Molecular and Cell Biology of Tumors

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Molecular and Cell Biology of Tumors

3. Mitogenic signaling I

Proto-oncogenes, oncogenes, signaling pathways, regulation of cell cycle

Hallmarks of cancer

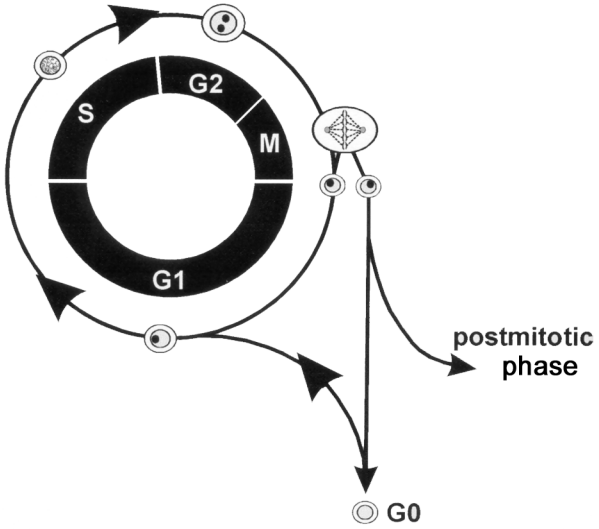
(1) Sustaining proliferative signaling
(2) Evading growth suppressors
(3) Resisting cell death
(4) Enabling replicative immortality
(5) Inducing angiogenesis
(6) Activating invasion and metastasis
(7) Genome instability and mutation
(8) Tumor-promoting inflammation
(9) Deregulating cellular energetics
(10) Avoiding immune destruction

Hanahan D. and Weinberg R.A., *Cell* 144 (2011) 646-674

Is cancer disease of the cell cycle?

- Deregulation of cell cycle is key factor in cell transformation
- Deregulation of cell cyle is not the only factor in cancerogesis, it is usually not fully transforming

Cell cycle progression



M - G1 - S - G2

M − DNA copies are separated; condensed chromosomes

G1 − DNA content: 2N

S − DNA replication

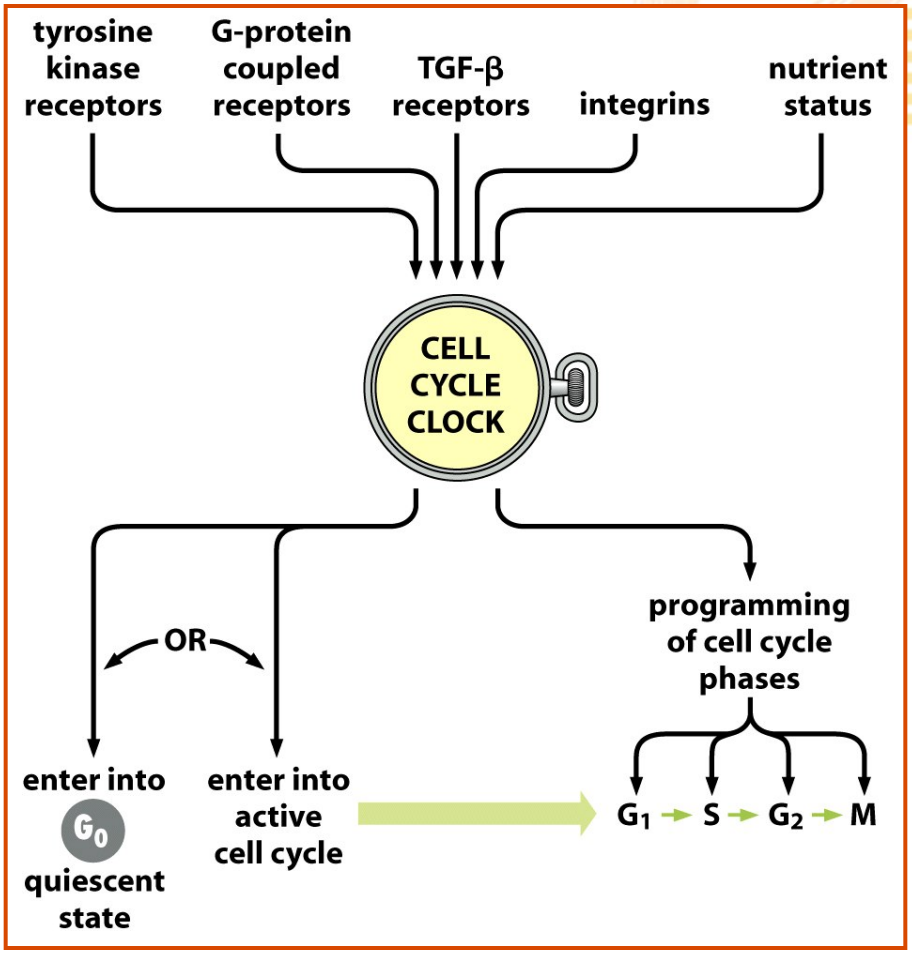
G2 − DNA content: 4N

G0 − senescent or non-dividing cells

Postmitotic phase − without re-entering cell cycle, linked with terminal differentiation

Cell cycle regulation

Term „**cell cycle clock**“ indicates that in a cell there is a molecular „device“ that integrates all signals coming from inside and from extracellular space and determines whether cell enters active cell cyle or quiscent state.



Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Key molecules regulating cell cycle

- **Cyclin-dependent kinases** (Cdk, Cdc; human - 13)
- Have catalytic and regulatory subunits, kinase activity is dependent on interaction with respective binding partners − cyclins
- **Cyclins** (human - 29)
- Their expression levels fluctuate during cell cycle
- They affect the specific phase of the cell cycle by activating respective CDK kinase, afterwards are quickly degraded
- Some have other functions not cycle dependent expression
- **Inhibitors** (CKIs, cyclin-dependent kinase inhibitors)
- **Ink4** family of CKIs (p16, p15, p18, p19) – target mainly Cdk4 and Cdk6
- **Cip/Kip** family (p21, p27, p57) – more promiscuous, often interfere with binding of cyclin to CDK

