the complement pathways. In all, the innate immune system is thought to recognize approximately 103 of these microbial molecular patterns.

1.7.1. Endocytic Pattern-Recognition Receptors

Endocytic pattern-recognition receptors are found on the surface of phagocytes and promote the attachment of microorganisms to phagocytes leading to their subsequent engulfment and destruction. They include:

(1) mannose receptors of phagocytes are C-type lectins binding mannose-rich glycans with mannose or fructose as the terminal sugar that are commonly found in microbial glycoproteins and glycolipids.

(2) scavenger receptors bind to bacterial cell wall components such as LPS, peptidoglyan and teichoic acids and stressed, infected, or injured cells. Scavenger receptors include CD36, CD68, and SRB-1.

(3) opsonin receptors bind microbes to phagocytes. One portion of the opsonin binds to a PAMP on the microbial surface and another portion binds to a specific receptor on the phagocytic cell. Acute phase proteins

like mannose-binding lectin (MBL), C-reactive protein (CRP) C3b and C4b complement factors, Surfactant proteins in the alveoli SP-A and SP-D and the antibody molecule IgG can function as opsonins.

(4) *N*-formyl Met receptors bind N-formyl methionine, the first amino acid produced in bacterial proteins since the f-met-tRNA in bacteria has an anticodon complementary to the AUG start codon

1.7.2. Signaling of Pattern-Recognition Receptors

Binding of microbial PAMPs to their PRRs promotes the synthesis and secretion of intracellular regulatory molecules such as cytokines that are crucial to initiating innate immunity and adaptive immunity. These include inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), and interleukin-12 (IL-12), as well as chemokines such as interleukin-8 (IL-8), MCP-1, and RANTES. Cytokines in turn, bind to cytokine receptors on other defense cells.

1.7.3. Extracellular TLRs

The Toll receptor was originally identified in Drosophila as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos. In 1996, Hoffmann and colleagues demonstrated that Toll-mutant flies were highly susceptible to fungal infection. This study made everyone aware that the immune system, particularly the innate immune system, has a skilful means of detecting invasion by microorganisms. Subsequently, mammalian homologues of Toll receptor were identified one after another, and designated as Toll-like receptors (TLRs) (Figure II.1-24). The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is now called the Toll/IL-1 receptor (TIR) domain.

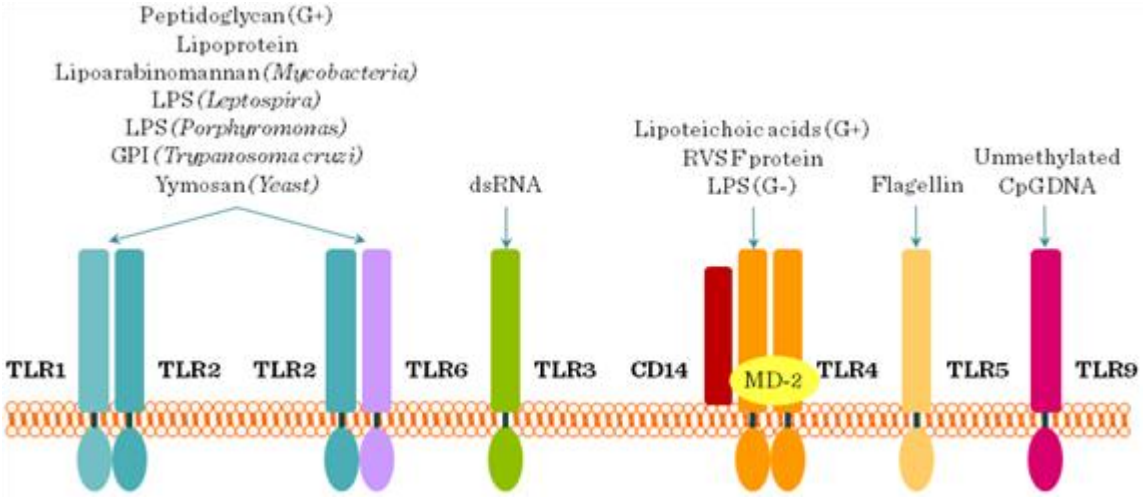


Figure II.1-24: Toll-like receptors-pattern recognition

1.7.4. Families of TLRs

A total of 13 mammalian TLR members containing a conserved TIR domain in their intracellular domain and an individual leucine-rich repeat domain in their extracellular domain have been identified. Despite of this similarity, the extracellular portions of both types of receptors are structurally unrelated (Figure II.1-24).

TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and TLR3, TLR7, TLR8, and TLR9 are expressed on the endosome-lysosome membrane.

1.7.5. TLR signaling and function

Upon the recognition of PAMPs, TLR signaling (Figure II.1-25) promptly induces potent innate immune responses that signal through adaptor molecules like myeloid differentiation factor 88 (MyD88), Toll/interleukin (IL)-1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing interferon (IFN) (TRIF), and TRIF-related adaptor molecule (TRAM) to activate transcription factors, nuclear factor (NFkB), activator protein 1 (AP-1), and interferon regulatory factors (IRFs) to induce antibacterial and antiviral responses. Thousands of genes are activated by TLR signaling, implying that this method constitutes an important gateway for gene modulation.

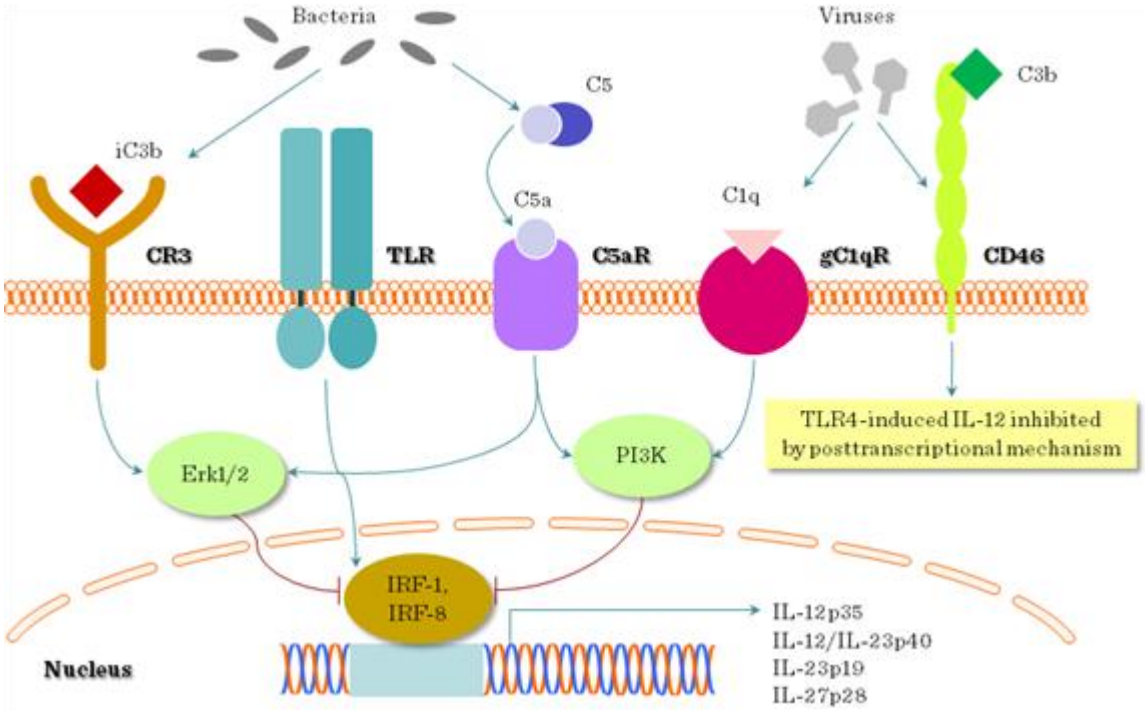


Figure II.1-25: Overview of complement receptor (CR) and Toll-like receptor signaling

1.7.6. Signaling PRRs found in the membranes of the endosomes /phagolysosomes

TLR-3, 7, 8 – bind single- or double-stranded viral RNA; TLR-9 – binds unmethylated cytosine-guanine dinucleotide sequences (CpG DNA). Most of the TLRs that bind to viral components trigger the synthesis of interferons that block viral replication within infected host cells.

1.7.7. Signaling PRRs found in the cytoplasm:

(1) NOD1 and 2 (NOD–nucleotide-binding oligomerization domain) are cytosolic proteins that allow intracellular recognition of peptidoglycan components (muramyl dipeptide of all bacteria) leads to the activation of genes coding for inflammatory cytokines such as IL-1, TNF-alpha, IL-8, and IL-12 in a manner similar to the cell surface TLRs.

(2) CARD-containing proteins (CARD–caspase activating and recruitment domain), such as RIG-1 (retinoic acid-inducible gene-1) and MDA-5 (melanoma differentiation-associated gene-5), are cytoplasmic sensors that detect both viral double-stranded and single-stranded RNA molecules produced in viral-infected cells and trigger the synthesis of cytokines called interferons that block viral replication within infected host cells in a manner similar to the endosomal TLRs. TLR signaling can be inhibited by several agents at different levels (Figure II.1-26).

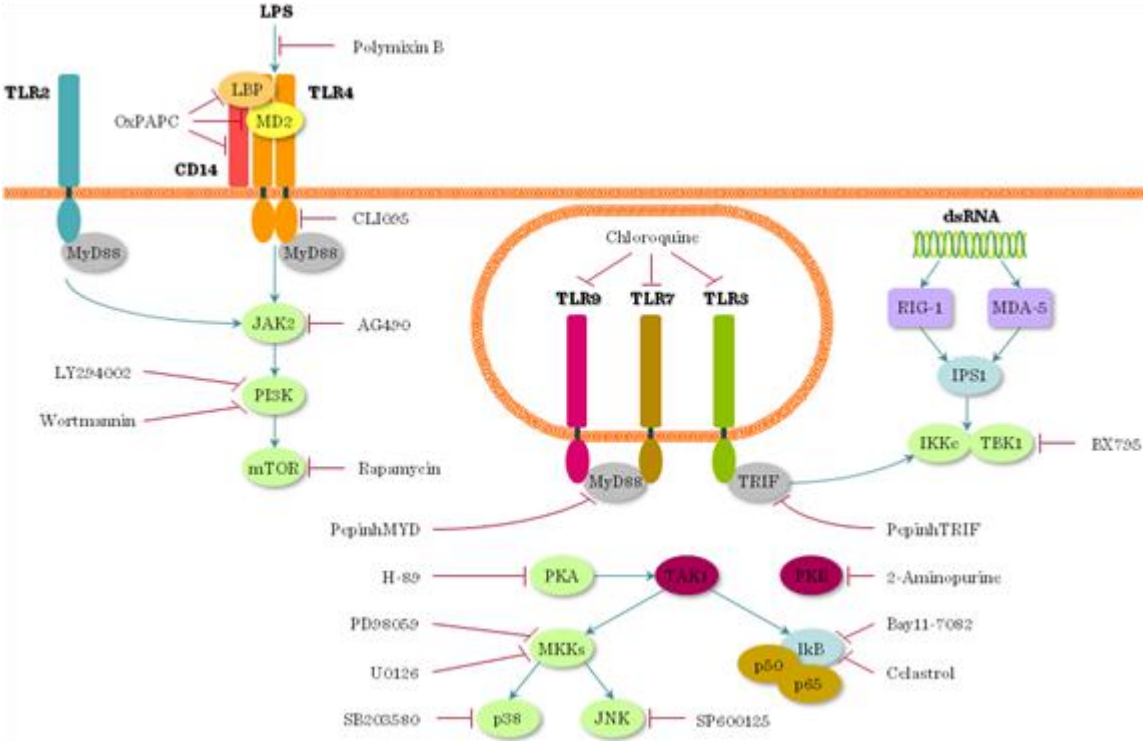


Figure II.1-26: Toll-like receptor inhibitors

1.7.8. Complement receptor signaling

There are several types of complement receptors (CR1-4, C3aR, C5aR) involved in a number of immunologic processes (Figure II.1-27). CR1 (CD35) is expressed by erythrocytes, monocytes, neutrophils and B cells, and its most important function is the clearance of immune complexes from the circulation. CR2 (CD21, EBV receptor) is mostly expressed on B cells and follicular dendritic cells, and serves as an activatory co-receptor in B cell activation.CR3 and CR4 consist of CD11b or CD11c and CD18, expressed mainly by neutrophils, NK cells and macrophages, and serve as PRRs, enhancing phagocytosis. C3aR and C5aR belong to the 7-TM receptor group and their signaling is G-protein dependent (for more details see Chapter I.2.2 and I.4.1).

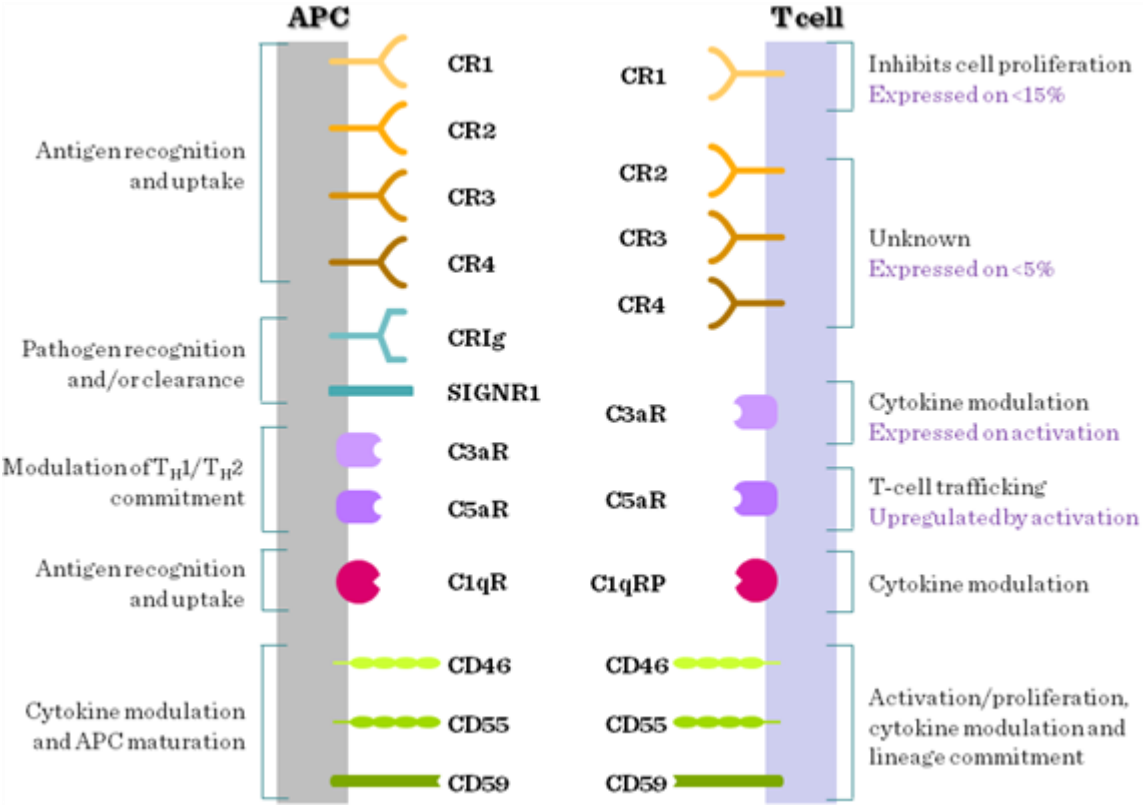


Figure II.1-27: Complement receptors

2. II.2 Hormone and growth factor signaling

2.1. II.2.1 Tyrosine kinase-linked receptors

2.1.1. II.2.1.1 Growth-factor signaling

2.1.1.1. Definition

Growth factors (GFs) are small molecular weight soluble mediators controlling proliferation, survival, metabolism and tissue differentiation. They have also important implications in tumor.

2.1.1.2. History

Their description and isolation were closely linked to the development of in vitro tissue/cell culturing techniques. Propagation of cells under in vitro conditions began at the turn of the 19th-20th century. Rous made experiments with chicken tumor (sarcoma) cells (RSV). Carrel's experiments showed that in simple buffered salt solution the cells did not proliferate, he made initial trials with diluted plasma/serum. Temin and Dulbecco worked out the precise requirements for tissue culturing and found reduced serum need of tumor cells which they interpreted as an enhanced capacity of tumor cells to respond to proliferation signals ("growth factors"). They also observed that serum supported cell growth better than plasma, which, as later turned out, was due to PDGF coming from activated platelets. R. Levi-Montalcini and S. Cohen described NGF and EGF, the first growth factors.

2.1.1.3. Groups

PDGF: platelet-derived growth factor; EGF: epithelial growth factor; NGF: neuronal growth factor; FGF: fibroblast growth factor; TGF: transforming growth factor, IGF: insulin-like growth factor (Figure II.2-1 and Table II.2-1).

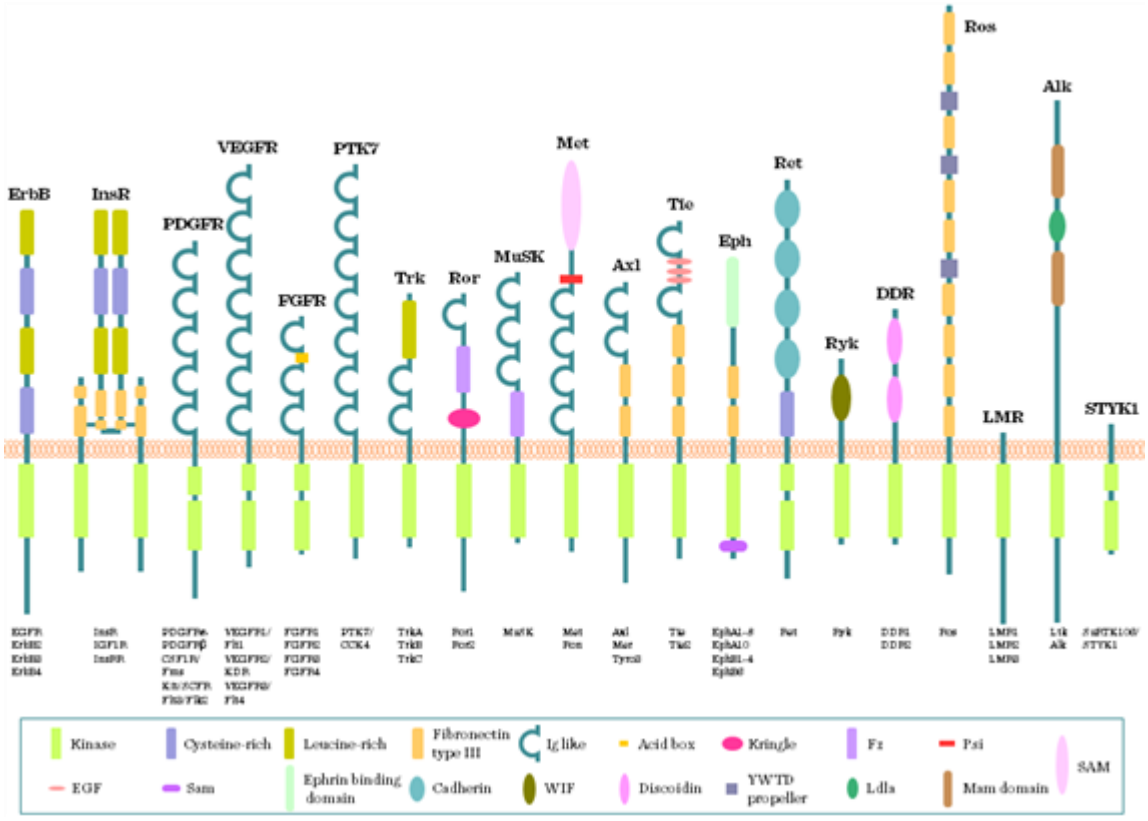


Figure II.2-1: Growth factor (GF) receptors

Table II.2-1: Receptor classes

Class	Examples	Structural Features of Class
I	EGF receptor; NEU/HER2, HER3	Cysteine-rich sequences
II	Insulin receptor; IGF-1 receptor	Cysteine-rich sequences; characterized by disulfide-linked heterotetramers
III	PDGF receptors, c-Kit	Contain 5 immunoglobulin-like domains; contain the kinase insert
IV	FGF receptors	Contain 3 immunoglobulin-like domains as well as the kinase insert; acidic domain
V	Vascular endothelial cell growth factor (VEGF) receptor	Contain 7 immunoglobulin-like domains as well as the kinase Insert domain
VI	Hepatocyte growth factor (HGF) and scatter factor (SF) receptors	Heterodimeric like the class II receptors except that one of the two protein subunits is completely extracellular. The HGF receptor is a proto-oncogene that was originally identified as the MET oncogene
VII	Neurotrophin receptor family (TRKA, TRKB, TRKC) and NGF receptor	Contain no or few cysteine-rich domains; NGFR has leucine rich domain

2.1.1.4. Receptor dimerization and signaling

Growth factor receptors belong to the receptor tyrosine kinase family (for the details of growth factor receptor signaling see chapter I.2.3.1 Receptor tyrosine kinases).