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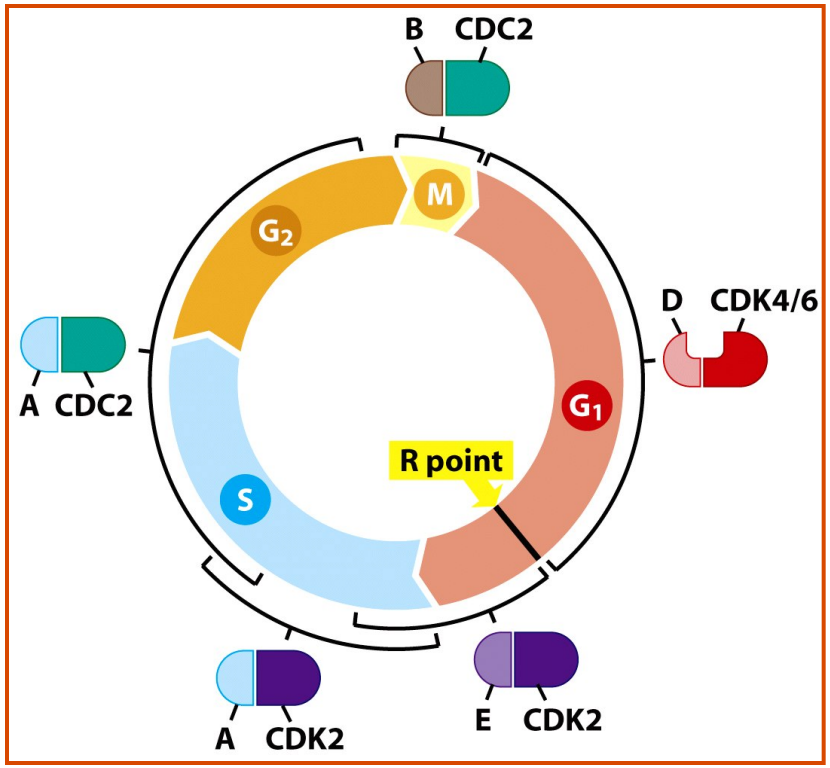
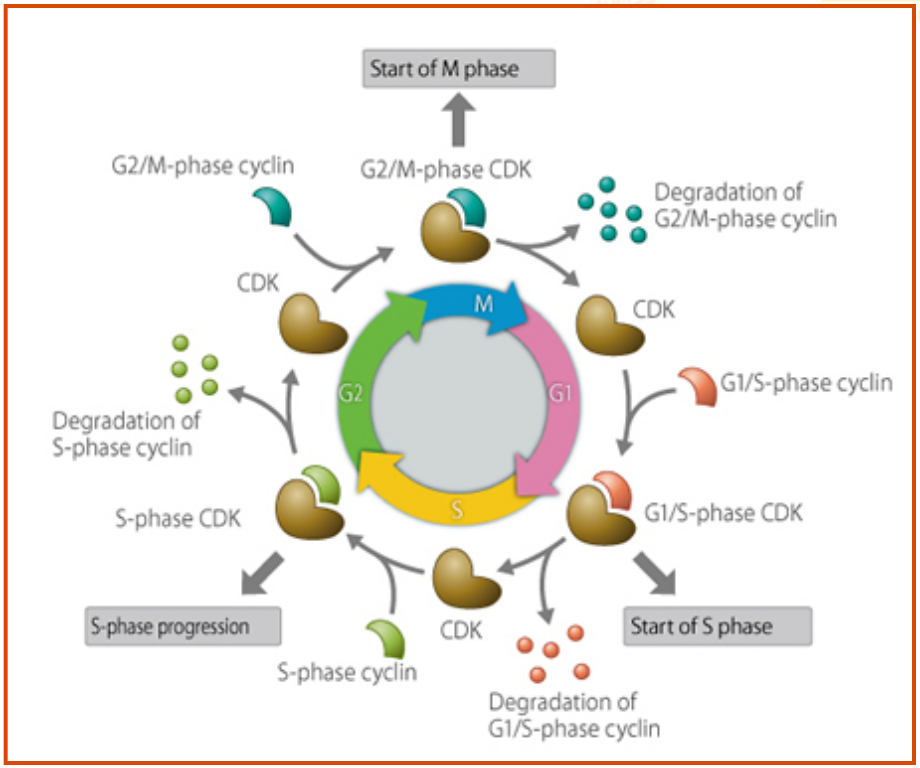
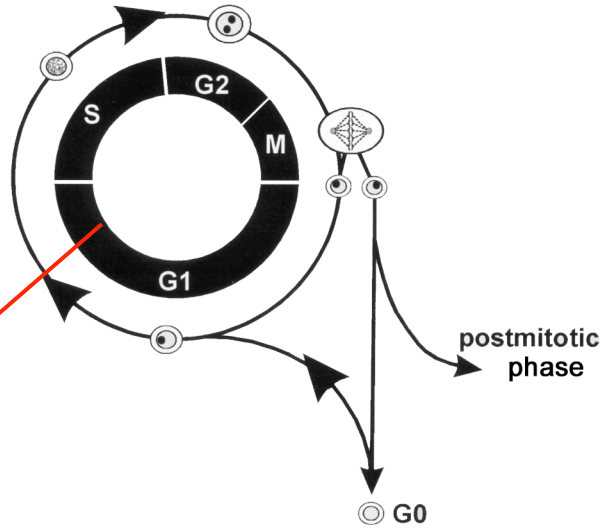




Restriction checkpoint

- mitogenic stimulation induces cell cycle progression
- antimitogenic stimulation blocks cell cycle

- Cell cycle is dependent of exogenous signals only during the part of G1 phase − until restriction checkpoint (or G1 checkpoint or Major checkpoint)



Restriction checkpoint: key regulatory point in cell cycle

pRB – trigger of cell cycle

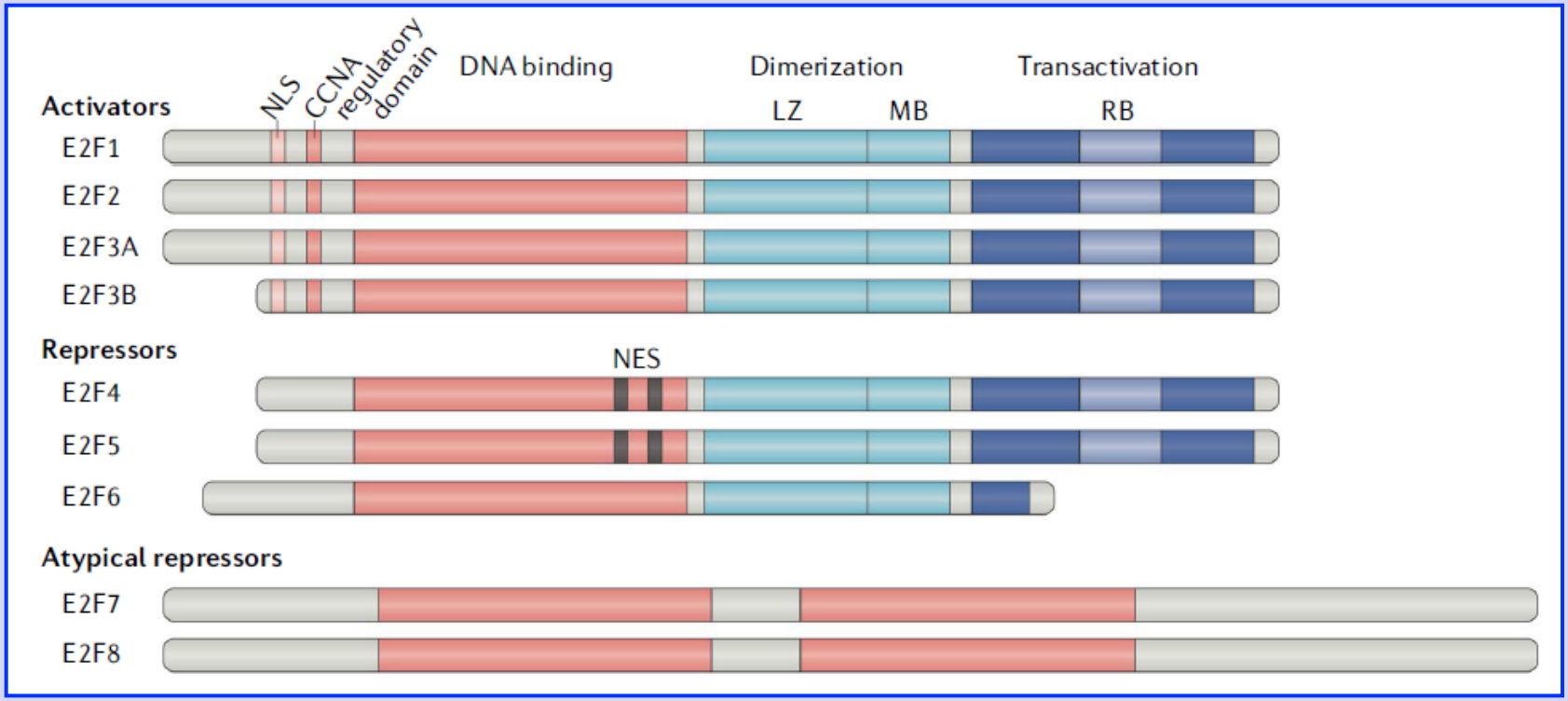
- **pRB** in hypophosphorylated state blocks the transition through the restriction checkpoint: it interacts with TFs of **E2F** family (8 genes) and thus prevent transactivation of their target genes (proteins encoded by these genes are required for S phase)