Ligand binding leads to receptor dimerization, which induces phosphorylation of the kinase domain and its activation (Figure II.2-2 – Figure II.2-5). Different receptors utilize different dimerization/activation strategies: for example PDGF is a dimer, which cross-links two cell surface PDGF receptor monomers; the binding of EGF to its receptor induces a conformational change, which promotes dimerization; FGF is complexed by heparin and cross links two FGF monomers; in case of insulin the receptor is already dimerized on the cell surface, ligand binding causes a conformational change and autophosphorylation (for more details on insulin signaling see next chapter).

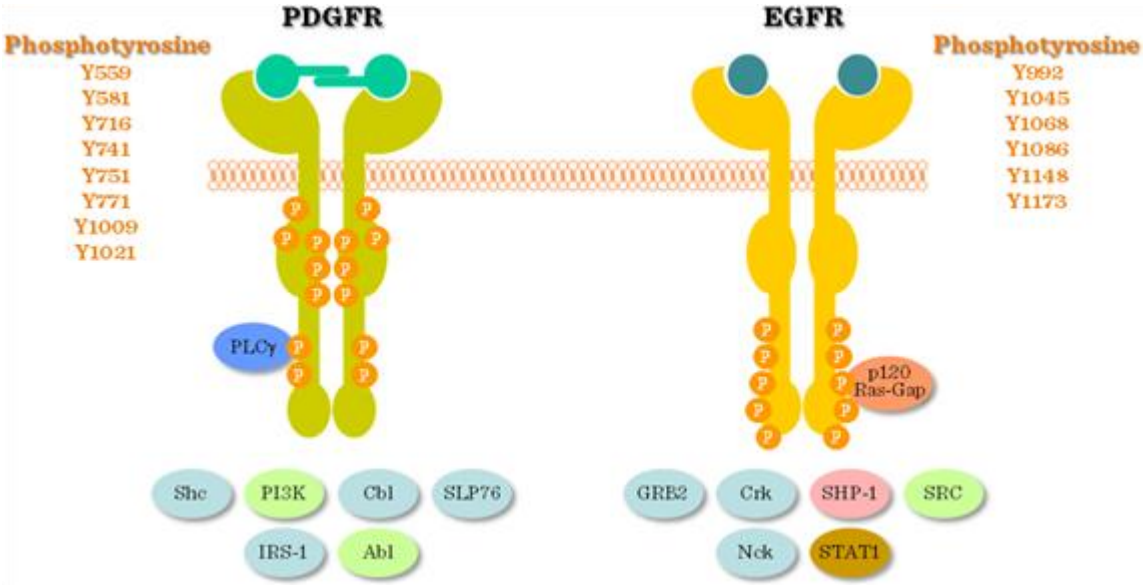


Figure II.2-2: Autophosphorylation of RTKs

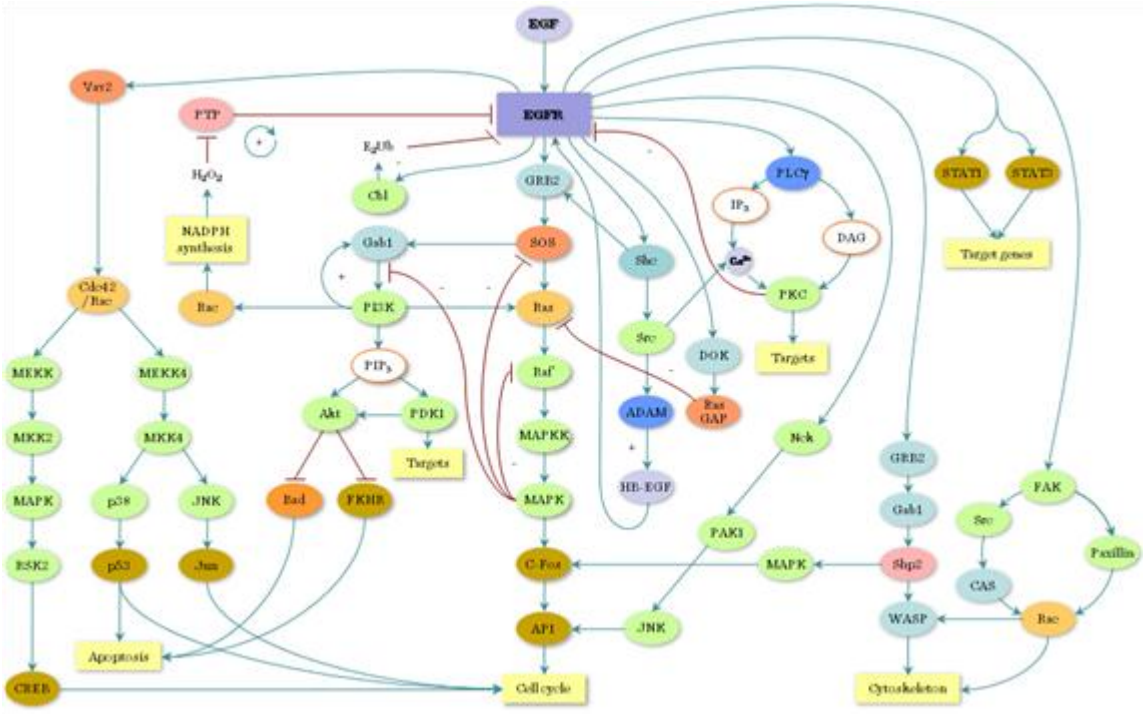


Figure II.2-3: Overview of EGF signaling

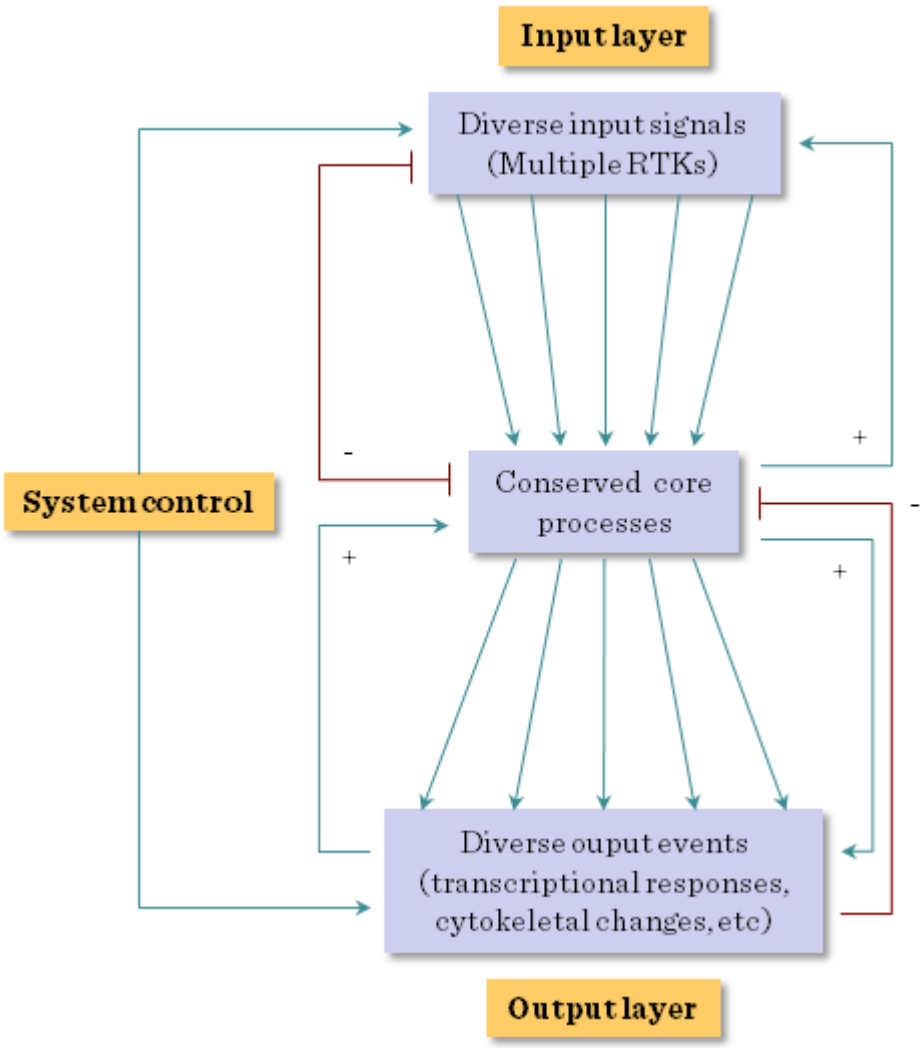


Figure II.2-4: General characteristics of GF signaling

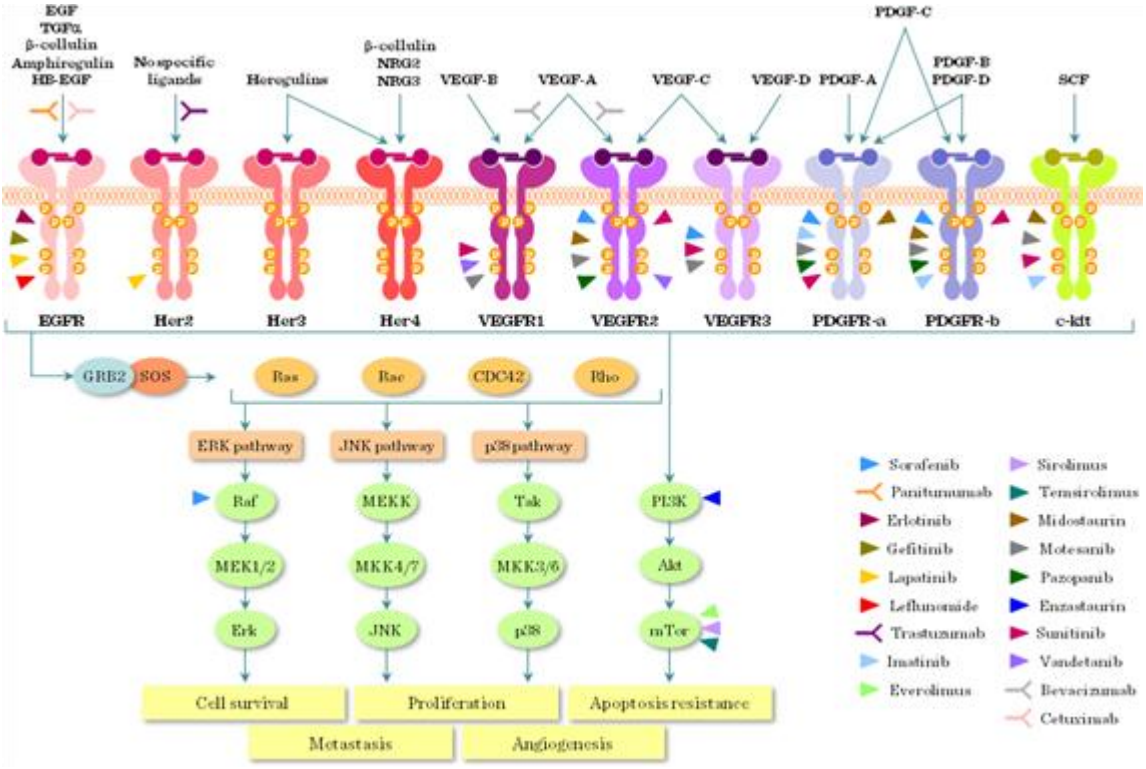


Figure II.2-5: GF receptors as therapeutic targets

2.1.1.5. Growth factor signaling in tumors

Growth factors and receptor tyrosine kinases and their signaling pathways are not only involved in the physiological regulation of cell proliferation and differentiation but also in the development of malignant tumors. They serve as pathogenic or prognostic markers and are also promising targets of tumor therapies. EGFR is expressed in several malignant tumor types e.g. non-small cell lung cancer (NSCLC), head & neck squamous cell carcinoma (SCCHN), colorectal carcinoma, glioblastoma, prostate-, ovarian- and breast cancer. For example, HER2 (human epidermal growth factor receptor-2) positive breast cancer can be successfully treated with a monoclonal antibody, produced against the receptor (Herceptin). The antibody inhibits EGF signal transduction and consequently the proliferation of the tumor. Signals mediated by EGFR are also important in the angiogenesis of the tumors, leading to tumor growth and higher metastasis ratio. PDGFR and VEGFR are also involved in tumor development; their inhibitors prevent tumor proliferation and inhibit angiogenesis.

2.1.1.6. II.2.1.2 Insulin signaling

Insulin is a hormone produced by pancreatic beta cells in response to elevated blood glucose level, which regulates carbohydrate and fat metabolism of the body. Insulin induces glucose uptake of liver, muscle and fat tissue cells from the blood and glycogen storage. In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver controlling the activities of a set of metabolic enzymes by phosphorylation and dephosphorylation events and also regulating the expression of genes encoding hepatic enzymes involved in gluconeogenesis. In the absence of insulin or when insulin-response is impaired („insulin resistance”) a serious metabolic disorder, diabetes mellitus develops.

Insulin like growth factor is a 7.6 kDa peptide secreted mainly by the liver stimulated by growth hormon.

Insulin receptor is a trans-membrane protein dimer consisting of 2 alpha and 2 beta chains covalently bound by disulfide bridges.

2.1.1.7. Insulin receptor signaling (PI3K-Akt/PKB pathway)

Ligand-induced tyrosine-phosphorylation of the insulin/IGF receptors leads to the cytoplasmic recruitment of Insulin receptor substrate 1 (IRS-1) through its SH2 domains. IRS-1 transmits signals from the insulin/IGF-1 receptors towards the PI3K/Akt and the ERK/MAPK pathways. IRS-1 is an important mediator of both

metabolic and growth promoting pathways: IRS-1-/- mice have only mild diabetes but pronounced growth retardation (50% of the weight of normal mice). IRS-1 overexpressing transgenic mice develop breast cancer.

PI3-kinases control an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. Many of these functions relate to the ability of class I PI 3-kinases to activate protein kinase B.

Akt/PKB, a serine/threonine protein kinase, is involved in multiple cellular processes for example glucose metabolism, proliferation, apoptosis, transcription and cell migration. Activated Akt phosphorylates glycogen synthase kinase 3 (GSK3). A major substrate of GSK3 is glycogen synthase, an enzyme catalyzing the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore the inactivation of GSK3 by Akt promotes glucose storage as glycogen.

2.2. II.2.2 G-protein-linked receptors (epinephrine,serotonin,glucagon)

2.2.1. II.2.2.1 Epinephrine (adrenaline)

Epinephrine (also known as adrenaline) is a catecholamine hormone produced by the adrenal medulla from phenylalanine or tyrosine. It increases heart rate, constricts blood vessels, dilates air passages and participates in the complex adaptation to danger situations (“fight-or-flight”). Epinephrine comes from epi- and nephros (Greek), whereas the term adrenaline comes from ad- and renes (Latin), both meaning “on the kidney” referring to the anatomic location of the adrenal glands.

2.2.1.1. History

Adrenal extracts containing adrenaline as well as other catecholamines were first isolated by the Polish physiologist N. Cybulski in 1895. J. Takamine K. Uenaka isolated adrenaline in 1901. Adrenaline was first synthesized by F. Stolz and H. D. Dakin, independently, in 1904.

2.2.1.2. Adrenergic receptors

Adrenaline receptors (Figure II.2-6) belong to the G-protein coupled receptors (7-TM); subtypes include α1/2, β1/2/3. α1 receptors are Gq coupled and activate PLC, α2 are Gi coupled, while β receptors are Gs coupled inhinibiting or activating adenylyl-cyclase, respectively. For a more detailed description of the G-protein coupled receptor signaling see Chapters I.2.2 and I.4.1.

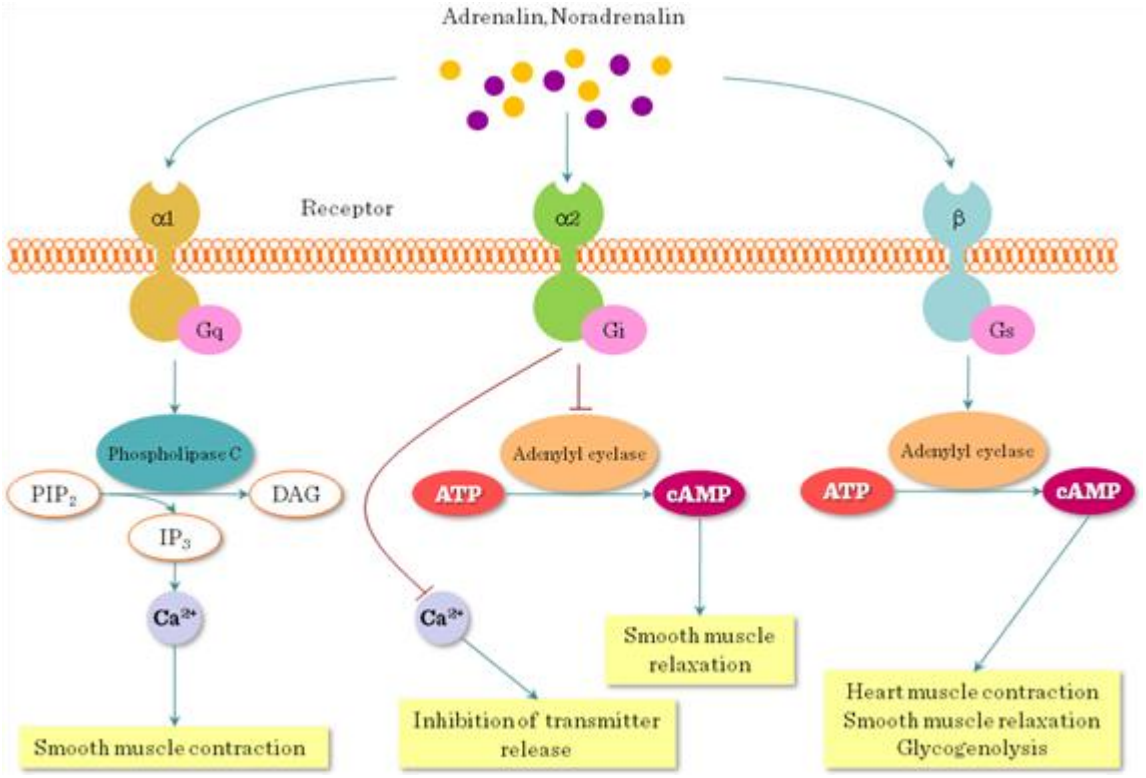


Figure II.2-6: Adrenergic receptors

2.2.2. II.2.2.2 Glucagon

Glucagon is a hormone produced by pancreatic α-cells in the Langerhans islets, which elevates blood glucose level, thus, has an opposing effect to insulin.

The glucagon receptor is a 62 kDa protein belonging to the G protein coupled receptor family (class B). The glucagon receptor associates with Gs, activating adenylyl –cyclase causing cAMP elevation and PKA activation. For a more detailed description of the G-protein coupled receptor signaling see Chapters I.2.2 and I.4.1.

2.2.3. II.2.2.3 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter (not hormone) which is synthesized from tryptophan. 5-HT can be found in a wide variety of tissues: gastrointestinal tract (source: enterochromaffin cells), platelets and the central nervous system. One of its important effects is the induction of positive feelings; hence its other name "happiness hormone". An important consequence is that the modulation of serotonin at synapses could be used to treat depression and other mood disorders. Moreover, 5-HT also participates in the regulation of appetite, sleep, muscle contraction, and some cognitive functions, including memory and learning.

Serotonin receptors, also known as 5-HT receptors, belong either to the G protein-coupled receptors (GPCRs) or the ligand-gated ion channels in the central or peripheral nervous system where they might exert either excitatory or inhibitory neurotransmission.

5-HT 1/5 receptors are Gi coupled, with inhibitory functions; 5-HT 2 is Gq coupled with excitatory function, 5-HT 4/6/7 receptors are Gs coupled with excitatory functions; 5-HT 3 receptor is a ligand-gated Na + /K + channel. For a more detailed description of the G-protein coupled receptor signaling see Chapters I.2 and I.4.1.

2.3. II.2.3 Intracellular/nuclear receptor signaling (steroidhormonesandthyroxin)

Intracellular/nuclear receptors are also considered as ligand-dependent transcription factors. Their structural organization is highly conserved, but their function is very diverse.

2.3.1. History

The first observation was made by the Scottish surgeon G.T. Beatson who found that inoperable breast tumors showed regression after ovaryectomy. Other observations included that castration of animals improves meat; ancient Chinese medicine used placental extracts in different diseases. Kendall and Reichstein described cortisone and thyroxine in 1926. Butenandt and Doisy discovered estrogen in the urine of pregnant women. The discovery of androsteron and progesteron (first isolated from the corpus luteum of pigs) followed. In 1961 Jensen described the estrogen receptor, in the 1980s: cloning of estrogen (ER), glucocorticoid (GR) and thyroxine (TR) receptors were done by Chambon, Evans and Vennström.

2.3.2. II.2.3.1 Intracellular receptor families

(Figure II.2-7)

Table II.2-2: Intracellular receptor families

Family	Receptor	Ligand(s)
Steroid hormone rec.	estrogen rec. (ER) glucocorticoid rec. (GR) mineralocorticoid rec. (MR) androgen rec. (AR) progesterone rec. (PR)	estradiol cortisol aldosterone testosterone progesterone
Thyroid hormone rec.	thyroid hormone rec. (TR)	T3
Retinoid rec.	retinoic acid rec. (RAR) retinoic acid X rec. (RXR)	all-trans-retinoic acid 9-cis-retinoic acid
Vitamin D rec.	vitamin D rec (VDR)	1,25-hydroxy-cholecalciferol
Lipid sensors	liver X rec. (LXR) farnesoid X rec. (FXR)	oxysterols bile acids
PPAR	peroxisome proliferator activated rec.	fatty acids, eicosanoids (e.g. LTs, PGs)

There are 48 known receptors in human, but 270 (!) in C. elegans; note: several orphan receptors.

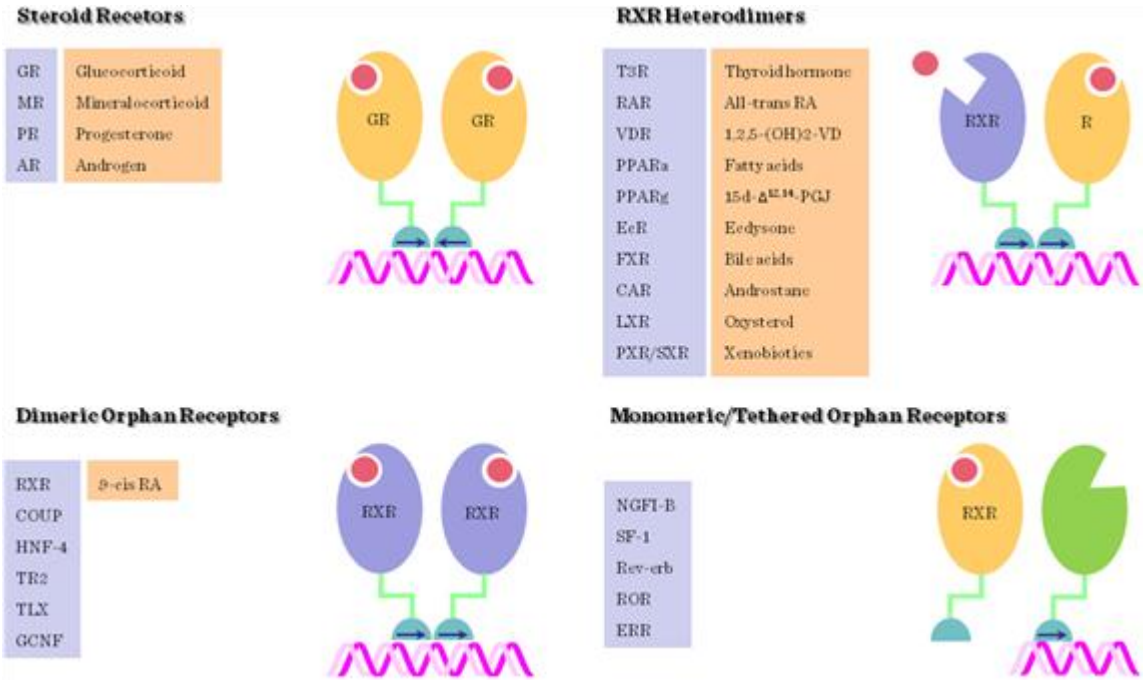


Figure II.2-7: Nuclear receptor superfamily

2.3.2.1. Structure of nuclear receptors

The receptors are made-up from 6 domains (Figure II.2-8). The N-terminal region (A/B domains) of the molecule is variable (50-500 AA); the central (C domain) DNA binding domain (DBD) is highly conserved (70 AA) double zinc finger. The moderately conserved (200-250 AA) ligand-binding domain (LBD; domain E) is situated between the hinge domain (D) and the C-terminal (F) domain of variable length. Activation function (AF)-1/2 sequences are found in the N-/C-terminal domains, with ligand-dependent or –independent regulatory functions, respectively. Many members of the nuclear receptor family form homo- or heterodimers, the DNA and the ligand binding domains are important in these processes.

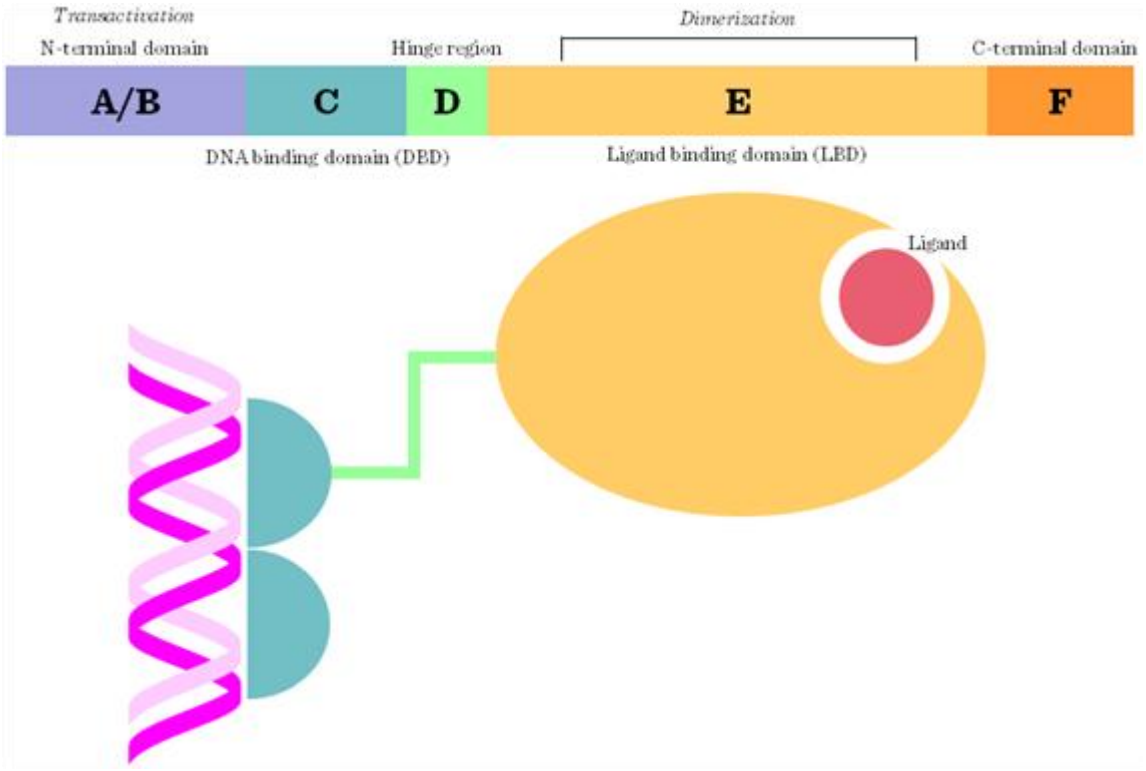


Figure II.2-8: Functional domains of transcription factors

2.3.2.2. Nuclear receptor mediated signaling

The inactive (unliganded) Class I receptors (e.g. GR) form a cytoplasmic receptor complex with heat shock proteins (Hsp90, 70, 40), co-chaperone p23 and immunophilins (e.g. FKBP52 which links the complex to dynein). In the absence of ligand there is dynamic assembly-disassembly of this complex. Upon ligand binding the receptor dissociates from the complex and transported to the nuclear pores along microtubules (Figure II.2-9).

Class II receptors (e.g. RXR, TR), on the other hand, localize in the nucleus, already in unliganded state.

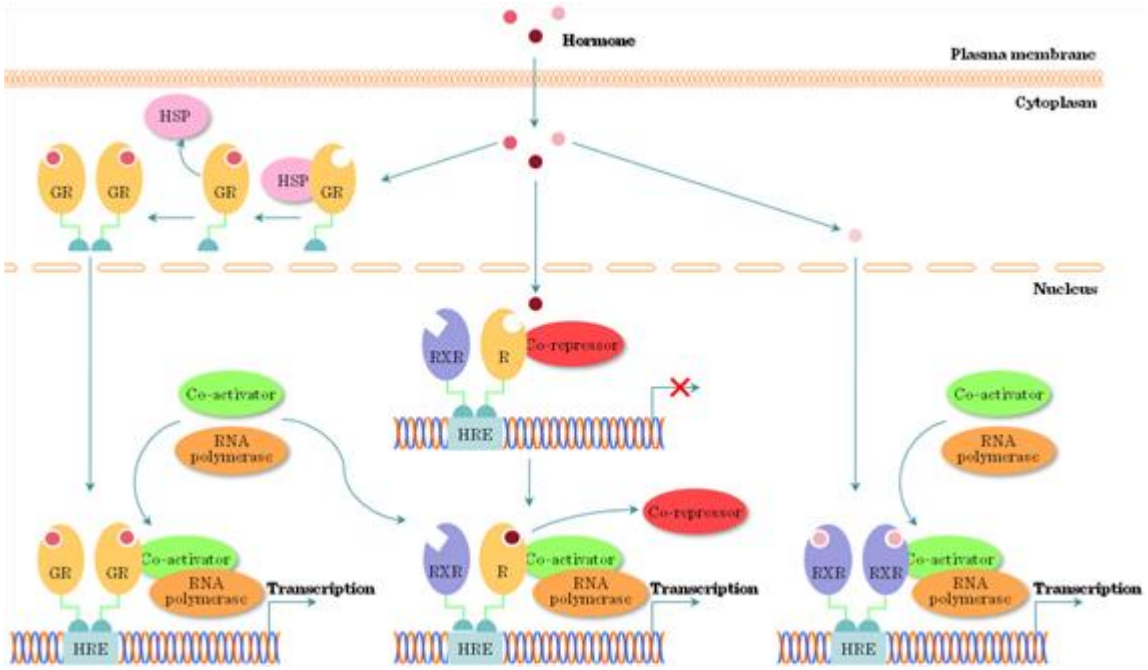


Figure II.2-9: Mechanism of steroid receptor action

2.3.2.3. DNA binding

DNA binding sites of intracellular receptors are called response elements (RE) usually comprise 2x6 base pair sequences. Members of the steroid receptor family form homodimers and bind to palindromic, inverted repeats separated by 3bp spacer (IR3)

(e.g. GR, MR, PR, AR: 5'-AGAACA-3'; ER: 5'-AGGTCA-3'). Non-steroid receptors bind to direct repeats of the sequence 5'-AGGTCA-3' (DRn, n=number of spacers), and can both form homodimers (e.g. TR, VDR) or heterodimers (e.g. TR, VDR, RAR, LXR, FXR, PXR, CAR, PPAR).

2.3.2.4. Regulation of transcription

Activated intracellular receptors can act as trans-activators (Figure II.2-10):

(1) The ligand-bound receptor recruits co-activators up-regulating transcription of the target genes through the interaction with the general transcription factors. Importantly, chromatin has to be "opened up" (ATP-dependent chromatin remodeling / histone acetylation) for the transcription initiation.

(2) Ligand binding can also lead to co-repressor dissociation, enabling co-activators to bind to the transcription initiation complex.

In case of trans-repression without ligand transcription proceeds constitutively, and ligand binding inhibits transcription. For more details on transcription factors see Chapter I.4.4.

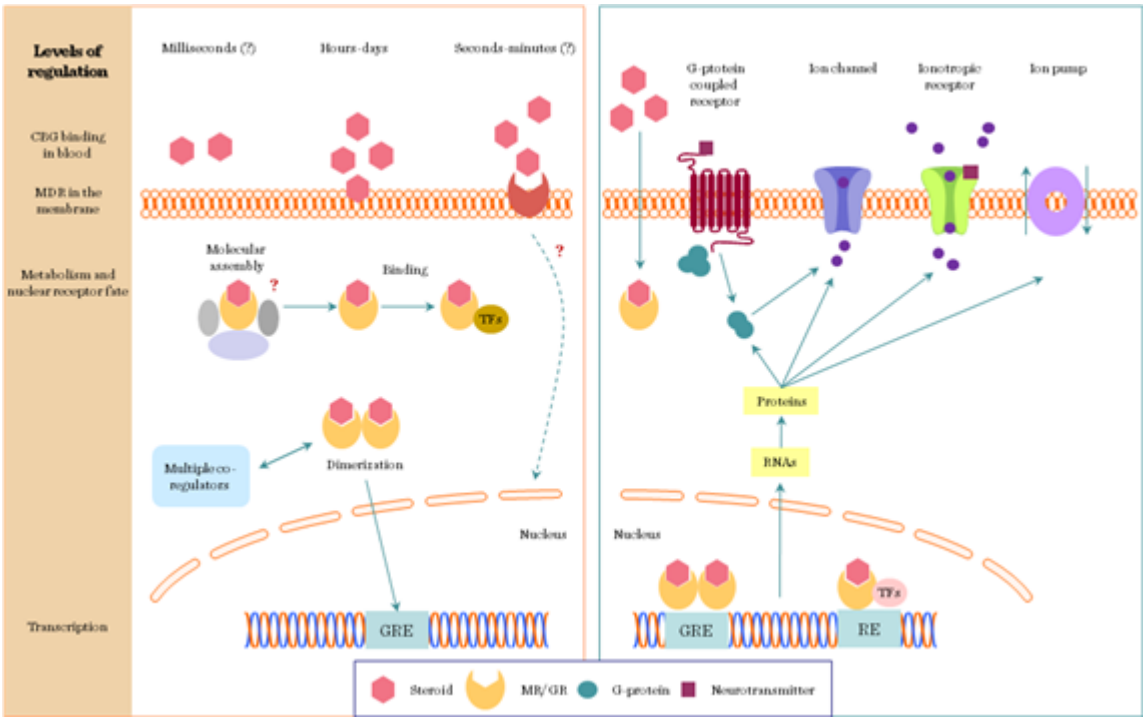


Figure II.2-10: Genomic steroid actions

2.3.2.5. Regulation of nuclear receptors

Transcriptional activity of intracellular receptors can be up-regulated by phosphorylation of Ser residues in the N-terminal A/B domains by cyclin-dependent kinases, PKC, PKA, ERK, PKB/Akt, JNK/SAPK, p38-MAPK. AF-1 can be phosphorylated by CDK, ERK, JNK, p38-MAPK, PKB, while AF-2 by Src in case of ER. Down-regulation of transcriptional activity can be caused by phosphorylation of the DBD by PKC or PKA.

2.3.2.6. Therapeutic implications – hormone analogues

Several hormone analogues are used for the treatment of a wide variety of diseases. Synthetic glucocorticoid analogues are used as anti-inflammatory, immunosuppressive drugs (e.g. autoimmune diseases, transplantation, some leukemias). Sex steroids are used as substitution therapy (endocrine diseases), birth control and breast cancer. Thyroxin can be used as substitution therapy after thyroidectomy, while Vitamin A/D to treat vitamin deficiency.

2.4. II.2.4 Non-genomic steroid hormone signaling pathways

2.4.1. Introduction

The above described intracellular receptor signaling pathway is considered as “classical” or genomic (see Chapter II.2.3), since it acts via the regulation of gene-transcription (Figure II.2-11). Relatively long time (hours) is needed from the translocation of the active hormone receptor into the nucleus and then the transcription and translation, so the net effect appears only slowly.

However, some steroid effects can already be detected within minutes e.g. ion-currents change, membrane changes, phosphorylation changes (Figure II.2-11). Importantly, glucocorticoid analogues are widely used for the treatment of acute conditions: asthma, allergies or shock where high dose steroids exert rapid effects. Accumulating evidence proves that the apoptosis-inducing capacity of glucocorticoid hormone within the thymus might be, at least partially, also independent from genomic effects. The rapid nature of these effects excludes the possibility that the classical, genomic pathway could mediate them. Hence, these steroid responses, appearing within minutes after hormone exposure, are mediated by “non-genomic” or “alternative” signaling pathways (Figure II.2-11). Most of our knowledge about non-genomic steroid effects was drown from research on glucocorticoids and estrogen.

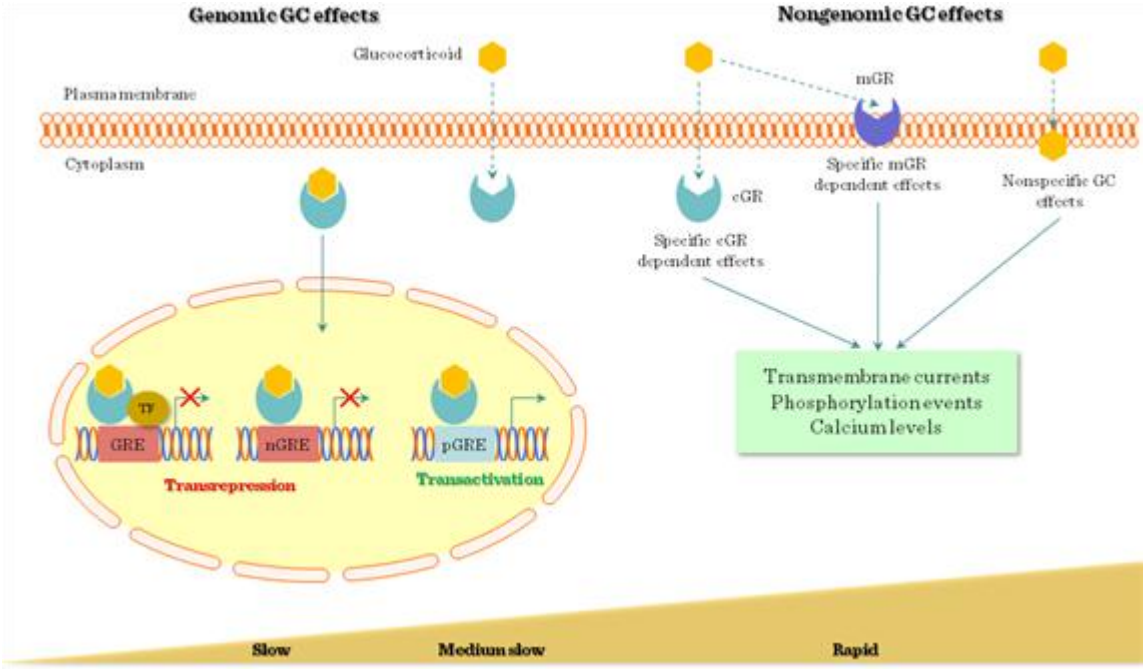


Figure II.2-11: Genomic and non-genomic GC effects

2.4.2. Non-genomic glucocorticoid receptor (GR) signaling pathways (Figure II.2-12)

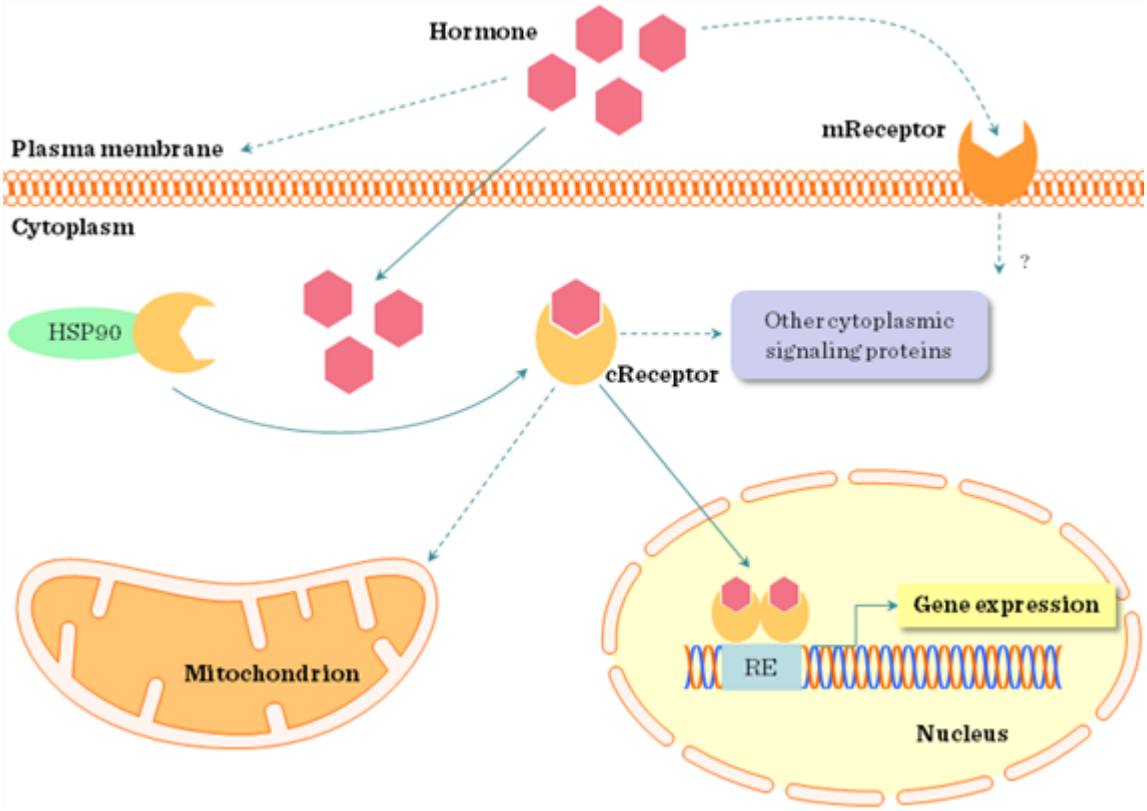


Figure II.2-12: Summary of genomic and non-genomic glucocorticoid effects

(1) Direct membrane effects

Glucocorticoids (GCs), especially at high doses, could change the physico-chemical properties of the plasma membrane due to their lipid soluble nature. Such effects were observed on human red blood cells. In a

mammary cancer cell line, high-dose steroid treatment influenced the membrane lipid mobility, and also increased membrane lipid mobility in LPS treated B lymphocytes. Inhibition of Na+ and Ca2+ transport through the plasma membrane and increased H+ uptake into the mitochondria was also described. In canine kidney epithelial cell system dexamethasone (a synthetic glucocorticoid analogue) had a direct effect on tight junction formation. 20 minutes of cortisol treatment caused changes in the excitability of principal basolateral amygdala neurons.