- When phosphorylated pRB losses the ability to bind with E2F and thereby the passage through the restriction checkpoint is possible

⇒ Regulation of the restriction checkpoint = regulation of pRB phosphorylation

E2F family of transcription factors

- 8 genes + alternatively spliced variants + multiple transcription start sites
- regulation: transcriptional, translational, posttranslational, protein degradation, biding of co-factors, cellular localization



Kent LN a Leone G. *Nat Rev Cancer* 19: 326-338, 2019

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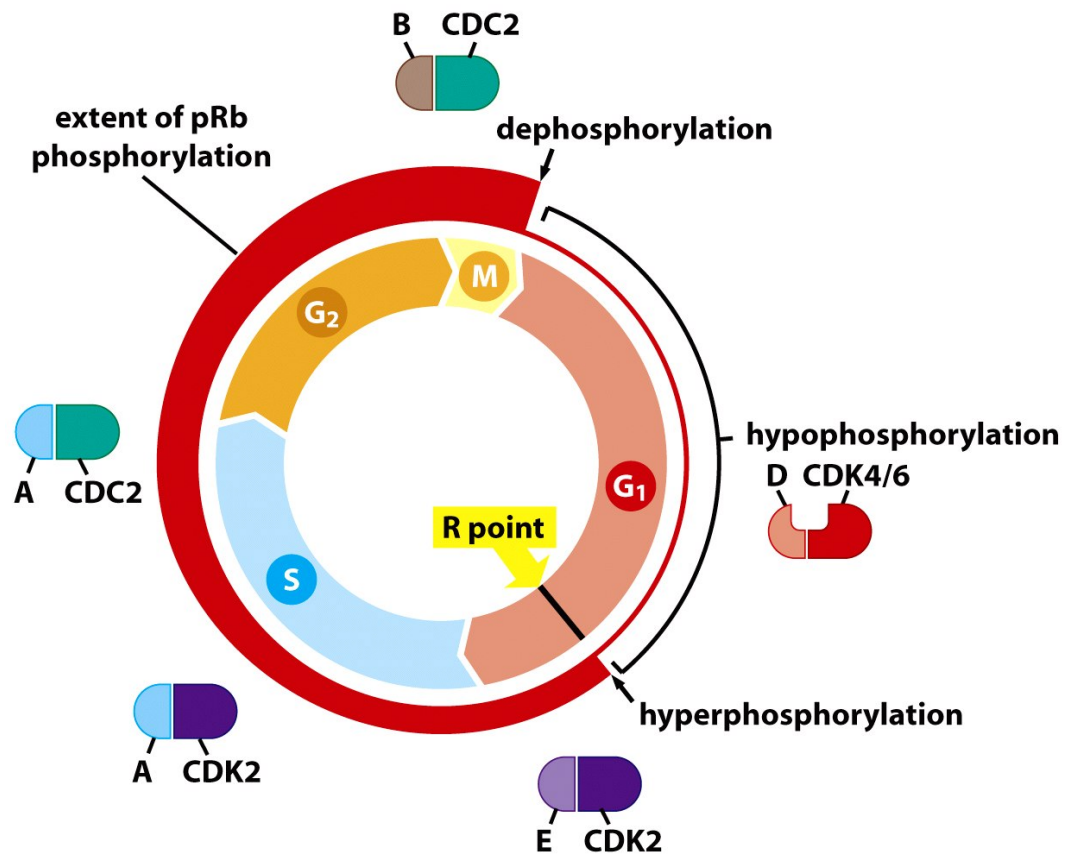
- **pRB**
- **E2Fs**