(2) Membrane GR

Membrane bound GR (mGR) was identified in rodent and human lymphoid cell lines and amphibian brain. Moreover, there was a correlation between the mGR expression and the cell cycle-dependent GC-induced apoptosis sensitivity of a human leukaemia cell line, so, the presence of the mGR correlates with GC-resistance of a cell type. mGR was also found on human blood monocytes and B cells; importantly, mGR+ monocyte frequency increased in rheumatoid arthritis, SLE and ankylosing spondylitis patients indicating that the mGR expression might have had pathogenetic consequences. However, intracellular signalling pathways activated by the mGR are still unknown.

(3) Interaction between the GR and other cytoplasmic signaling proteins

As discussed in Chapter II.2.3, the unliganded GR forms a multimolecular complex in the cytoplasm. Recent studies in human T cells showed that, besides the chaperon molecules (heat shock proteins and immunophilins), the GR associates with cytoplasmic signaling proteins, too. For example, the ligand bound glucocorticoid receptor associates or increases its association with many signaling molecules of the T cell receptor-signaling pathway (e.g. Lck, Fyn or ZAP-70). Moreover, this association can induce phosphorylation changes in Lck, Fyn or ZAP-70, for example. This cross-talk between the GR and the TcR signaling pathway could account for the immunosuppressive action of some glucocorticoid analogues.

(4) Mitochondrial GR

Upon ligand binding the glucocorticoid receptor can directly translocate to the mitochondria in both lymphoid and non-lymphoid cells where it can initiate the apoptotic cascade. The ligand-induced mitochondrial GR translocation showed a close correlation with the GC-induced apoptosis sensitivity of several cell types. In case of CD4+CD8+ (DP) thymocytes the GR translocates to the mitochondria rather than to the nucleus upon short-term in vitro GC treatment correlating with their high GC-induced apoptosis sensitivity. In the mitochondria, the GR might act through diverse mechanisms:

a) Acts as mitochondrial transcription factor.

b) Interaction with other mitochondrial transcription factors.

c) Interaction with pro- and anti-apoptotic proteins (e.g. Bcl-2 family proteins).

d) Decreases the mitochondrial membrane potential.

2.4.3. Non-genomic effects of other steroid hormones

Estrogens have been shown to induce multiple changes in intracellular signaling cascades. Membrane estrogen receptor (mER) was also identified and structural data support that it is a G-protein coupled receptor. Mitochondrial translocation of the ER has also been described.

Progesterone might influence cell membrane permeability and stimulate progesterone membrane component 1 or its complexes. Progesterone receptor localized near the plasma membrane induces phosphorylation and intracellular calcium level changes. Membrane bound progesterone receptor has also been identified.

Androgens can activate the MAPK cascade through non-receptor tyrosine kinase c-Src, and might act through PKA as well. The membrane bound form of testosterone receptor is thought to take part in non-genomic androgen actions.

Non-genomic aldosterone induces phosphorylation and calcium level changes, and influence the Na+-K+-2Cl- -transporter. The membrane aldosterone receptor is also thought to be a G-protein coupled receptor.

Thyroid hormones and Vitamin-D can both induce phosphorylation of signaling molecules and elicit intracellular calcium signal.

3. II.3 Signaling in tumor cells (EGF-R,Her-2R,adhesionmolecules)

3.1. Introduction

In normal cells, proliferation/cell division is a tightly controlled process. As discussed earlier, pathways initiated by growth factor receptors are only active for a limited time and are down-regulated/stopped promptly by several mechanisms (for details see Chapter II.2.1.1; Growth-factor signaling). Continuous activation of these pathways leads to uncontrolled cell proliferation: tumor diseases. In case of malignant tumors, further genetic modifications appear through a course of mutations: cells loose their polarity and adhesion to their original extracellular matrix, they express new adhesion molecules, break through the basal membrane of the original tissue, and become invasive; thus, metastatic tumor cells reach far parts of the body through the blood stream or lymph vessels.

Normally, continuously appearing tumor cells are controlled by immune surveillance mechanisms: for example NK cells and cytotoxic lymphocytes or macrophages. When these defense mechanisms decline or the tumor cells evade them, the transformed cells might “escape” from the immune system and cause a systemic disease (Figure II.3-1 and Figure II.3-2). Tumor escape mechanisms include modified MHC-I expression, expression of pro-apoptotic molecules or inhibitory co-stimulatory molecules by the tumor cells. Tumor cells might produce thick extracellular matrix, which prevents them physically from being reached by immune cells. TGFβ has a central role in tumor growth: it induces the surrounding cells to produce proteases; it has angiogenic properties, and suppresses immune cells (Figure II.3-3). Moreover, tumor cells might become apoptosis resistant, for example by the loss of Fas sensitivity (Figure II.3-4).

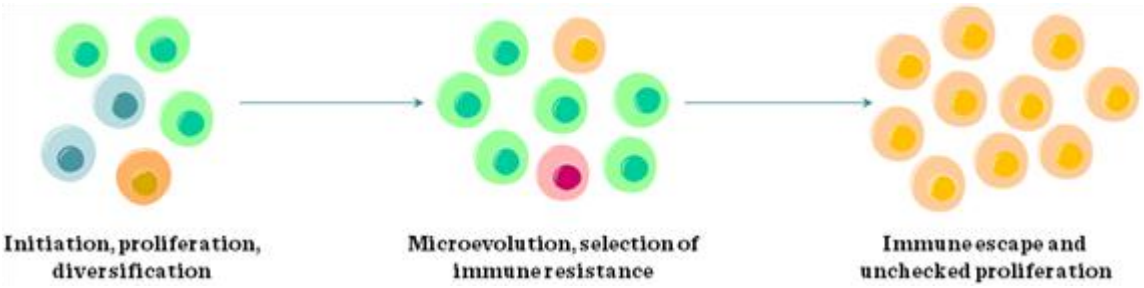


Figure II.3-1: Immune selection in the development of cancer: no two tumors are alike

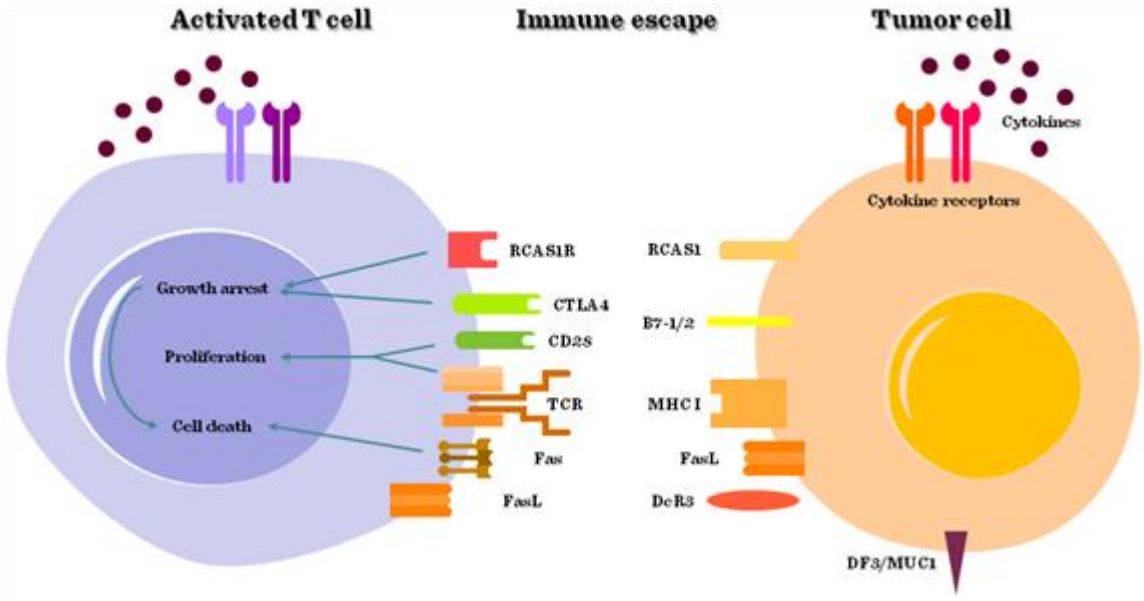


Figure II.3-2: Tumor and activated T cells

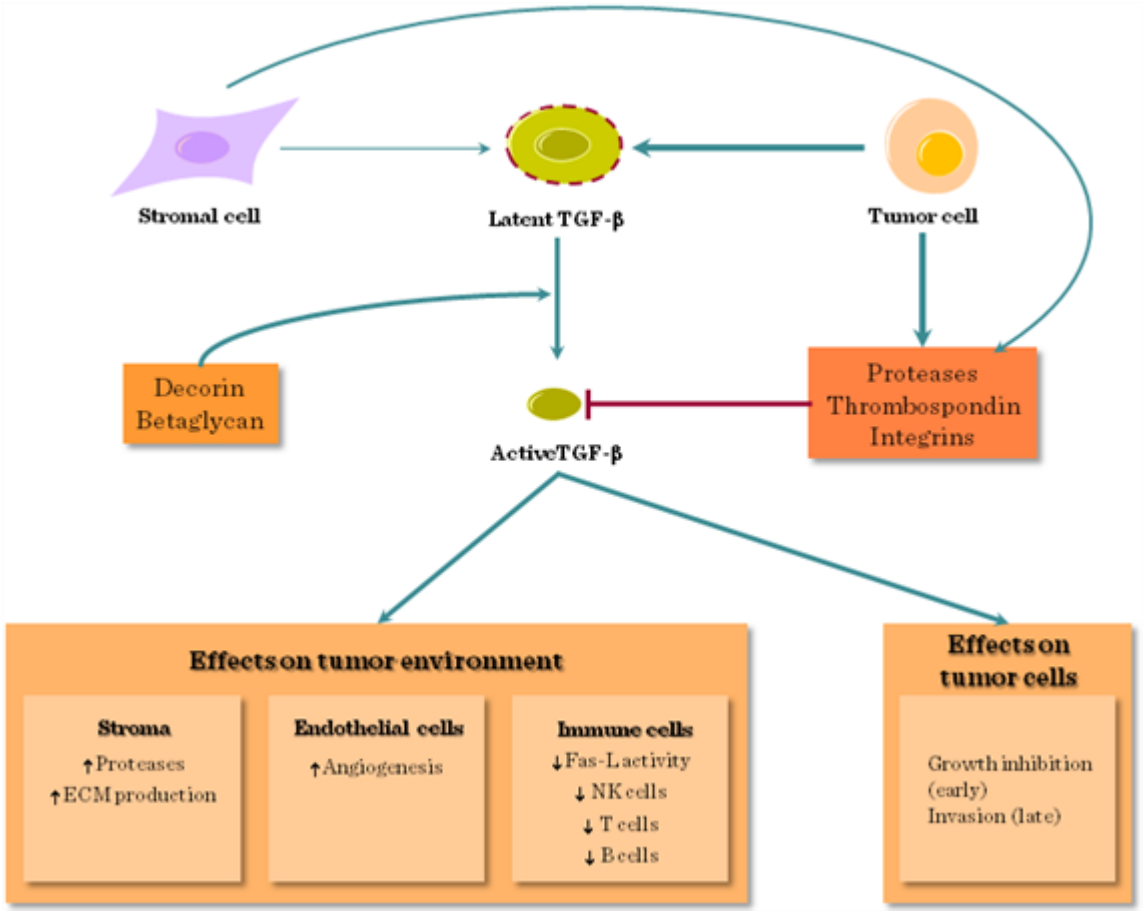


Figure II.3-3: TGF-b signaling in tumor signaling and cancer progression

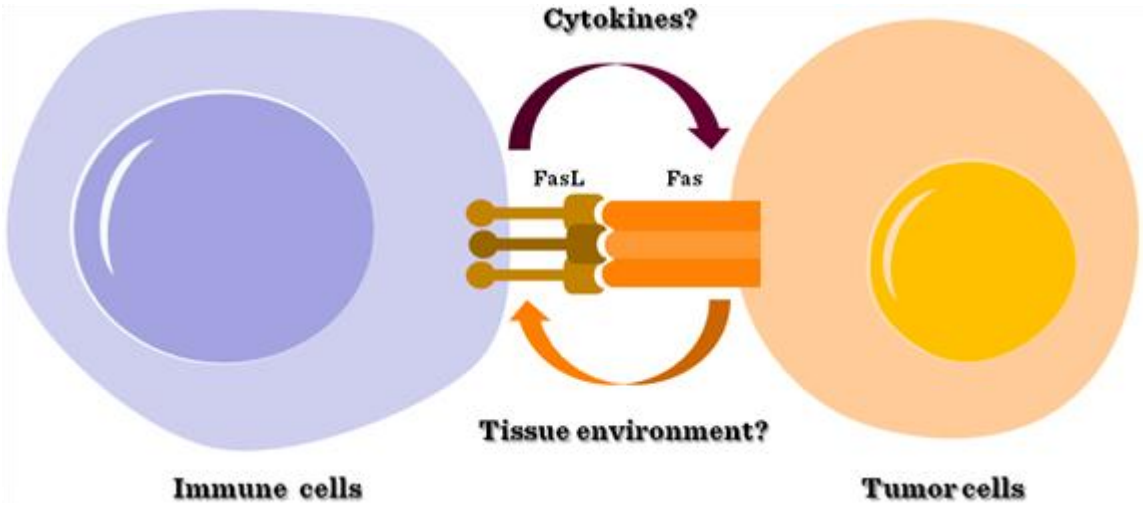


Figure II.3-4: What happens when Fas-stimulated immune cells resist to die?

3.2. EGFR, HER-2

The role of EGFR in malignant tumors first was shown in metastatic breast cancer. This disease, which otherwise has very poor prognosis, can be very effectively treated with a monoclonal antibody against the EGFR (HER-2). Trastuzumab (Herceptin) binds to the receptor inhibiting the binding of the natural ligand (EGF) to it, and also prevents receptor dimerization (Figure II.8-5). This blocks the signaling pathways driving the

continuous proliferation (MAPK pathway, PI3K/Akt). However, patients should be screened first, whether their tumor overexpresses HER-2.

Amplification of HER-2 gene has been shown in gastric, ovarian and endometric cancers. HER-2 mutations were found in lung adenocarcinomas, head-neck tumors, colorectal carcinomas and melanoma.

Ras controls 75% of the EGFR pathways (for more details see chapter II.2.1.1); therefore mutation in this key molecule might lead to a shift towards alternative pathways, for example the PI3K, Akt or PKC activation. Importantly, such Ras mutations might lead to a therapy resistant tumor cell phenotype.

3.3. Kidney cancer

Von-Hippel Lindau (VHL) tumor suppressor gene mutations occur frequently in kidney cancers. The VHL protein is involved in ubiquitination processes; a particularly important target protein is hypoxia inducible factor 1 (HIF-1). Loss of function mutation of VHL leads to increased HIF levels, causing decreased apoptosis and the production of angiogenic factors (e.g. VEGF), both of which contribute to the tumor growth.

3.4. Integrin signaling

Physiologically, integrins anchor cells to extracellular (ec.) matrix molecules and transmit signals from important ec. matrix components like collagen or fibronectin. Integrin signaling proceeds through integrin-linked kinase (ILK), focal-adhesion kinase (FAK) and Src kinase and regulates cell survival, apoptosis, differentiation and proliferation. Integrin signaling modulates growth factor receptor signaling through NCK and PINCH.

4. II.4 Apoptosis signaling

4.1. Introduction

Apoptosis (programmed cell death) occurs in multicellular organisms leading to characteristic changes in cell morphology (blebbing, cell membrane changes, cell shrinkage, nuclear DNA condensation, fragmentation etc), and death of the cell. The resulting membrane bound cell fragments called “apoptotic bodies” are recognized, engulfed and quickly removed by phagocytes before the contents of the cell can spill out, so preventing tissue damage and inflammation.

4.2. Initiation of the cascade

The process of apoptosis is controlled by a diverse range of cell signals. To initiate the apoptotic enzyme cascade (caspases) several proteins are involved, but two main methods of regulation have been identified: targeting mitochondria functionality, or directly transducing the signal via adaptor proteins to the apoptotic mechanisms.

4.3. Extrinsic apoptosis pathway

Extracellular/extrinsic inducers: toxins, hormones, growth factors, nitric oxide or cytokines (Figure II.4-1).

The engagement of TNF-receptor family members (TNF-R1, Fas receptor – FasL) induce direct signal transduction and initiation of apoptosis via the intermediate membrane proteins, TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). The death-inducing signaling complex (DISC), then forms, which contains the FADD, caspase-8 and caspase-10 (Figure II.1-20 and Figure II.1-21).

4.4. Intrinsic apoptosis pathway

Intracellular/intrinsic inducers: stress, glucocorticoid hormone, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration, for example, by damage to the membrane, can all trigger the release of intracellular apoptotic signals by a damaged cell (Figure II.4-1).

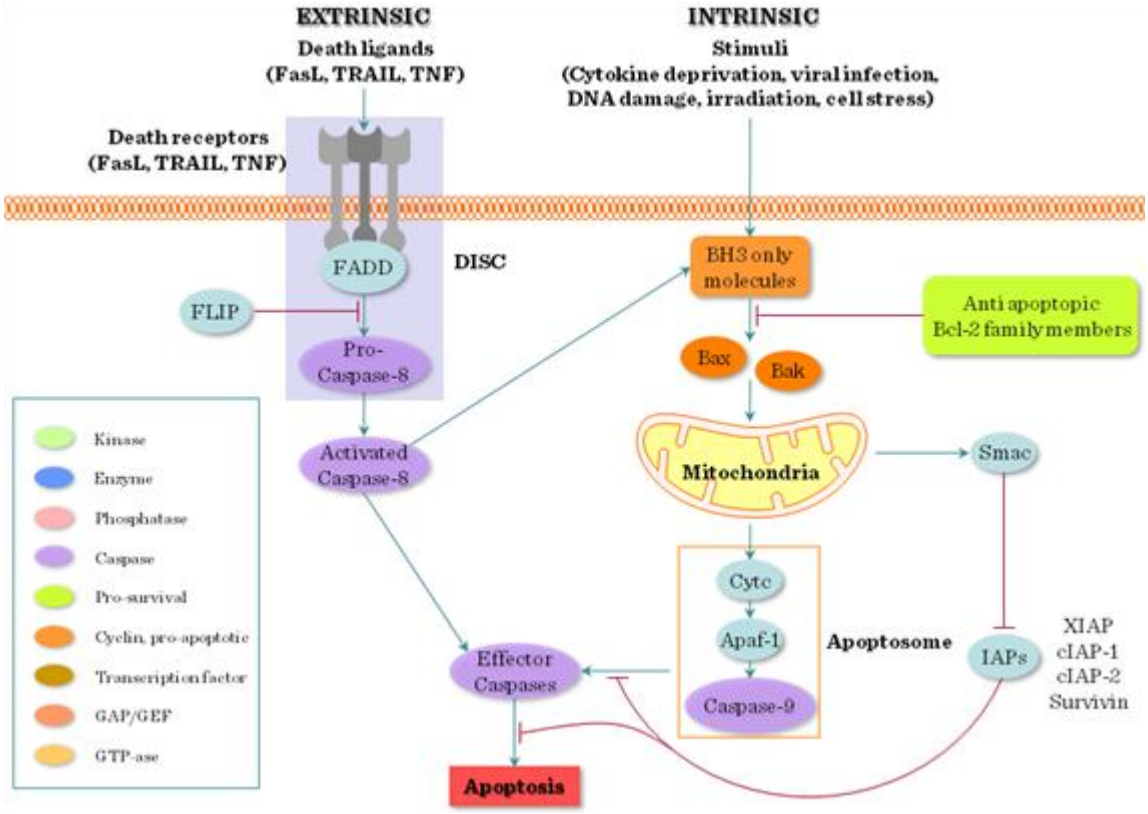


Figure II.4-1: Apoptosis pathways

4.5. The mitochondrial pathway:

Apoptotic proteins may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out (Figure II.4-2). Mitochondrial Outer Membrane Permeabilization Pore (MAC) is regulated by various proteins of the Bcl-2 family (Figure II.4-3), which are able to promote or inhibit apoptosis by direct action on MAC/MOMPP. Bax and/or Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibit pore formation. Second, mitochondria-derived activators of caspases (SMACs) are released into the cytosol following an increase in permeability. SMAC binds to inhibitor of apoptosis proteins (IAPs) and deactivates them. Cytochrome c is also released from the mitochondria, which binds to Apoptotic protease activating factor-1 (APAF-1) and to pro-caspase-9 to create a protein complex called apoptosome (Figure II.4-4). When caspase-9 is activated, it will in turn activate the effector caspase-3.

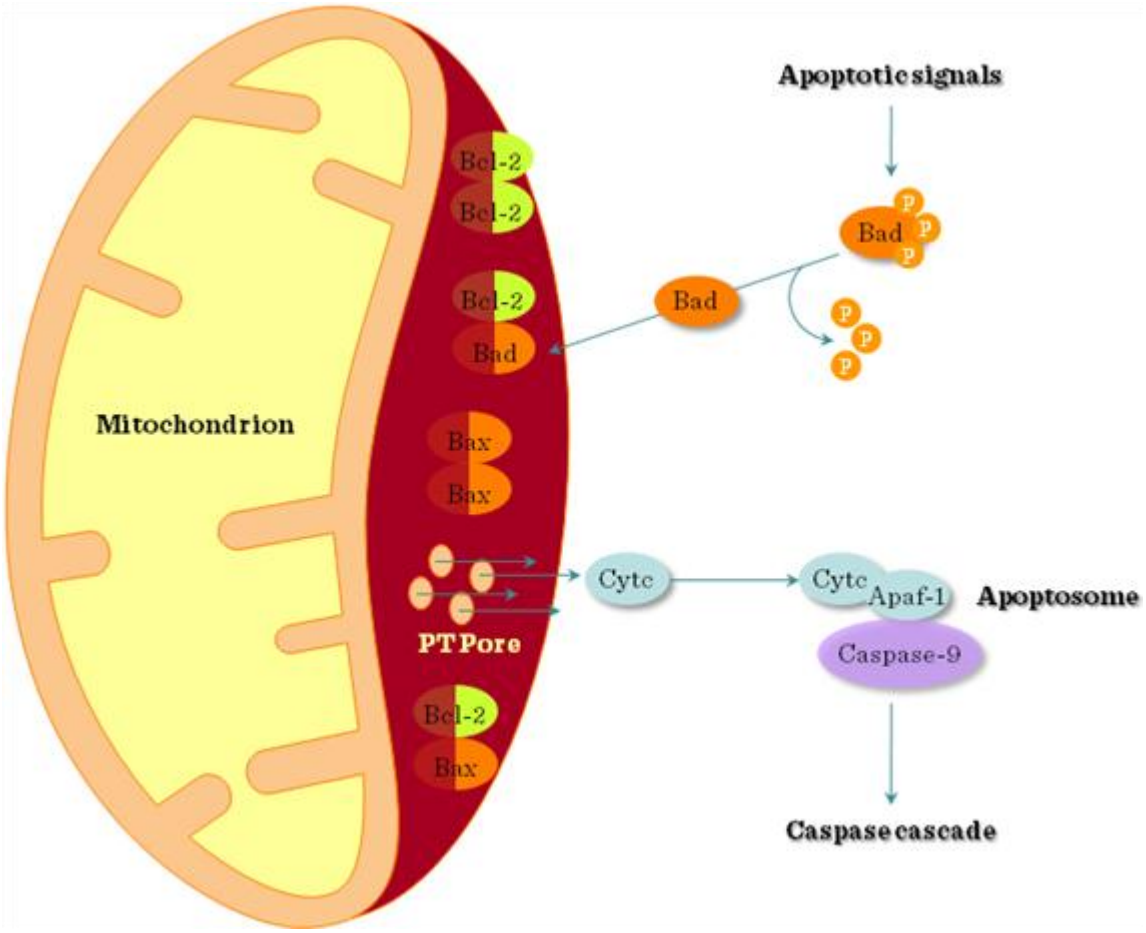


Figure II.4-2: Mitochondrial apoptosis pathway

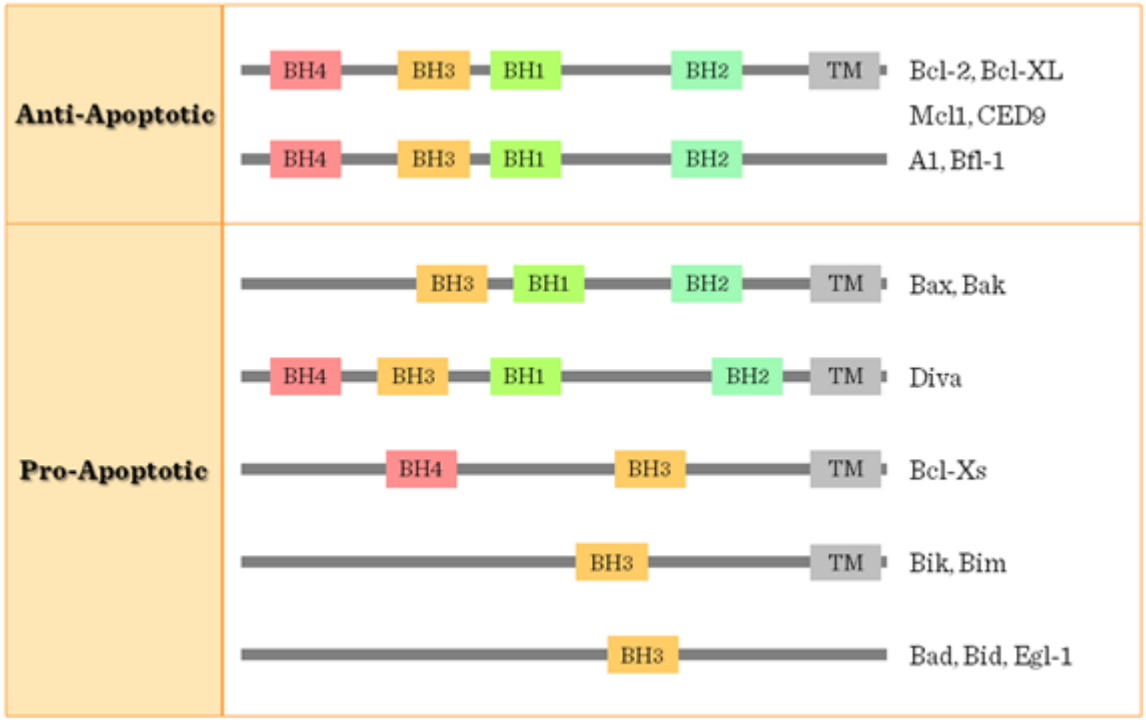


Figure II.4-3: Bcl-family

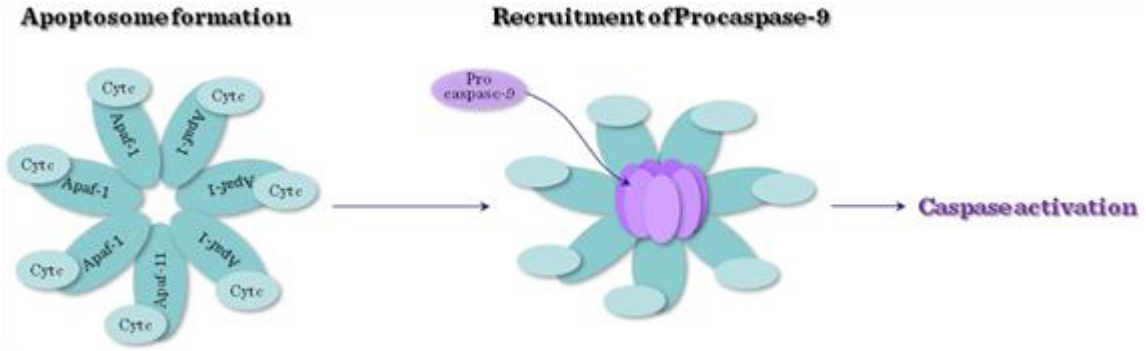


Figure II.4-4: Apoptosome

4.6. Caspase cascade

Caspases are a family of cysteine-dependent aspartate-directed proteases (cysteine proteases) which can be rapidly activated. Among the 12 known human caspases the initiator caspases (e.g., Caspase-2,-8,-9, and -10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g., Caspase-3, -6,-7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process.

They are first synthesized as inactive pro-caspases, that consist of a prodomain, which contain either a CARD domain (e.g., caspases-2 and -9) or a death effector domain (DED) (caspases-8 and -10) that enables the caspases to interact with other molecules that regulate their activation.

The caspase cascade can be activated by:

(1) Granzyme B: (released by Tc and NK cells) known to activate caspase-3 and -7

(2) Death receptors: (Fas, TRAIL receptors and TNF receptor), which can activate caspase-8 and -10

(3) Apoptosome (regulated by cytochrome c and the Bcl-2 family), which activates caspase-9.

Some of the final targets of caspases include:

(1) nuclear lamins

(2) ICAD/DFF45 (inhibitor of caspase activated DNase or DNA fragmentation factor 45)

(3) PARP (poly-ADP ribose polymerase)

(4) PAK2 (P 21-activated kinase 2).

Apoptosis cascades might be influenced at several points with some approved or experimental drugs (Figure II.4-5).

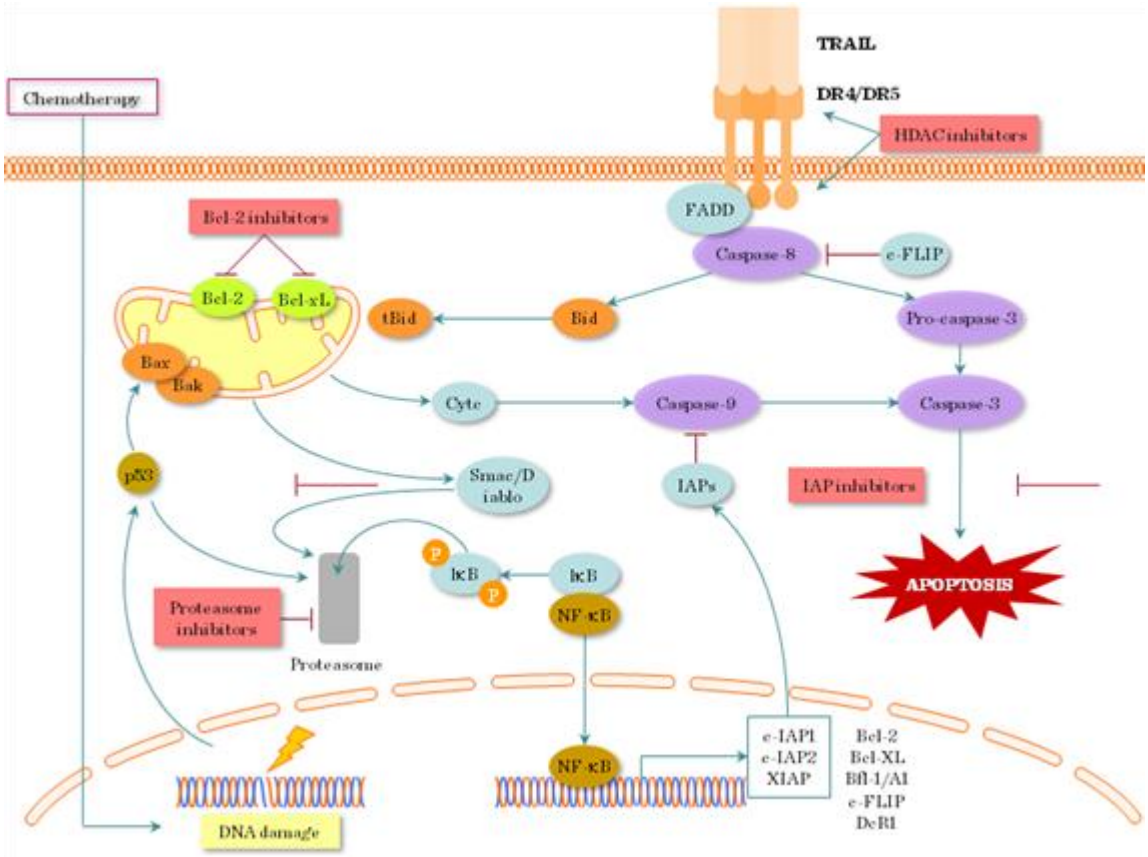


Figure II.4-5: Apoptosis signaling intervention

5. II.5 Receptor interactions, signaling “cross-talk”

5.1. Introduction

As discussed in the previous chapters, during evolution distinct signaling pathways have developed. Although there is a need for the precise separation of certain pathways to maintain the specificity of signals, upon complex physiological stimuli more pathways might be activated parallel, creating a basis for interaction between them. Importantly, signaling pathways form a functional network; some proteins can participate in multiple pathways modifying each other’s function (synergism/antagonism). Such networks can be based on direct protein interactions for example in case of large signaling complexes, organized by scaffold proteins and the cytoskeleton. Functional interactions include post-translational modifications like phosphorylation/dephosphorylation. From the practical point-of-view, even the most pathway-selective drugs (for more details on Intervention with signaling pathways see the next chapter) could have side effects due to signal “cross-talk”.

5.2. “Levels” of signal “cross-talk”

(1) Interactions between receptors

Cell surface receptors form multimeric complexes influencing each other’s function. The crucial role of receptor-receptor interactions in signal-integration is the filtration of incoming signals as well as integration of coincident signals. The basic molecular model for the known intramembrane receptor/receptor interactions among G protein-coupled receptors (GPCRs) was suggested to be heterodimerization, based on receptor-specific interactions between different types of receptor monomers.

After GABABheterodimer receptors were discovered, the discovery of heterodimerization of several 7-TM/GPCRs such as δ/κ opioid receptors followed increasing the impact of this field rapidly. Distinct types of GPCRs like somatostatin SSTR5/dopamine D2and adenosine A1/dopamine D1heterodimerizations were other milestones on the field of receptor interactions. These heterodimeric complexes are either established via direct

intramembrane receptor/receptor interactions, or sometimes indirectly via adapter proteins. GPCRs might also associate with ion channel receptors, for example in GABAA/dopamine D5receptor heterodimerization. Such receptor mosaic complexes in the central nervous system might have special integrative functions responsible for the molecular basis of learning or memory. Growth factor receptors (receptor tyrosine kinases) often interact with integrins specialized for the binding of extracellular matrix proteins (Figure II.5-1). Cross talk between innate immune receptors complement receptor 3 and Toll-like receptors have been also described.

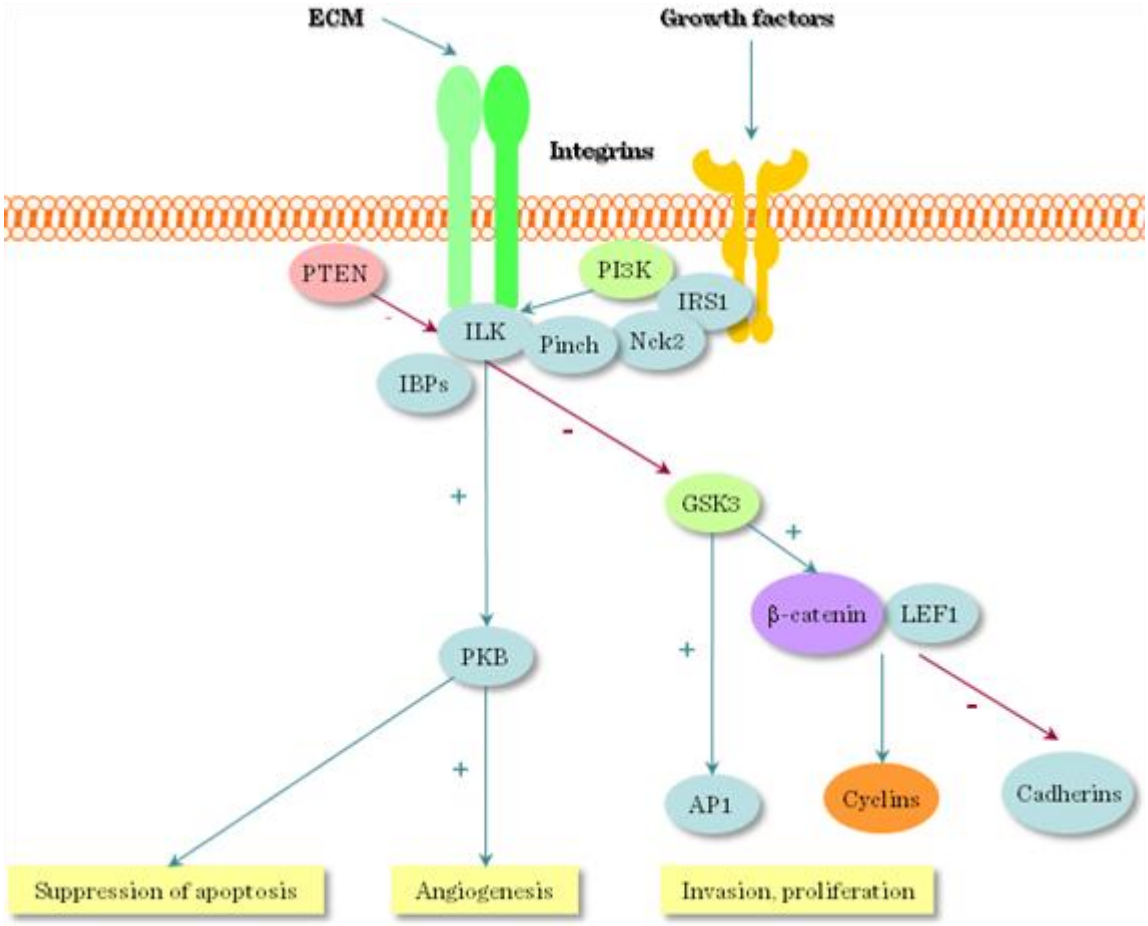


Figure II.5-1: Growth factor receptor – integrin signaling interaction

(2) Plasma membrane proximal signaling complexes

A common theme in some signaling pathways is the build-up of plasma membrane proximal signaling complexes upon receptor engagement. Often these complexes are hold together by adapter and scaffolding proteins. Examples include the initial steps of BcR/TcR signaling and growth factor receptor signaling.

(3) Cytoplasmic signaling complexes, pathway branching / merging

Scaffold proteins, chaperones and the cytoskeleton organize cytoplasmic signaling complexes. Branching and merging of certain pathways are common at this level (Figure II.5-2). For example Ras serves as an important junction in the growth factor receptor mediated signaling network. The glucocorticoid receptor (GR) and ZAP-70 also form cytoplasmic complexes in T cells (for more details see Non-genomic glucocorticoid signaling in Chapter II.2.4). Kinase-anchoring proteins (AKAP) integrate 3 pathways: Ras-MAPK, cAMP-PKA and Ca2+-signaling.