Phosphorylation of pRB during cell cycle

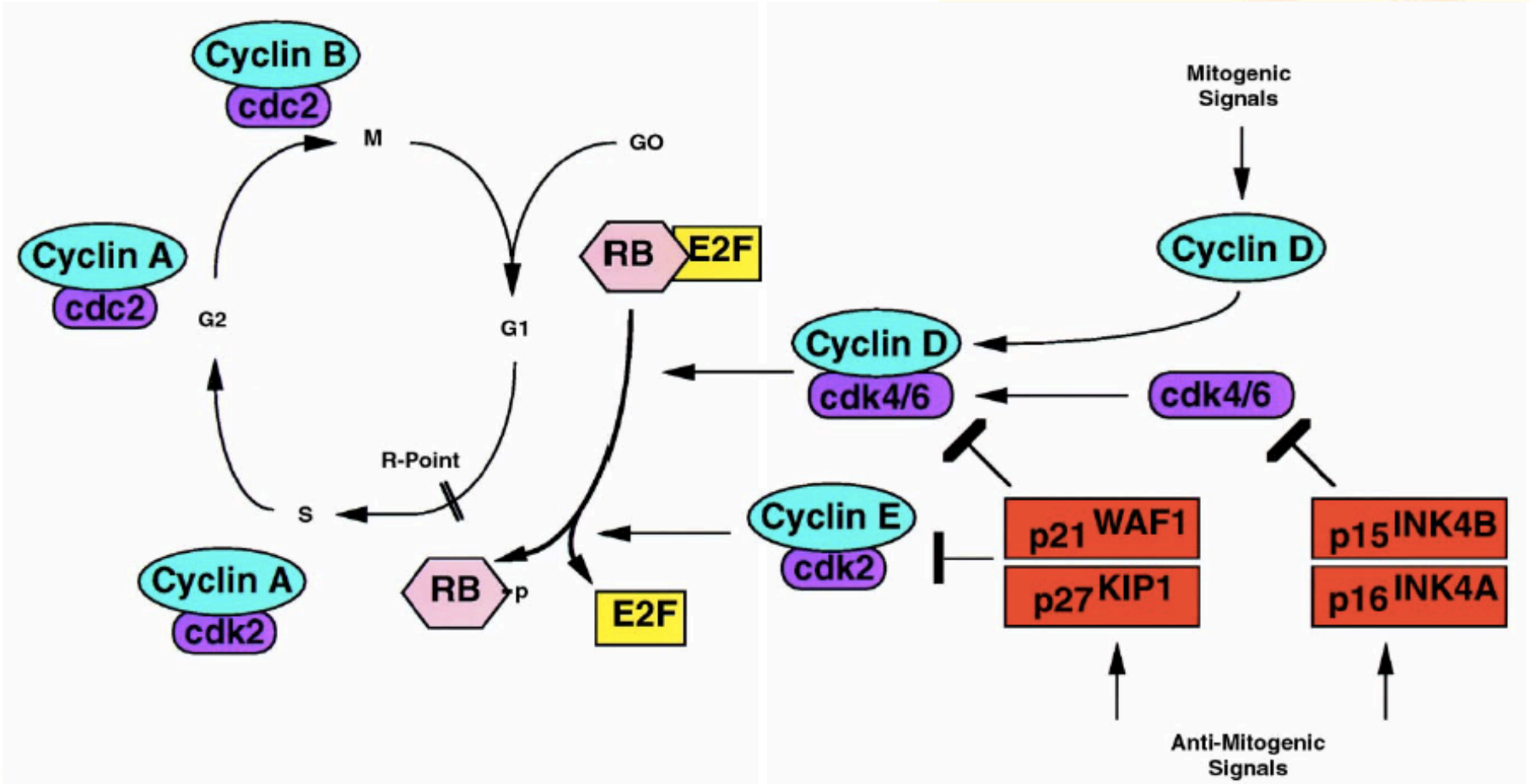


Extent of pRB phosphorylation is tightly controlled during cell cycle: during M/G1 transition all phosphate residues are removed from pRB, after restriction checkpoint the extent of pRB phosphorylation gradually increases with peak at M phase

(pRB is phosphorylated at serine and threonine amino acids)

Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Cell cycle regulation



Lundberg AS F a Weinberg RA, *Eur J Cancer 35:* 1886-1894, 1999

Regulation of pRB phosphorylation

Phosphorylation of pRB

- Positivelly regulated by:
  - complexes of cyclins D (D1, D2 and D3) and CDK4/CDK6
  - complexes of cyclin E and CDK2
- Negativelly regulated by:
  - inhibitors of cyclin:CDK complexes: $p21^{WAF1}$ and $p27^{KIP1}$
  - inhibitors of CDKs: $p15^{INK4B}$ a $p16^{INK4A}$

Mitogenic signaling leads to increased expression of cyclins D and donwregulation of protein levels of inhibitors

Antimitogenic signaling leads to overexpression of inhibitor(s) and decreased expression of cyclins D

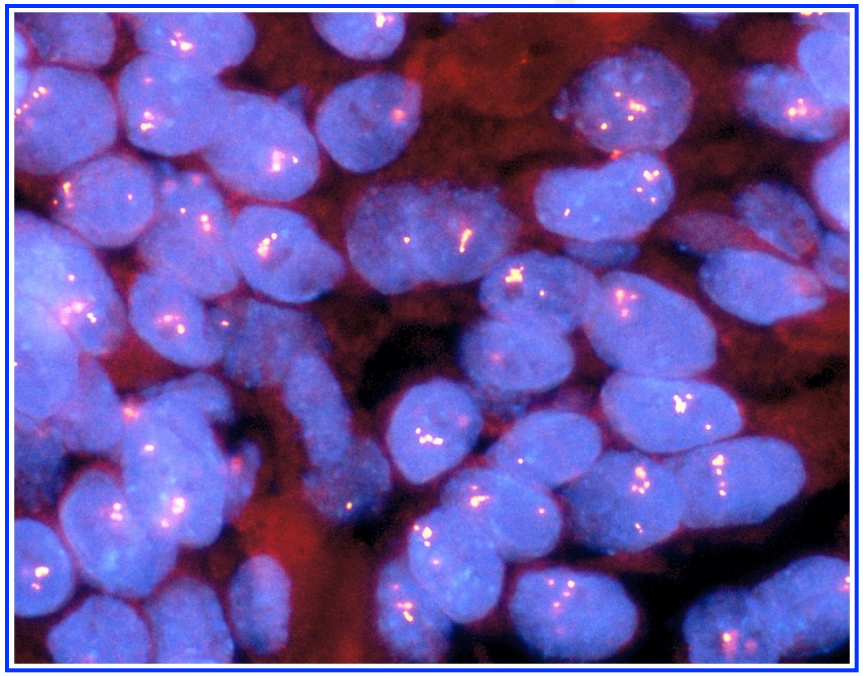
What is damaged in transformed cells?

- Tumor suppressor **pRB** (retinoblastoma, ..)
- Tumor suppressor **p16^{INK4A}** (melanoma, ..)
- protooncogenes **cyclins D**
  - amplification or overexpression of cyclin D1 in more than 50% of breast cancer, HNSCC
  - Chromosomal translocations including cyclin D1 locus in mantle cell lymphomas: t(11;14)

- „**periferal players**“ − components of mitogenic and anti-mitogenic pathways

Amplification of cyclin D1 locus in head and neck cancer

- Example of detection of ***CCND1*** amplification (**11q13**) in head and neck squamous cell carcinoma (HNSCC) by FISH. There are 3 to 5 copies per cell.
- Approx 1/3 of HNSCC have amplification of *CCND1* and thereby enhanced expression level of cyclin D1. As result regulation of pRB phosphorylation is disrupted