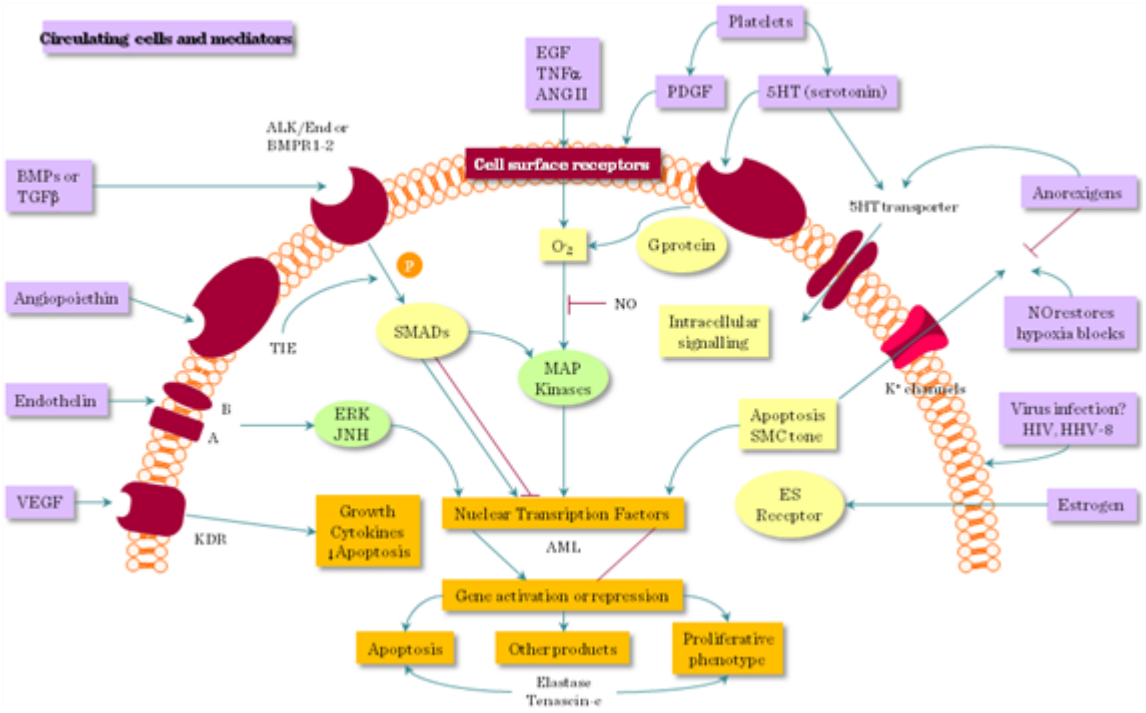


Figure II.5-2: Convergence of signaling pathways

(4) Transcription factors

Finally, as we have already discussed in Chapter I.4.4, transcription factors interact to control the activity of the transcription machinery. For example, the GR can interact with AP-1, CREB, NFκB, T-bet, STATs and PU.1. TNFR and GR signaling also merge at the level of transcriptional regulation.

6. II.6 Wnt receptor signaling

6.1. Overview

Wnt (originates from “wingless” mutation described in Drosophila) signaling plays key roles throughout the whole lifespan of an organism from embryonic development through different types of cancers to various processes of aging. Wnt signals control a wide range of developmental events and cellular functions in many organs, implying the need for tight regulation of the highly complex Wnt-related intracellular signaling events. The following table summarizes several phenotypes of Wnt mutations in mouse, Drosophila, and C. elegans.

Table II.6-1: Wnt signaling

Gene	Organism	Phenotype
Wnt-1	Mouse	Loss of midbrain and cerebellum
Wnt-2	Mouse	Placental defects
Wnt-3A	Mouse	Lack of caudal somites and tailbud
Wnt-4	Mouse	Kidney defects
Wnt-7A	Mouse	Ventralization of limbs
wingless	Drosophila	Segment polarity, limb development, many others
Dwnt-2	Drosophila	Muscle defects, testis development
lin-44	C. elegans	Defects in asymmetric cell divisions
mom-2	C. elegans	Defects in endoderm induction and spindle orientation

(After: A. Wodarz and R. Nusse; Annu. Rev. Cell Dev. Biol. 1998. 14:59–88)

The role of Wnt signaling has been implicated in the control of cell adhesion as well as the pathogenesis of Alzheimer's disease (Figure II.6-1 – Figure II.6-3). Wnt signaling interacts with a range of other signaling cascades, for example Bmp/Noggin, Notch/Delta, fibroblast growth factors (FGF), epidermal growth factors (EGF) and Hedgehog, thereby participates in the regulation of complex cellular processes.

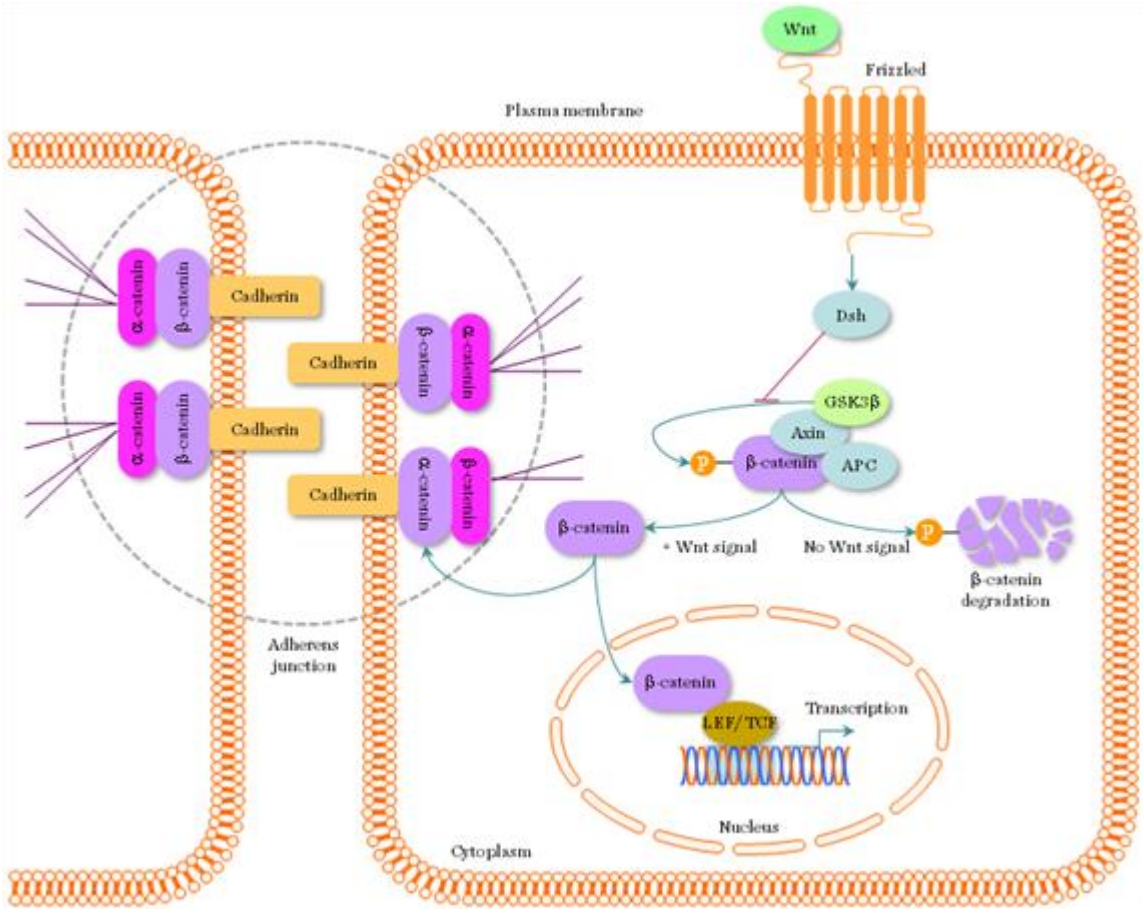


Figure II.6-1: b-catenin in cellular adhesion

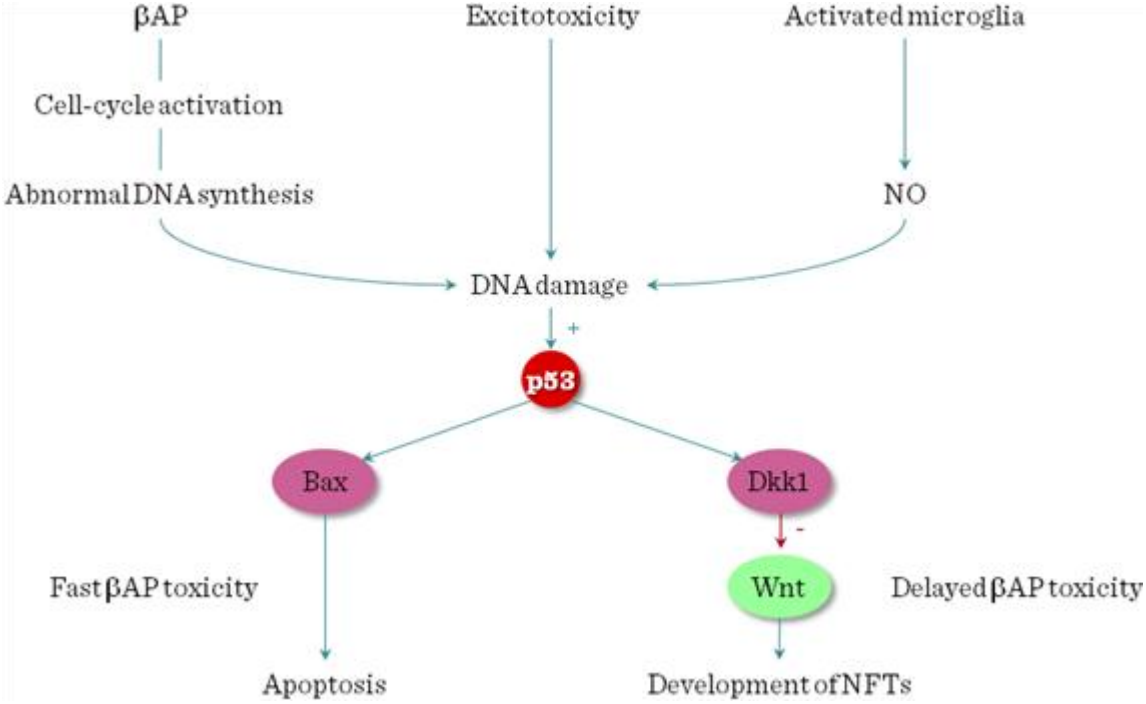


Figure II.6-2: Alzheimer's disease I

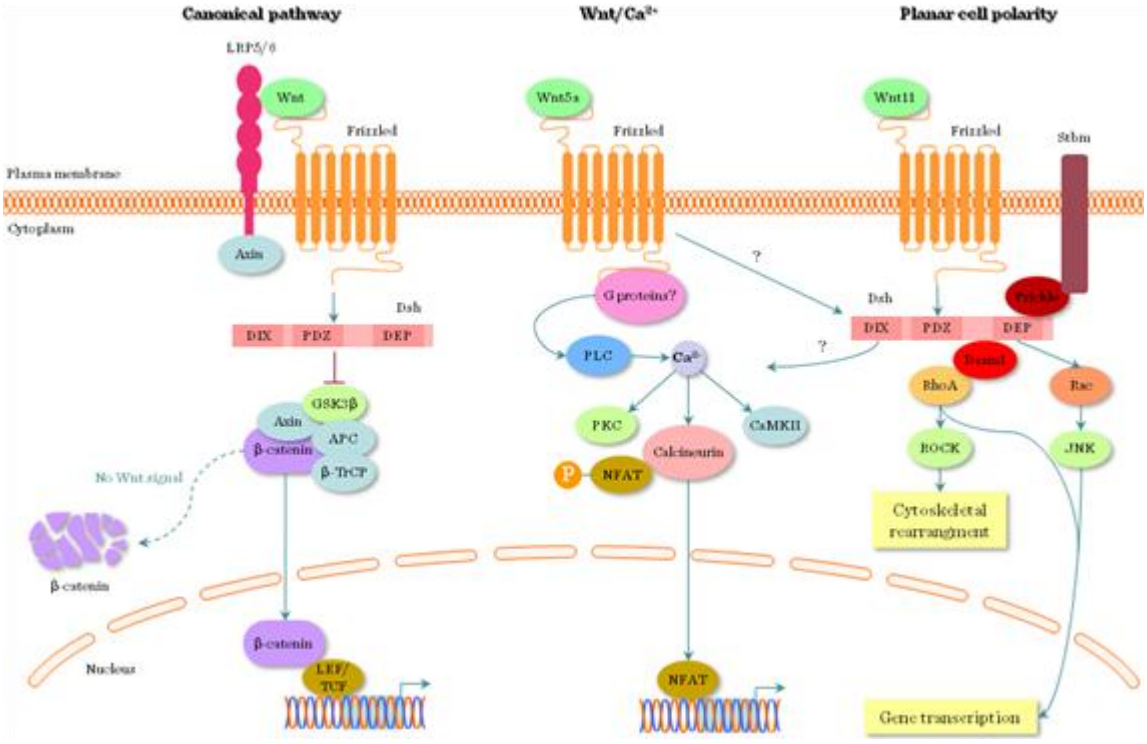


Figure II.6-3: Wnt signaling pathways

The Wnt family comprises of 19 secreted glycoproteins controlling a variety of developmental processes including cell fate specification, cell proliferation, cell polarity and cell migration. Their receptors, the Frizzled (Fz) family are 7-TM receptors; however, assembly of an active Wnt-Fz receptor complex also requires the presence of co-receptors, the low-density lipoprotein related protein 5 and 6 (LRP5/6).

Two main signaling pathways are involved in the signal transduction process from the receptor complex (Figure II.6-4): the canonical or b-catenin dependent, and the non-canonical pathway. Based on their ability to activate a particular Wnt pathway, Wnt molecules have been grouped as canonical (Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b,

Wnt8) and non-canonical pathway activators (Wnt5a, Wnt4, Wnt11) although promiscuity is a feature of both ligands and receptors.

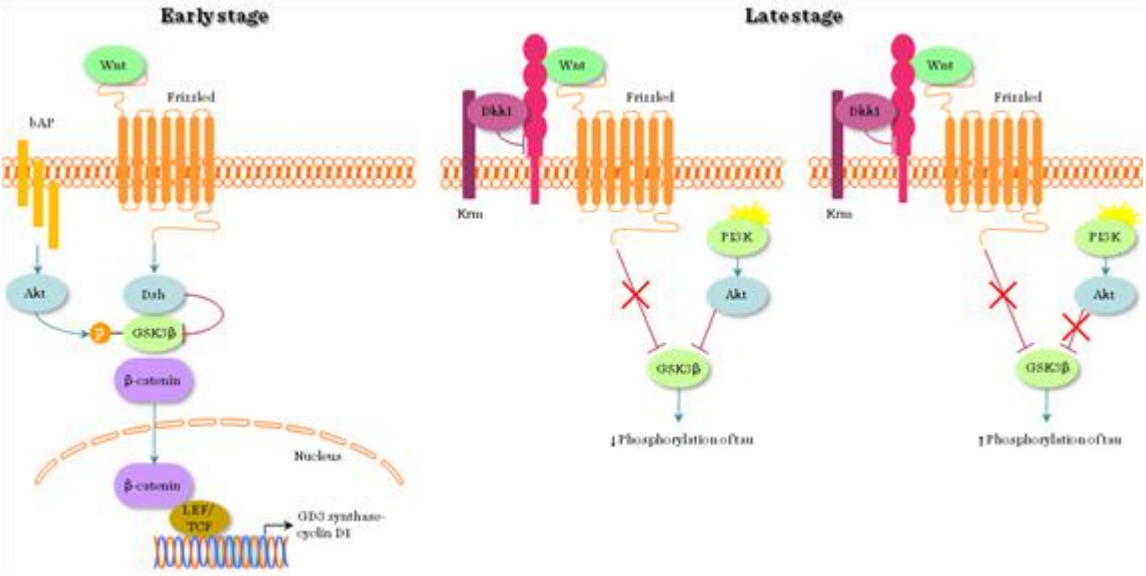


Figure II.6-4: Alzheimer's disease II

6.2. Canonical pathway

The canonical or b-catenin/Tcf dependent Wnt pathway (Figure II.6-5) is extensively investigated, and has been shown to be present in many kinds of cell, for example in developing thymocytes or in thymic epithelium. Generally, the absence of canonical Wnt-s keeps glycogen synthase kinase-3b (GSK-3b) active leading to the phosphorylation of b-catenin in the scaffolding protein complex of adenomatous polyposis coli (APC) and axin (Figure II.6-4). Phosporylated b-catenin is targeted for ubiquitination and 26S proteasome-mediated degradation, therefore, the cytosolic level of b-catenin decreases. In the presence of Wnt-s, on the other hand, signals from the Wnt-Fz-LRP6 complex lead to the phosphorylation of three domains of Dishevelled (Dvl), a family of cytosolic signal transducer molecules. Activation of Dvl ultimately leads to phosphorylation and consequently inhibition of GSK-3b. Inhibition of GSK-3b results in stabilisation and consequent cytosolic accumulation of b-catenin, which then translocates into the nucleus, where it forms active transcription complexes with members of the T-Cell Factor (LEF1, TCF1, TCF3, TCF4) transcription factor family and transcription initiator p300. Successful assembly of the transcription complex leads to the activation of various target genes including cyclin-D1, c-myc, c-jun, Fra-1 VEGFR, etc. (Further target genes can be viewed at Nusse's Wnt website: http://www.stanford.edu/~rnusse/wntwindow.html).

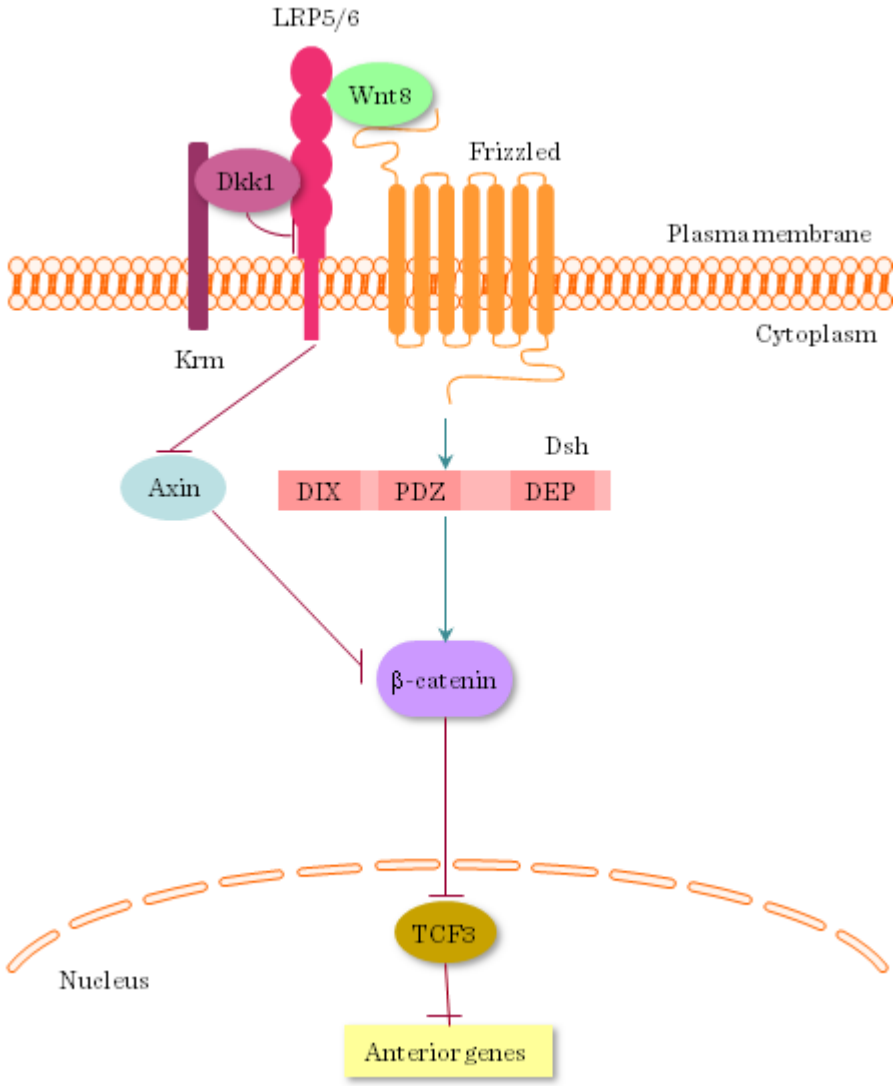


Figure II.6-5: Canonical Wnt pathway

6.3. Non-canonical pathway

The non-canonical pathways (Figure II.6-4) are independent from b-catenin and branches into the polar cell polarity (PCP) or c-Jun-N Terminal Kinase (JNK)/Activating Protein (AP1) dependent and the Ca2+ or Protein kinase C (PKC)/Calmodulin Kinase (CaMKII)/Nuclear Factor of Activating T- cells (NFAT) dependent pathways.

6.4. The role of Wnt-s in T-cell development

Manipulation of the levels of some Wnt-s and soluble Fz-s causes perturbation of T cell development highlighting the importance of Wnt dependent signalling for central T cell differentiation. Differential expression of Wnt ligands and receptors in thymic cell types shed light on that T-cell development may be influenced by indirect events triggered by Wnt signalling within the thymic epithelium. Cortical and medullary epithelial subsets express a wide range of Wnt-s and Fz-s. While Fz-9 is absent in the cortex, the medulla expresses all known Wnt receptors. In contrast, medullary epithelial cells show increased non-canonical Wnt expression, Wnt5a and Wnt11 in particular, suggesting that differential Wnt expression may play an important role in the control of thymic epithel differentiation, too.