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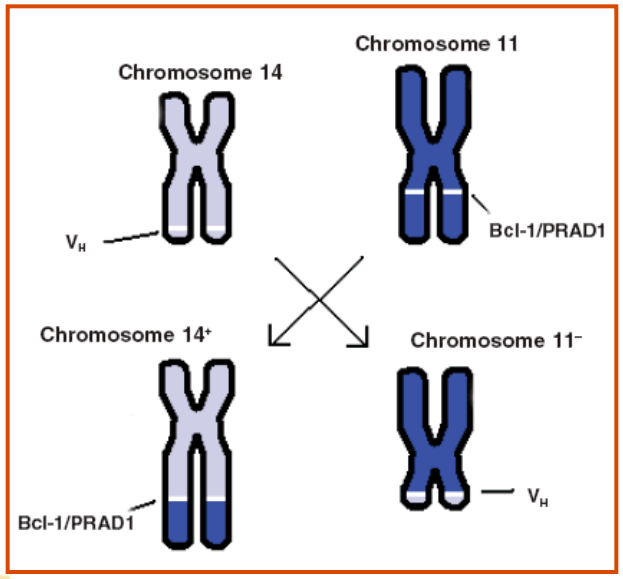
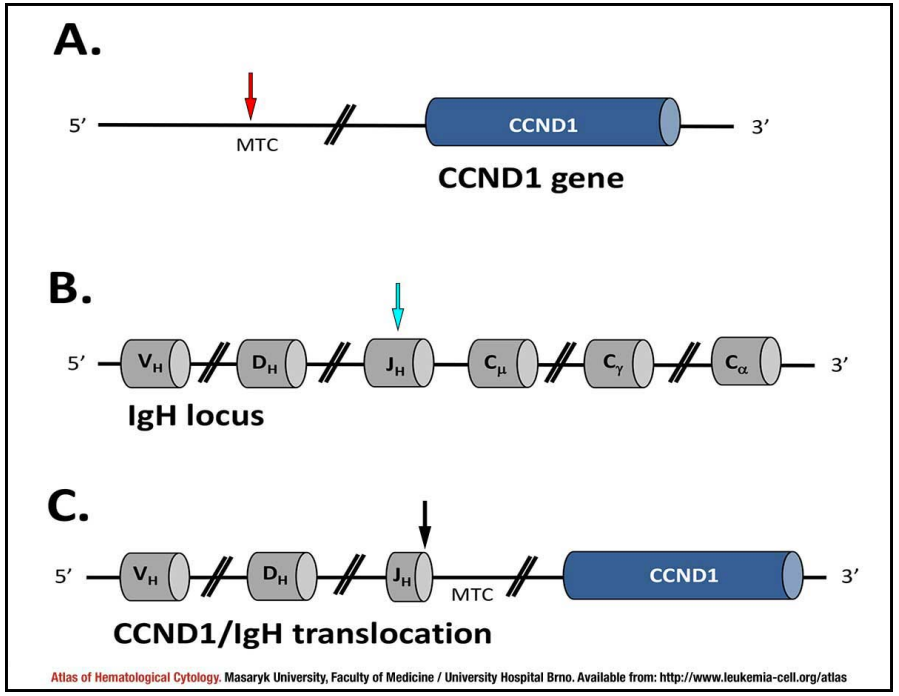
Cyclin D1 and mantle cell lymphoma (MCL)

MCL is characterised by t(11;14)(q13;q32) translocation which juxtaposes Ig heavy chain gene (IGH) sequences (enhancer) with the *CCND1* locus, leading to up-regulation of cyclin D1 expression.

Note: *CCND1=BCL1=PRAD1*





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- „**periferal players**“ − components of mitogenic and anti-mitogenic pathways

Mitogenic signaling

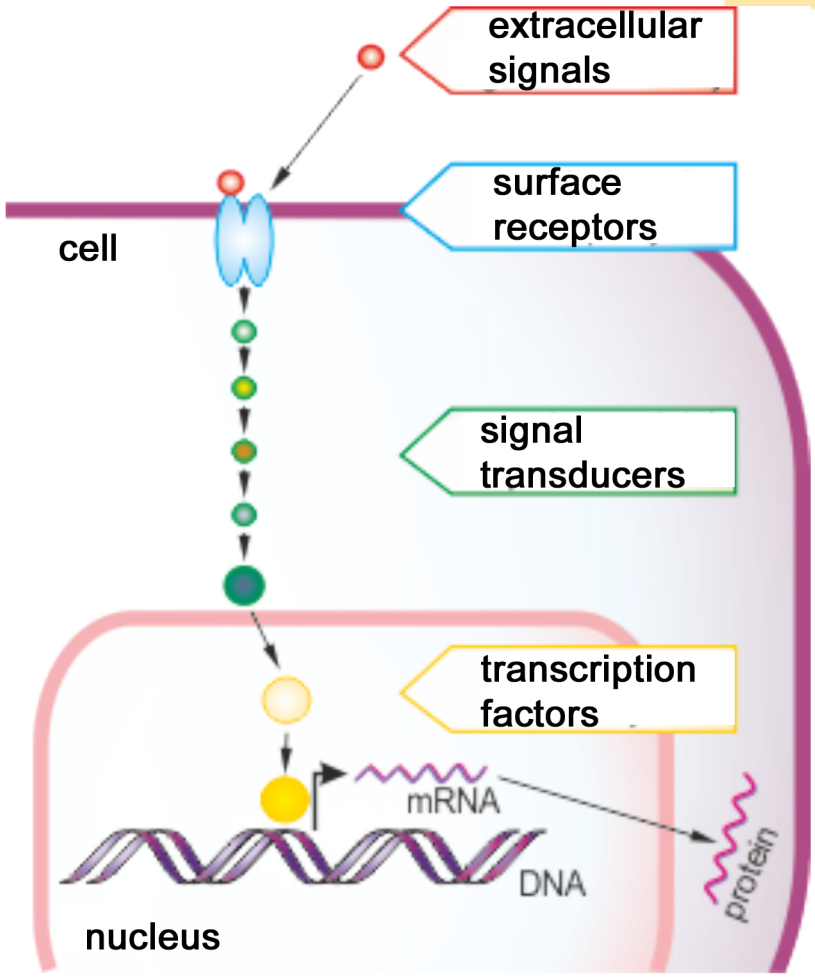
Classification of proto-oncogenes I

I. **Growth factors**
II. **Receptors for growth factors**
III. **Proteins Ras**
IV. **Non-receptor tyrosine kinases**
V. **Transcription factors**

This classification reflects steps of signaling pathways:

1. Binding of ligand (growth factor) with receptor
2. Activation of receptor protein kinase
3. Signal transduction to nucleus via cascade of protein kinases
4. Activation of transcription factor

Schematic outline of signaling pathway



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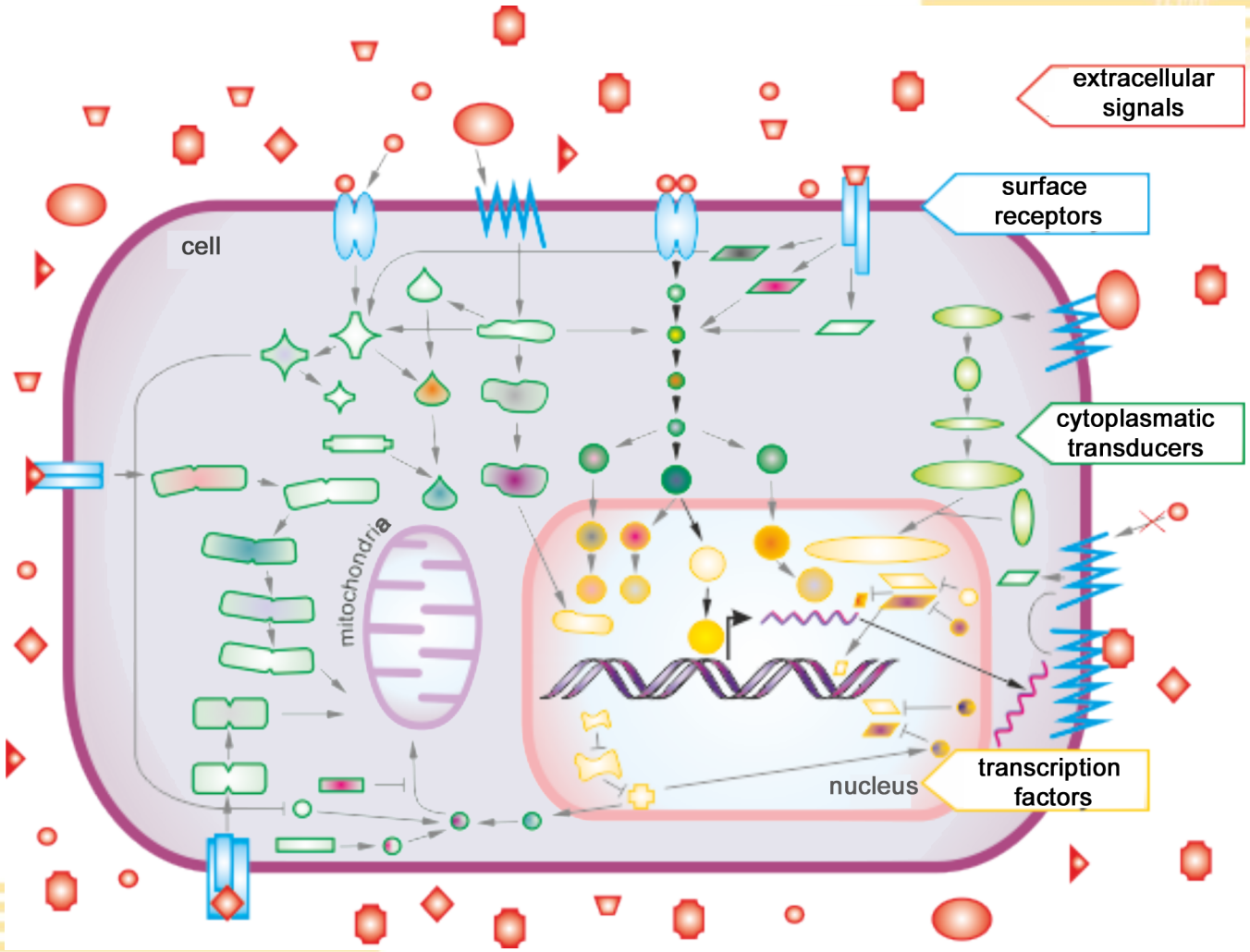
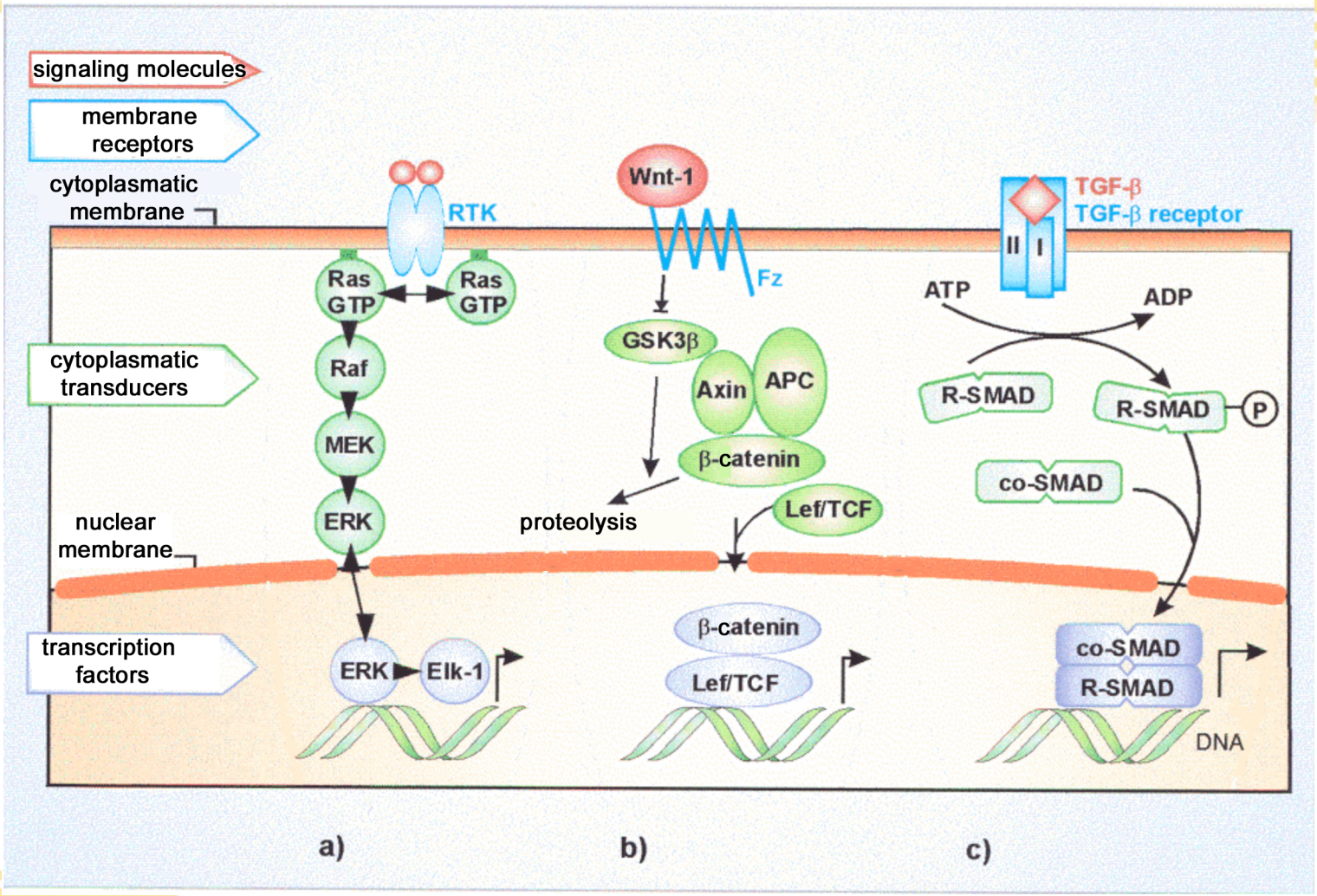
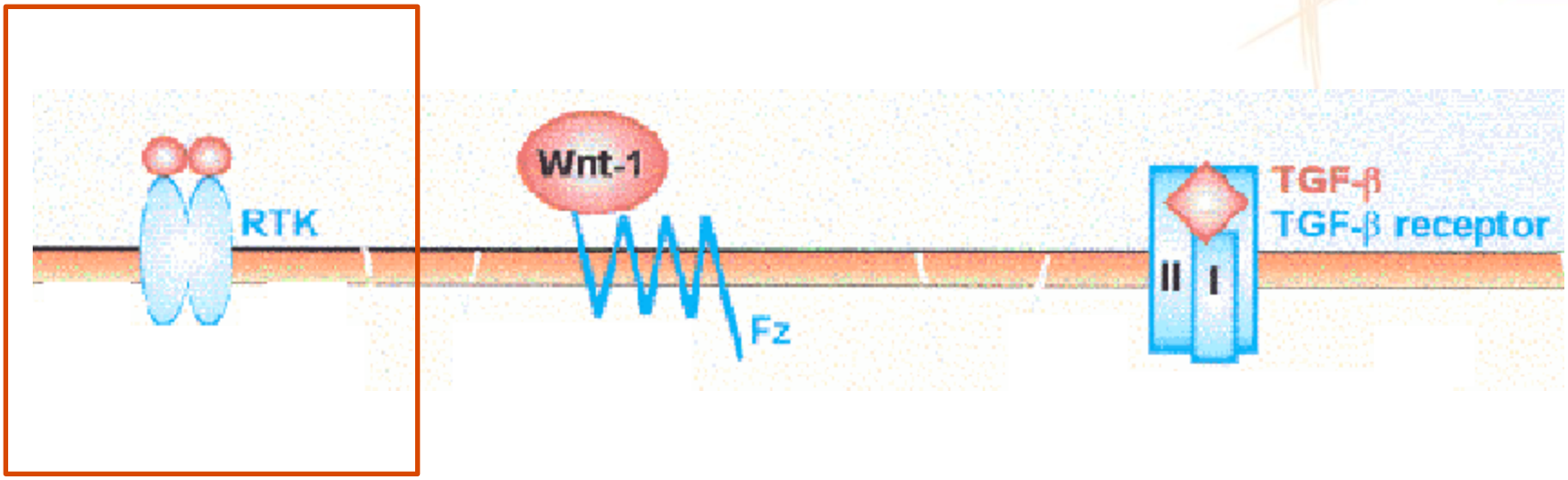