7. II.7 Signaling in the nervous system

Neurotransmission is the mechanism of signal transmission between two neurons. Action potential spreads continously along the plasma membran of the presynaptic neuron until it reaches the limits of the cell. In order to transmit the signal to the postsynaptic neuron, neurotransmitters are released into the synapse. These chemical messengers “bridge” the physical gap between the two neurons and guarantee that the signal is transmitted to the postsynaptic cell (Figure I.2-3).

Neurotransmitters bind to two main types of receptors: ligand-gated ion channels or metabotropic receptors (ususally from the 7-TM family) (Figure II.7-1) (for more details of receptor types see Chapter I.2). Ligand-gated ion channels are a combination of receptors, responsible for ligand binding, and ion channels. When an ionotropic receptor is activated, it opens a channel that allows ions such as Na+, K+, or Cl- to flow. Metabotropic receptors, on the other hand, usually activate G-proteins leading to the modulation of ion channels by intricate intracellular second messengers and signaling cascades. Others are tyrosine kinases or guanylyl cyclase receptors.

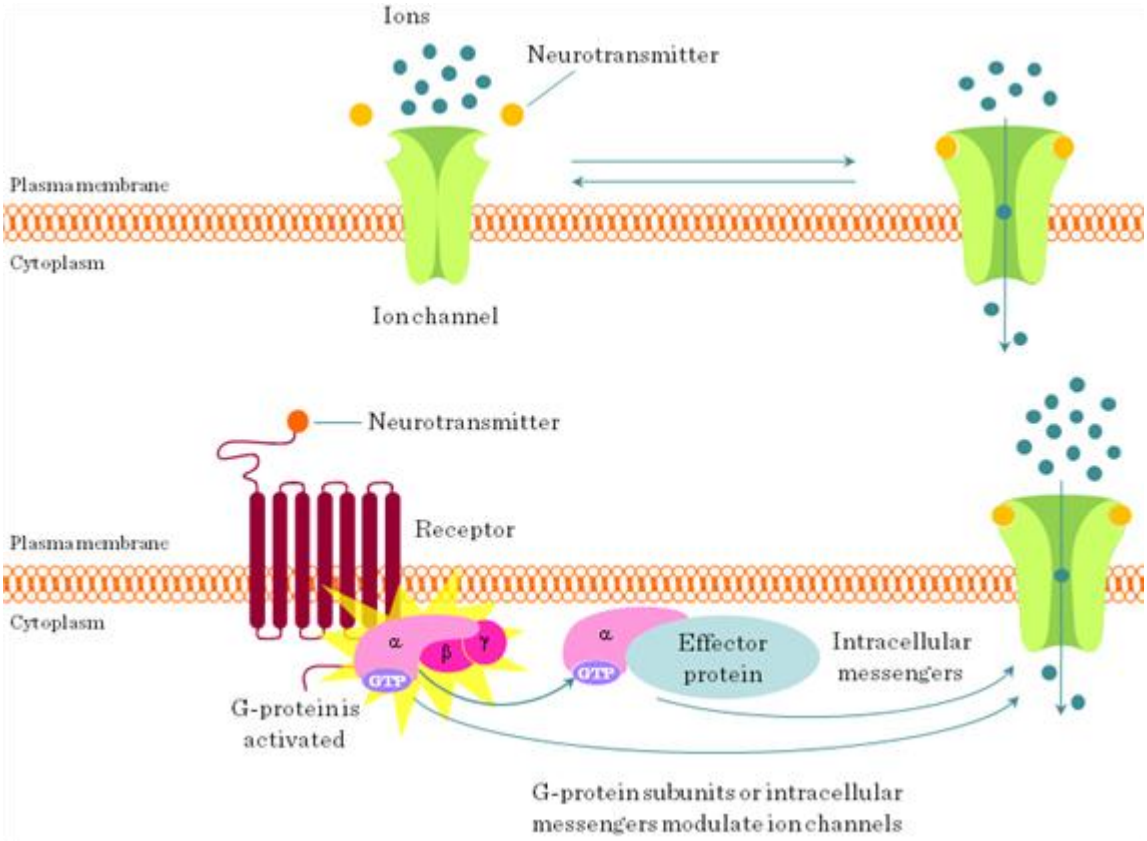


Figure II.7-1: Neurotransmission

7.1. II.7.1 Acetylcholine (Ach)

Ach is synthesized by the enzyme choline acetyltransferase (CAT) by the addition of an acetate group (derived from acetyl coenzyme A) to choline. Choline is taken up into cholinergic nerves by a high affinity transport process (sodium-choline cotransport) that is indirectly coupled to the Na+/K+ ATPase. Ach is stored as preformed neurotransmitter in vesicles found in the end plate of neurons; its release is depolarization-dependent, directly mediated by the influx of calcium ion. Ach acts through muscarinic or nicotinic receptors (Figure II.7-2 and Figure II.7-3).

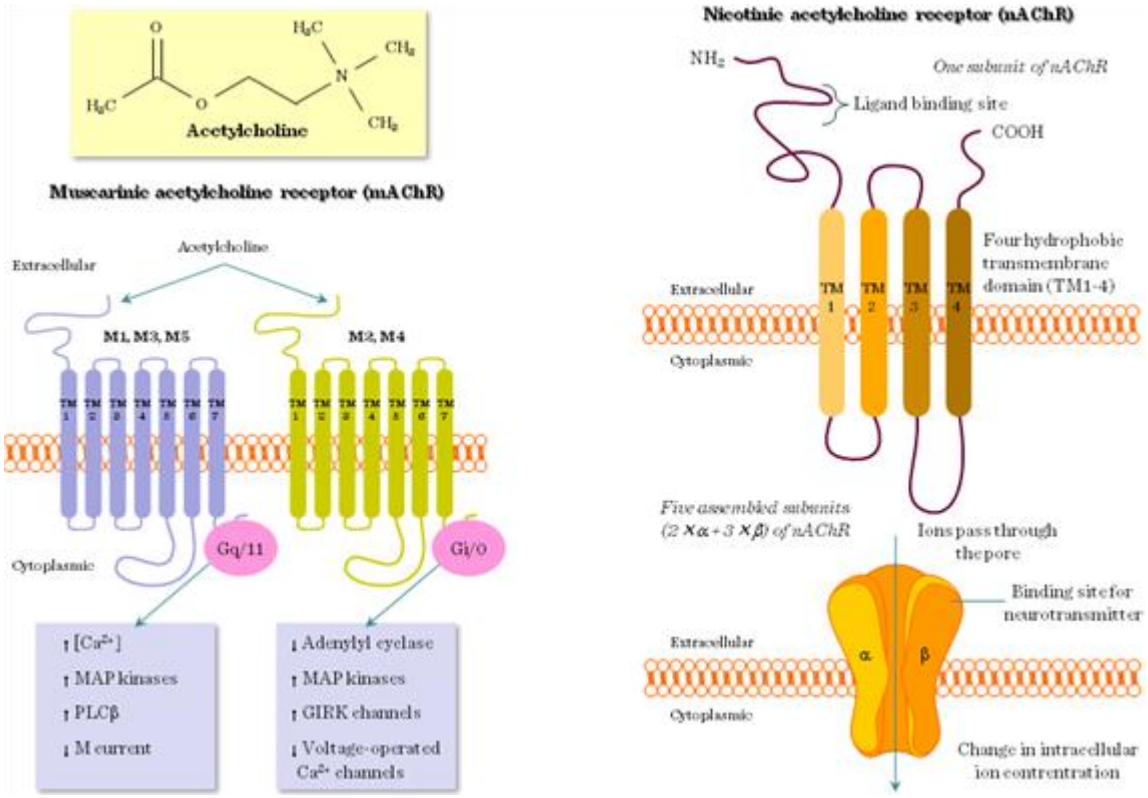


Figure II.7-2: Acetylcholine

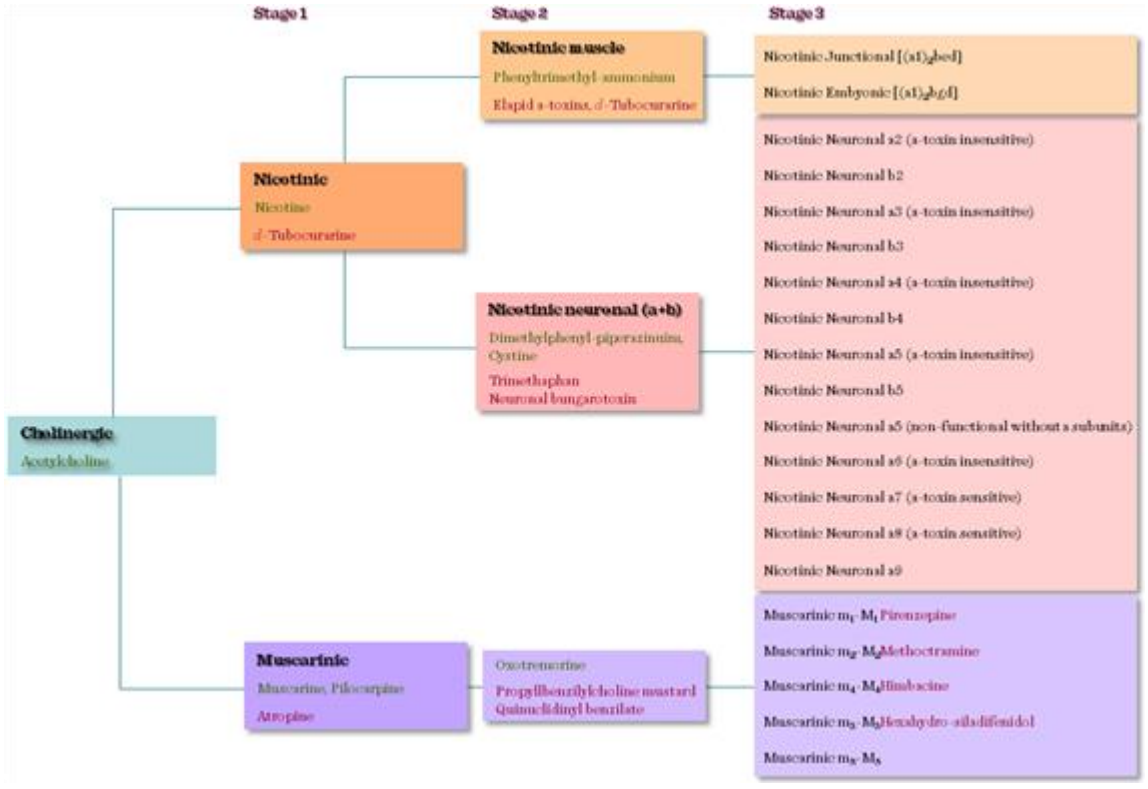


Figure II.7-3: Acetylcholine receptors

Muscarinic receptors belong to the 7-TM receptor family, which consist of seven transmembrane regions, with three extra and three intracellular loops (Figure I.2-4 and Figure I.2-5). Agonists bind to the region formed by the extracellular parts of the transmembrane region together with neighboring regions of the extracellular loop. Muscarinic receptors associate with G-proteins through the third intracellular loop. Five types of muscarinic

receptors are known: M1, M2, M3, M4 and M5. M2 and M4 activate Gi proteins, which, through the inhibition of adenylyl-cyclase, leads to a decrease in cAMP level. M1, M3 and M5 act through Gq activating phosphoinositide-specific phospholipase C, which leads to IP3 mediated calcium release from the endoplasmic reticulum.

The nicotinic receptor (Figure I.2-3) is a pentameric molecule built up of 2α, a β, γ (ε in the skeletal muscle) and δ subunit forming together the ion channel. The α subunits are responsible for Ack binding; both α subunits have to bind one acetylcoline molecule in order to open the ion channel. Nicotine receptors have four transmembrane (TM1, TM2, TM3, TM4) domains, both the N- and C-terminal is extracellular. TM2 builds up the wall of the channel, and amino acids in this region determine the conductance and ion selectivity of the molecule. Near to the N-terminal we can find a relatively big extracellular domain, which is responsible for the Ack binding. Nicotinic Ack receptor stimulation increases the intracellular calcium level. The increase in intracellular calcium can activate adenylyl-cyclase, protein kinases A and -C, calcium–calmodulin-dependent protein kinase (CaM-kinase) and phosphatidylinositol 3-kinase (PI3K). In turn, these phosphorylate downstream targets, mitogen-activated protein kinases, which lead to the activation of transcription factors.

7.2. II.7.2 Noradrenalin (NA)

During its synthesis, tyrosine is first oxidized to L-DOPA (by tyrosine 3-monooxygenase), which is decarboxylated to dopamine and oxidized into NA. An additional methylation step by phenylethanolamine N-methyltransferase (PNMT) in the cytosol of adrenergic neurons and cells of the adrenal medulla to adrenalin (A). Adrenaline or noradrenaline act on α- and β receptors (Figure II.2-6). Molecular cloning definitively identified the existence of three α1 subtypes: α1A, α1B, and α1D; three α2 subtypes: α2A, α2B, and α2C; and three β-AR subtypes: β1, β2, and β3. For more details on adrenergic receptor signaling see Chapter II.2.2.1.

7.3. II.7.3 Dopamine (D)

Dopamine is biosynthesized as described above. Dopamine receptors are members of the 7-TM G protein-coupled receptor family. There are at least five subtypes of dopamine receptors, D1, D2, D3, D4, and D5. The D1 and D5 receptors are coupled to adenylyl-cyclase activating Gs protein, whereas D2/D3/D4 receptors inhibit adenylyl-cyclase activity via coupling to an inhibitory Gi.

7.4. II.7.4 Serotonin (5-HT)

Serotonin acts through 5-HT receptors. For more details on 5-HT receptor and their signaling see Chapter II.2.2.3.

7.5. II.7.5 GABA

GABA is one of the major inhibitory neurotransmitter in the mammalian central nervous system. GABA is synthesized in neurons by the decarboxylation of L-glutamic acid by L-glutamic acid decarboxylase.GABA receptors are classified as GABA A, GABA B and GABAC. GABAA and GABAC are ligand gated ion channels (Figure I.2-2), while GABAB acts through G-protein coupled receptors.

7.6. II.7.6 Glutamate

At least four types of glutamate receptors can be distinguished in the brain. We distinguish between the ionotropic and metabotropic glutamate receptors. NMDA, AMPA and kainate receptors belong to the ionotropic receptors, which are ligand-gated nonselective cation channels allowing the flow of K+, Na+ and sometimes Ca2+ in response to glutamate binding. There are eight different types of metabotropic receptors divided into groups I, II, and III all of them G protein coupled receptors.

7.7. II.7.7 Glycine

Glycine can act as a neurotransmitter as well. Glycine receptors are composed of five distinct glycosylated integral membrane proteins consisiting of α and β subunits (Figure I.2-2). In the absence of the β subunit, α subunits are able to form homodimeric glycine receptors. When glycine binds to the receptor, the pore opens allowing Cl^- to diffuse passively across the membrane.

7.8. II.7.8 ATP

ATP receptors can be either ionotropic P2X or G-protein coupled receptors P2Y. Ionotropic receptors influence cellular functions through calcium signaling, whereas G-protein coupled receptors mediate signals through IP3 and DAG.

8. II.8 Pharmacological influence of the signaling

8.1. Introduction

As we have seen so far, a highly complex network in the cell is responsible for signal transduction. In most cases, from the cell surface receptors to the specific target genes, the signal is transmitted through several molecules. This complexity offers several targets for therapeutic interventions (Figure II.8-1, Figure II.8-2 and Table II.8-1). Advances in biotechnology in recent years provided us several monoclonal antibodies and other molecules that might interfere with certain pathways. For example, HER2 signaling might be effectively inhibited with monoclonal antibodies against the receptor, kinase inhibitors, Hsp-90 inhibitor, or on the DNA level with sequence specific antisense oligonucleotides (Figure II.8-3).

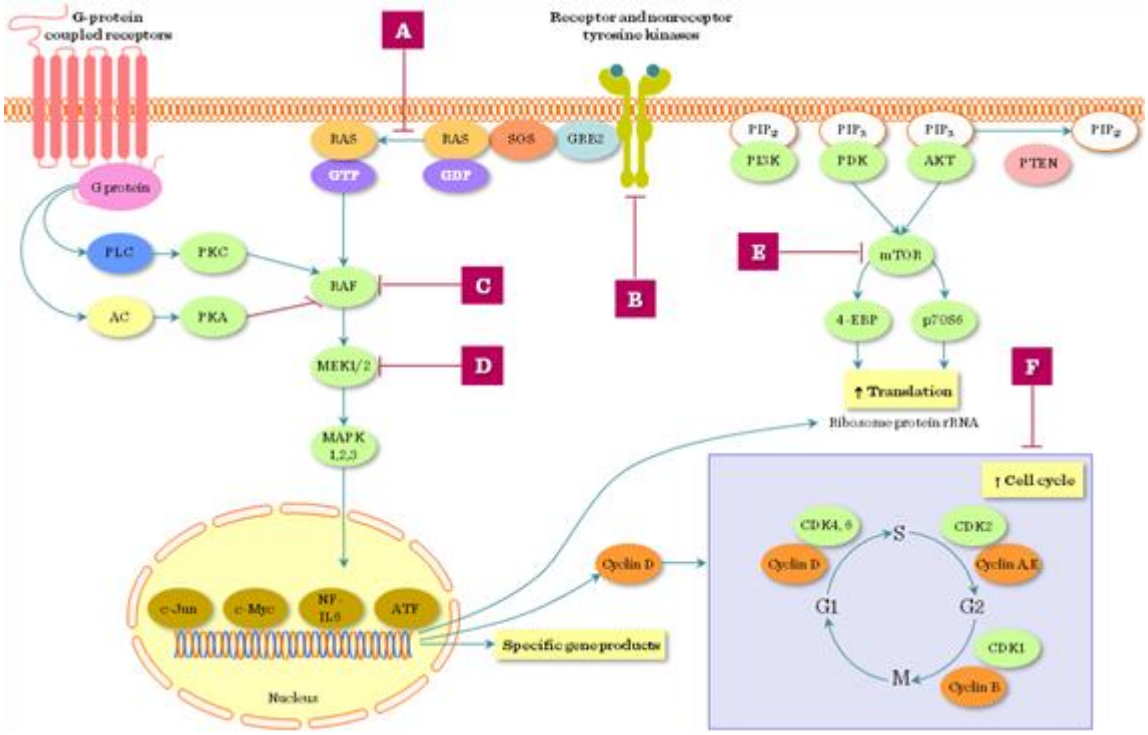


Figure II.8-1: Potential drug targets in signaling pathways

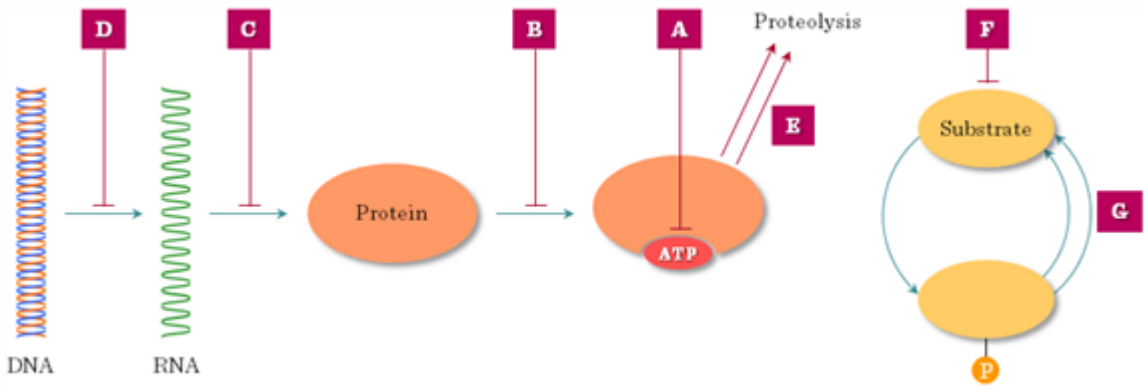


Figure II.8-2: Various levels of intervention

Table II.8-1: Selected kinase inhibitors in clinical development

	Target	Agent	Structure	Development stage
Growth-factor-receptor inhibitors	EGFR	IMC-C225 cetuximab (Erbitux; Imclone) ABX-EGF (Abgenix) EMD 72000 (Merck KgaA Darmstadt) RH3 (York Medical Bioscience) ZD1839 gefitinib (Iressa; AstraZeneca) MDX-447 (Medarex/Merck KgaA) OSI-774 erlotinib (Tarceva; OSI-Pharmaceuticals) CP-358,774 (Pfizer) EKB-569 (Wyeth Ayerst) GW2016/572016 (GlaxoSmithKline)	Monoclonal antibody Monoclonal antibody Monoclonal antibody Monoclonal antibody Monoclonal antibody; bivalent Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor	Phase III Phase II Phase I Phase II Phase I Phase III Phase III Phase II Phase I Phase I
	HER2/neu	Trastuzumab (Herceptin; Genentech) MDX-210 (Medarex/Novartis) 2C4 (Genentech) 17-AAG (Kosan)	Monoclonal antibody Monoclonal antibody Monoclonal antibody ethanamycin derivate inhibits HSP90	Registered Phase I Phase I Phase I
	PDGFR/c-Kit/BCR-ABL	Imatinib (STI571/Glivec/Novartis)	Small-molecule tirosine inhibitor	Registered
Ras inhibitors	Ras	ISIS 2503 (Isis Pharmaceuticals) R115777 (Johnson and Johnson) SCH66336 (Schering-Plough) BMS214662 (Bristol-Myers Squibb)	Antisense oligonucleotide Farnesyltransferase inhibitor Farnesyltransferase inhibitor Farnesyltransferase inhibitor	Phase II Phase II/Phase III Phase II Phase II
Raf inhibitors	Raf	ISIS 5132/CGP69846A L-779,450 (Merck) BAY 43-9006 (Onyx/Bayer)	Antisense oligonucleotide Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor	Phase II Phase II
MEK inhibitors	MEK	PD 184352 (CI-1040) (Pfizer) PD-0126 (Pharmacia)	Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor	Phase II Phase I
mTOR inhibitors	mTOR	CCI-779 (Wyeth) RAD001 (Novartis) Rapamycin/sirolimus (Wyeth)	Inhibits mTOR kinase by binding to FKBP12 Inhibits mTOR kinase by binding to FKBP12 Inhibits mTOR kinase by binding to FKBP12	Phase II Phase I as a cancer therapeutic Phase II/III as an immunosuppressant Registered as an immunosuppressant
Cyclin-dependent-kinase inhibitors	CDK	Flavopiridol (HMR-1275 (Aventis) UCN-01 (7-hydroxystaurosporine) CYC202 (Cyclacel) BMS-387032 (Bristol-Myers Squibb)	Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor Small-molecule tirosine inhibitor	Phase II Phase I Phase I Phase I
Other targets and agents	PKC	ISIS 3521/LY900003 Affinitak (Isis Pharmaceuticals) CGP41251/PKC412 (Novartis) Bryostatin-1 (GPC Biotek) UCN-01 (Kyowa Hakko Kogyo)	Antisense oligonucleotide Staurosporine analogue Small-molecule tirosine inhibitor Staurosporine analogue	Phase III Phase II Phase II Phase I/II
	PKC-b	LY333531 (Eli Lilly)	Small-molecule tirosine inhibitor	Phase I oncology Phase II diabetic neuropathy
	PDK1	UCN-01 (Kyowa Hakko Kogyo)	Staurosporine analogue	Phase I/II