Signaling pathways: examples



Growth factors and receptor tyrosine kinases (RTK)



Signaling via **tyrosine phosphorylation** is used mainly in **mitogenic** signaling. Other signaling pathways use serine and threonine residues.

Growth factors and receptor tyrosine kinases (RTK)

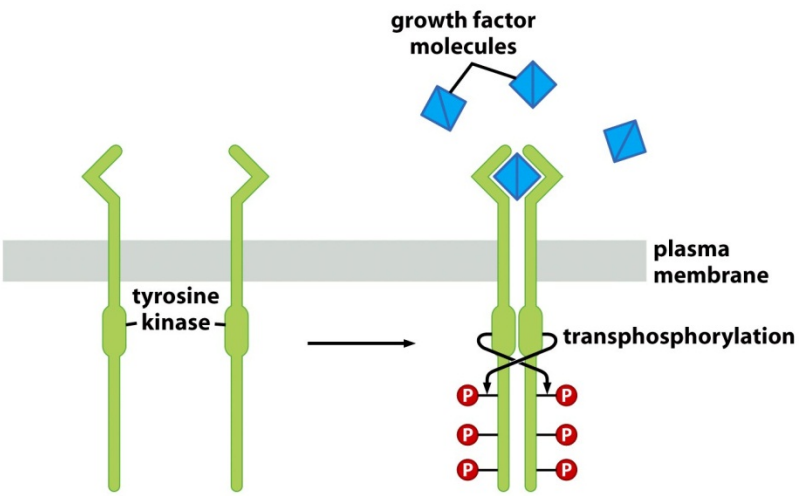
Growth factors: polypeptides that are produced by cells and triggers signaling for either start or stop of proliferation, differentiation and survival by binding to their receptors at the cell surface

- Evidence for involvement of extracellular signaling molecules in cell transformation: factors secreted by cancer cells are able to induce neoplastic transformation *in vitro*

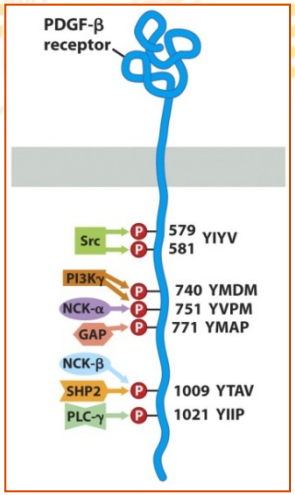
Stimulation:

- **autocrine** − growth factor stimulates producing cell
- **paracrine** − growth factor stimulates neighbourging cells
- **endocrine** − stimulation of distant cells

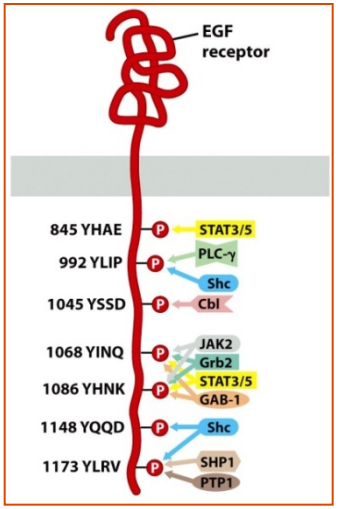
Signal transduction by RTK (EGFR)



After ligation with signal molecues receptors form dimers (all but IGF receptor that is convalently bound homodimer) and autophosphorylation is induced.



Phosphorylation induces conformation change − thus catalytic domain is opened and substrates may bind to the binding domain. Binding of the substrates is mediated mostly by **SH2** and **SH3** (*Src-homology*) domains.

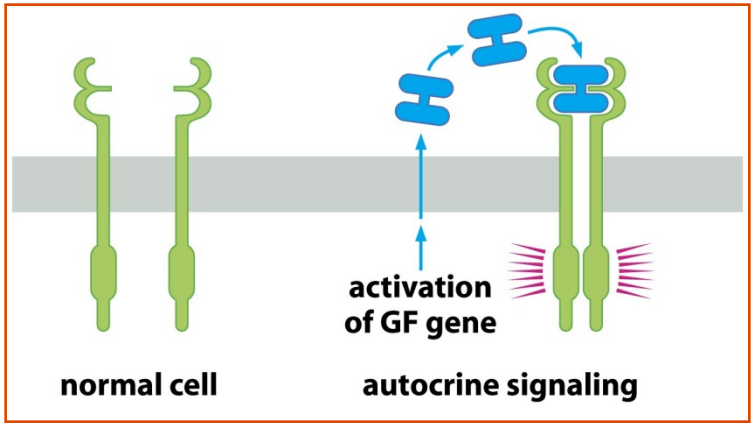


Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Type of mutations affecting growth factors and RTK

- Overproduction of growth factors (→ autocrine stimulation)
- Amplification of receptor: (a) concentration of receptors at the cell surface is so high that they cluster randomly and dimerize without interaction with ligand; (b) extremely efficient capturing of the extracellular signal: cells with high levels of receptor are stimulated for cell division even though the concentration of signal molecule is low and normally insufficient to activate mitogenic pathway
- Structural changes of receptor: activation is not dependent on binding of ligand (point mutations, short deletions, shortened forms as results of gene fusions, etc.)