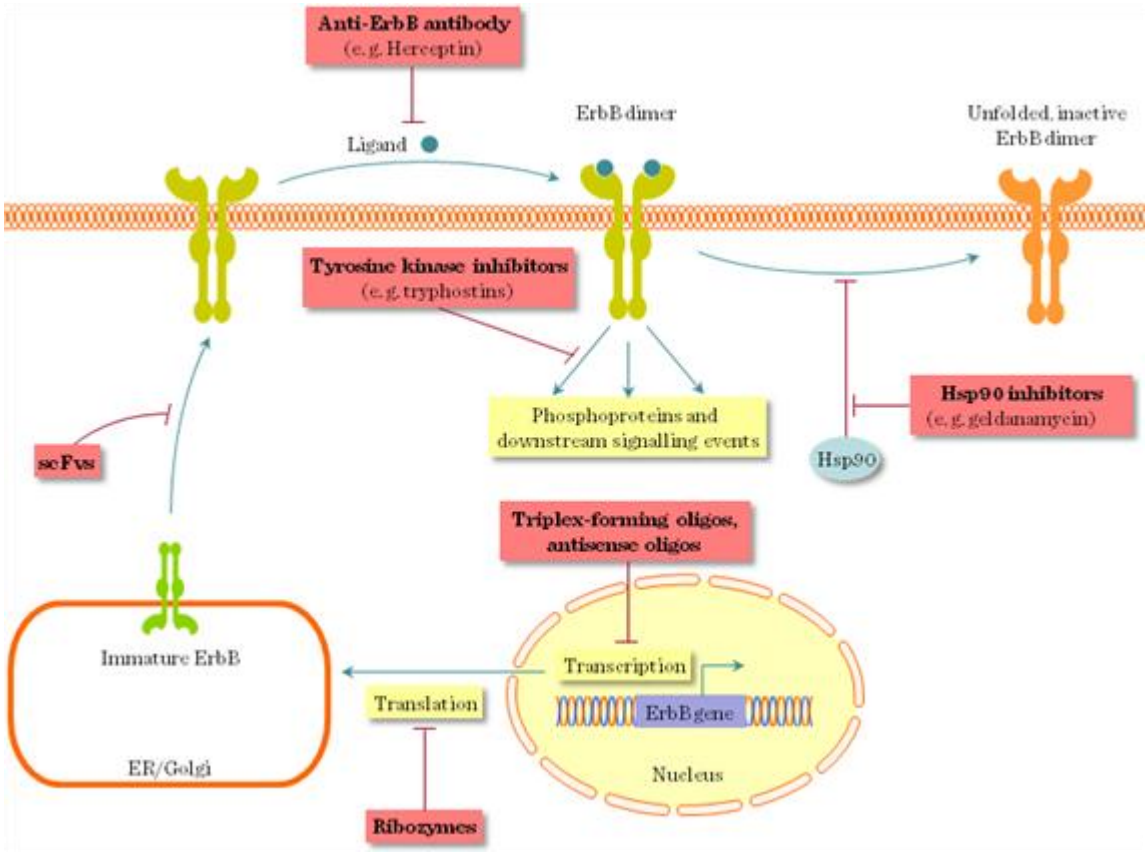


Figure II.8-3: ERB signaling intervention

In general, we can influence signaling pathways at multiple levels (Figure II.8-1, Figure II.8-2 and Table II.8-1): (1) blockade of cell surface receptors; (2) inhibition of signal transmission (e.g. kinase inhibitors); or (3) interference with the turnover of signaling proteins (e.g. proteosomal degradation, siRNS) are of interest. Some drugs are highly selective, i.e. they inhibit only one specific molecule, others, on the other hand, exert a more general effect either by acting on more molecules parallelly, or only one molecule that is involved in more pathways.

8.2. Growth factor receptor inhibitors

The following monoclonal antibodies are available against the EGFR: cetuximab, gefitinib, erlotinib. Trastuzumab (mAb) blocks HER2, thus, interfering with the continuous EGF driven proliferation in certain tumors (for more details see chapter II.3).

8.3. Kinase inhibitors

Kinase inhibitors (Figure II.2-4 and Table II.8-1) are promising molecules in the treatment of malignant tumors. They block kinases, which participate in tumor cell signaling, and consequently inhibit cell growth, proliferation or invasion. In CML, the increased activity of BCR-c-ABL fusion protein is inhibited by imatinib (Gleevec, Novartis). Other small molecule kinase inhibitors are available to Ras, Raf, MEK, PKC and Cyclin-depenedent kinase (CDK).

8.4. Calcineurin blockade

CyclosporinA and Tacrolimus (FK-506) are calcineurin (for details see Chapters I.4.3, II.1.1 and II.1.2) inhibitors. Blockade of this molecule inhibits T- and B cell activation, thus, they are efficient immunosuppressive agents (Figure II.8-4). The phosphatase activity of calcineurin is abrupted by CyclosporinA and FK-506 inhibitory complexes with CypA and FKBP-12, respectively.

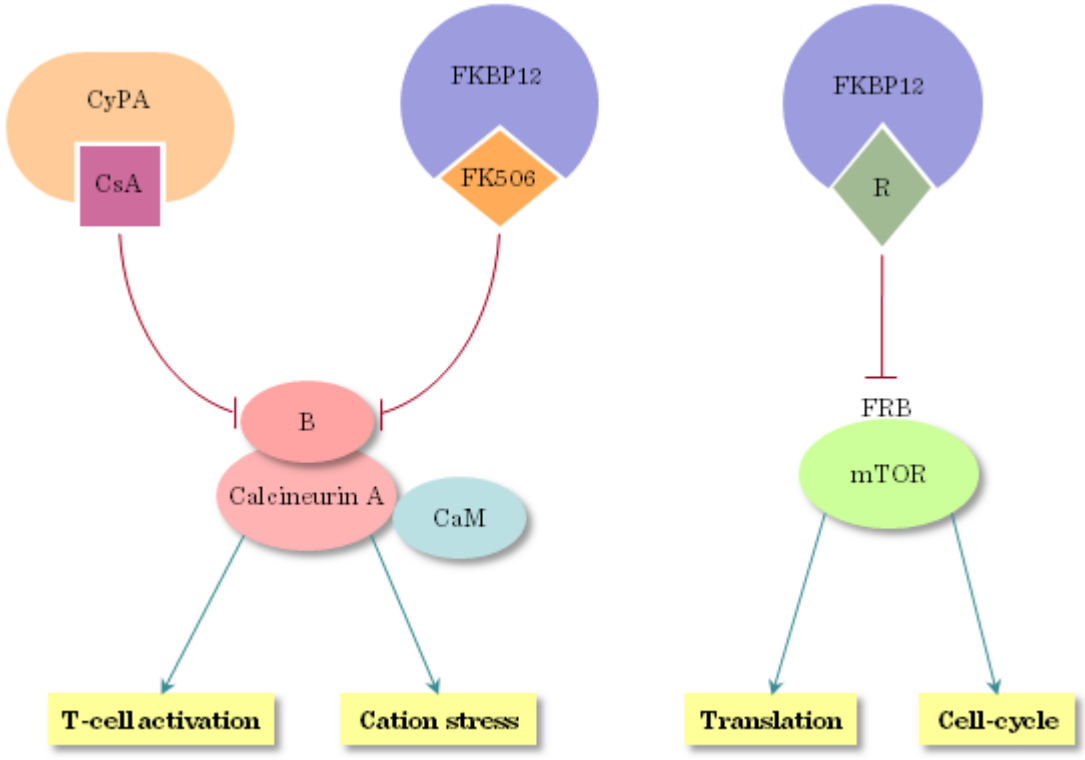


Figure II.8-4: Calcineurin and rapamycin

8.5. Inhibitors of mTOR

The immunosuppressive effect of Sirolimus (or Rapamycin) is based on its binding to FKBP-12 and the inhibition of mTOR (mammalian target of Rapamycin) (Figure II.8-4 and Figure II.8-5). TOR was first

described in Sacharomyces cerevisiae, while mTOR participates in the PI3K-PKB (Akt) signaling pathway. Wortmannin and LY294002 are inhibitors of PI3K, which is the upstream regulator of mTOR.

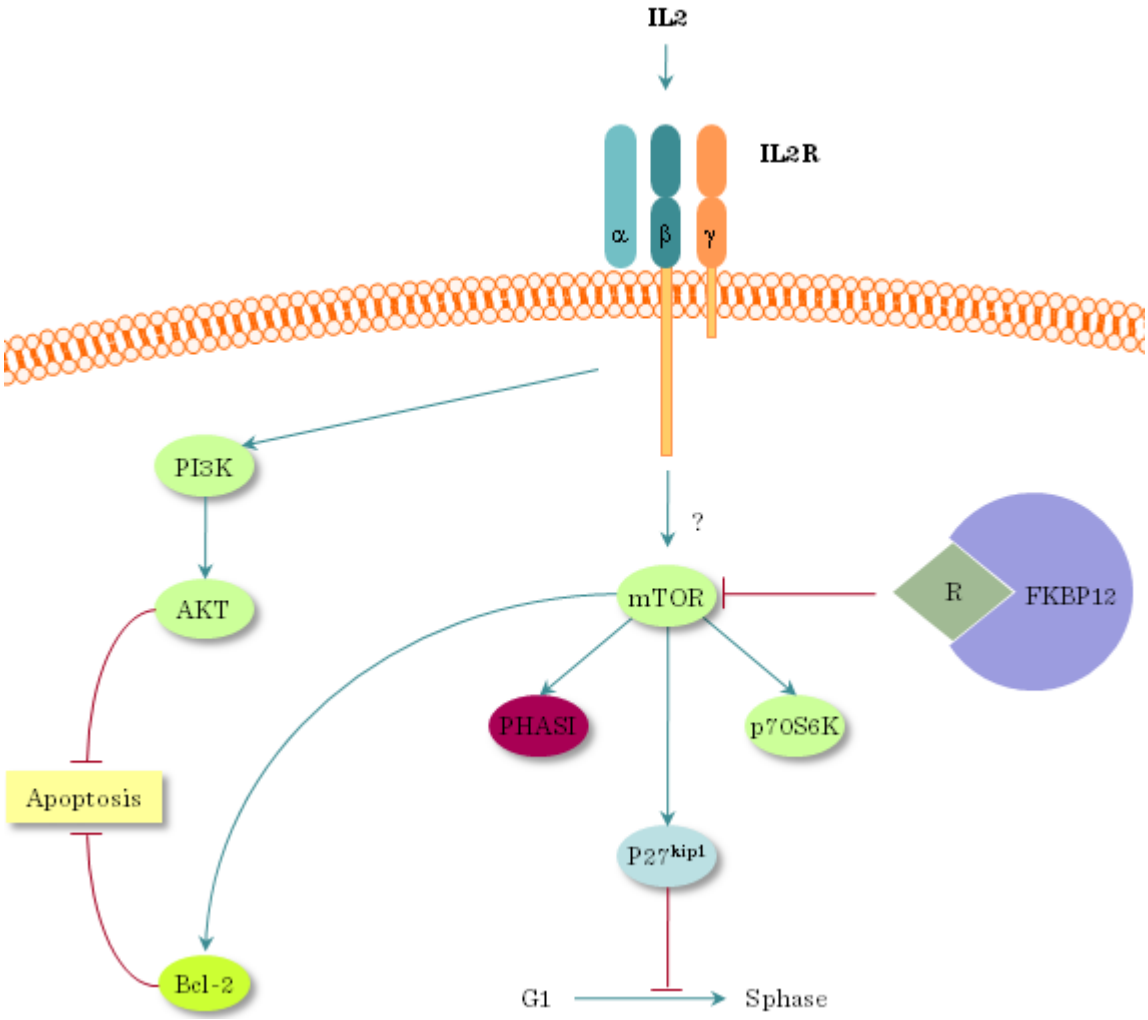


Figure II.8-5: Rapamycin

8.6. Proteosome inhibitors

The proteasome complex is responsible for degrading ubiquitinated intracellular proteins, which process is of central importance in the normal turnover of cellular proteins. Proteasomal degradation of signaling molecules is a potential regulatory mechanism; moreover, it is a promising target for intervention (Figure II.8-6). Bortezomib is a proteasome inhibitor used for the treatment of multiple myeloma and mantle cell lymphoma. It has been shown that Bortezomib interferes with the proteasomal degradation of IκB, thus, inhibits NF-κB signaling (Figure II.8-5).

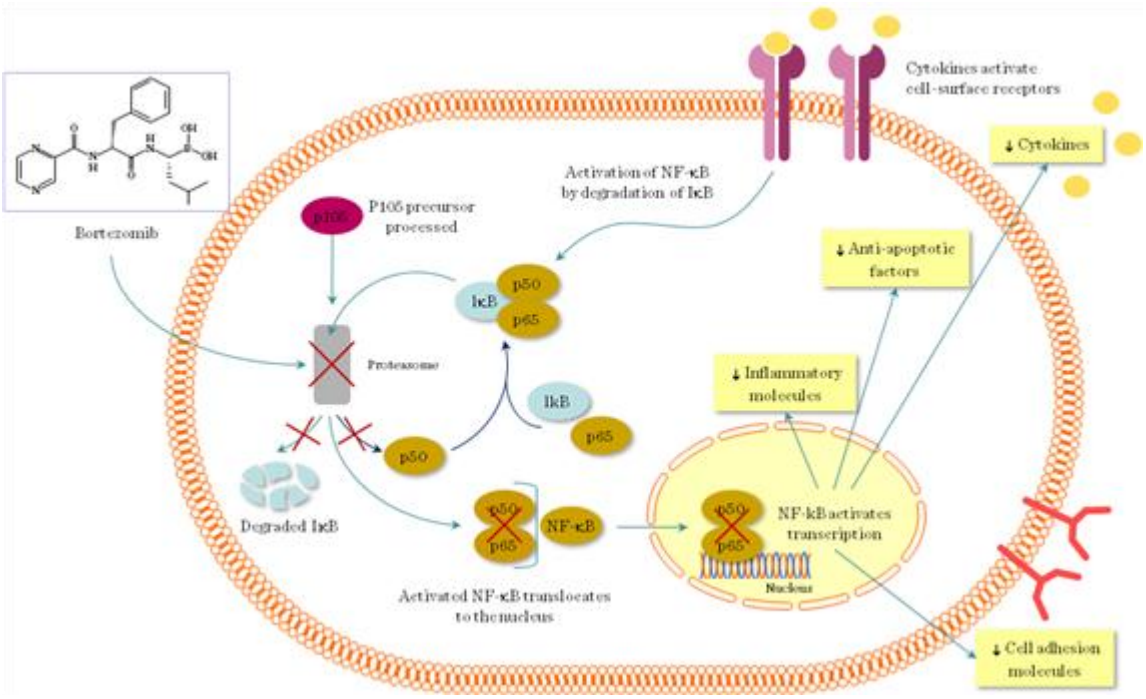


Figure II.8-6: Proteosome inhibitors-Bortezomib

8.7. Blocking Hsp-90

Hsp-90 serves as an important cytoplasmic chaperon protein organizing many structural and signaling proteins. Geldanamycin binds to the ATP-binding site of Hsp-90, inhibiting the binding to specific client proteins, which leads to their subsequent ubiquitination and proteasomal degradation (Figure II.8-7). For example, in tumor cells breakdown of mutated v-Src, Bcr-Abl and p53 proteins have beneficial effects.

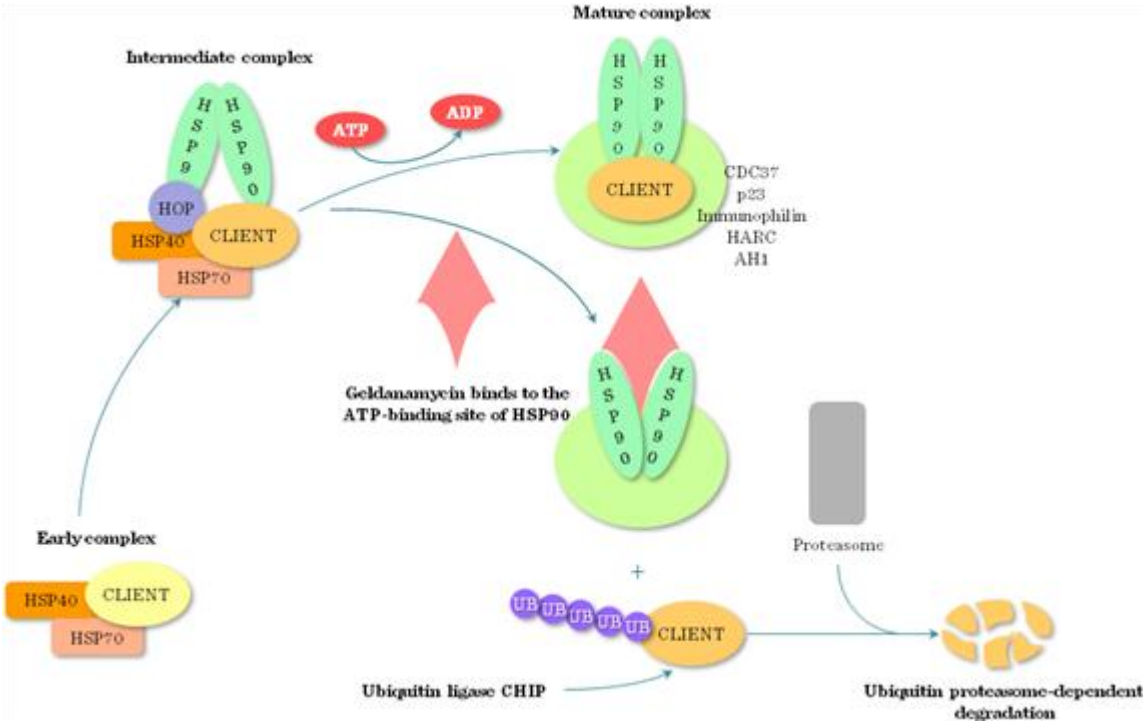


Figure II.8-7: HSP-90 inhibitors

Chapter 4. Further reading

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