Alterations of RTK



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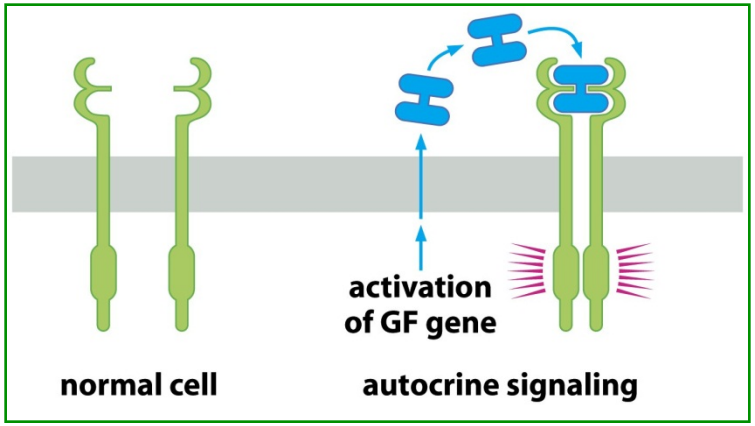
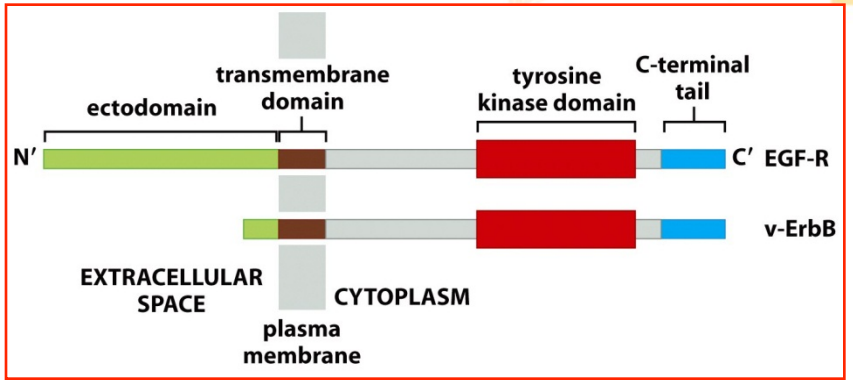


(1984 – discovery of the structural similarity of EGFR and v-ErbB)



v-ErbB has unlike EGFR shortened extracellular domain ⇒ it transduces mitogenic signal constitutively (without the interaction with ligand)

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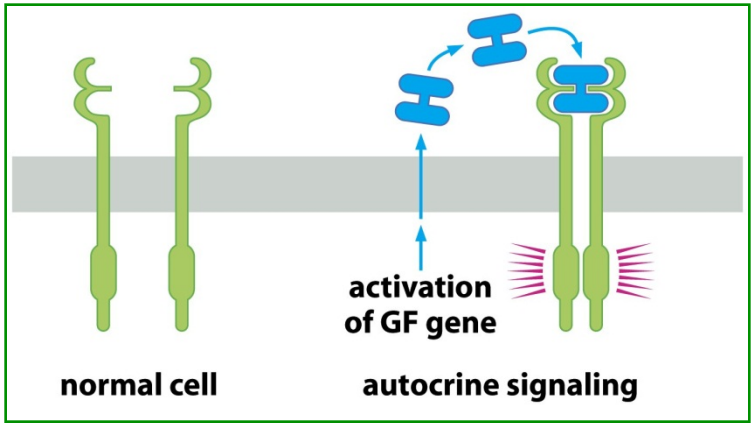

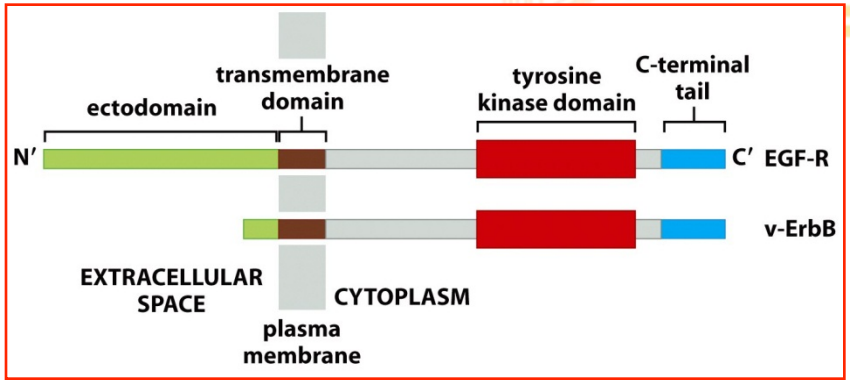

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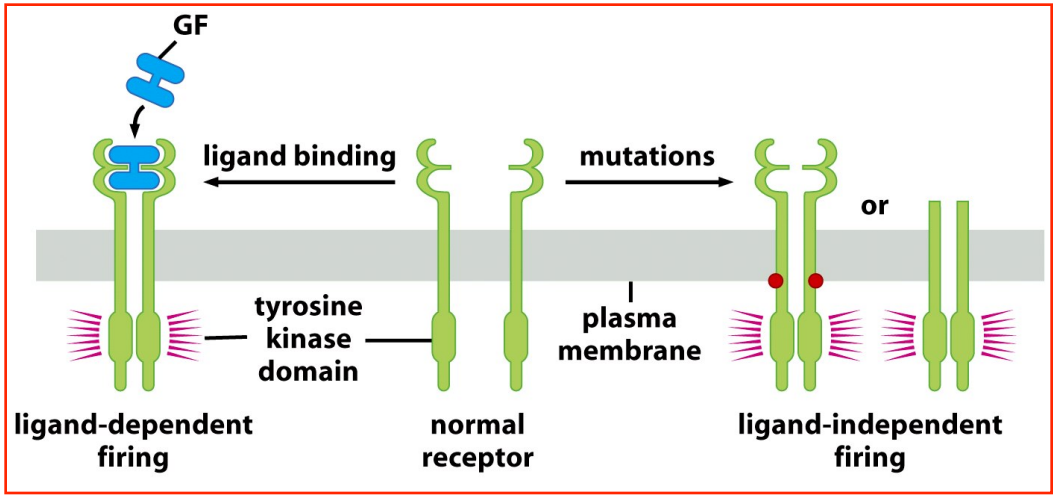

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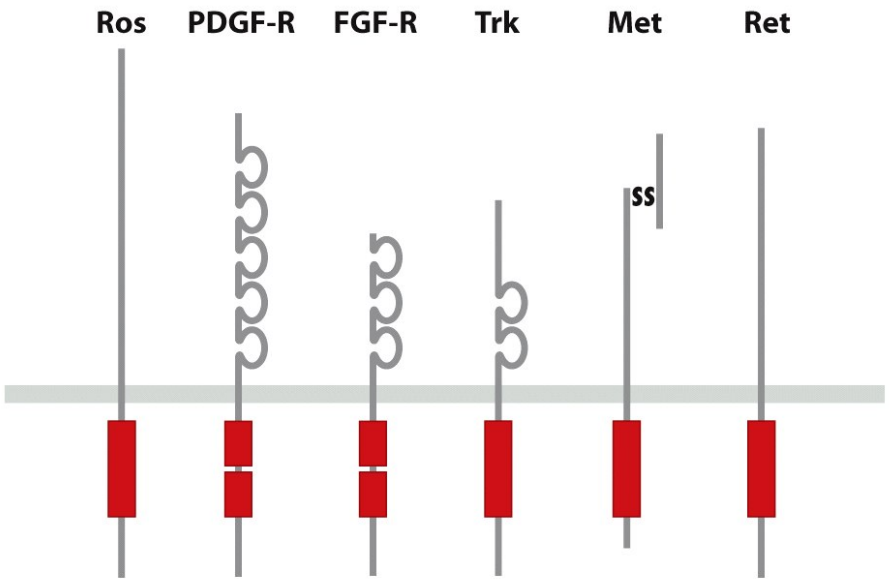
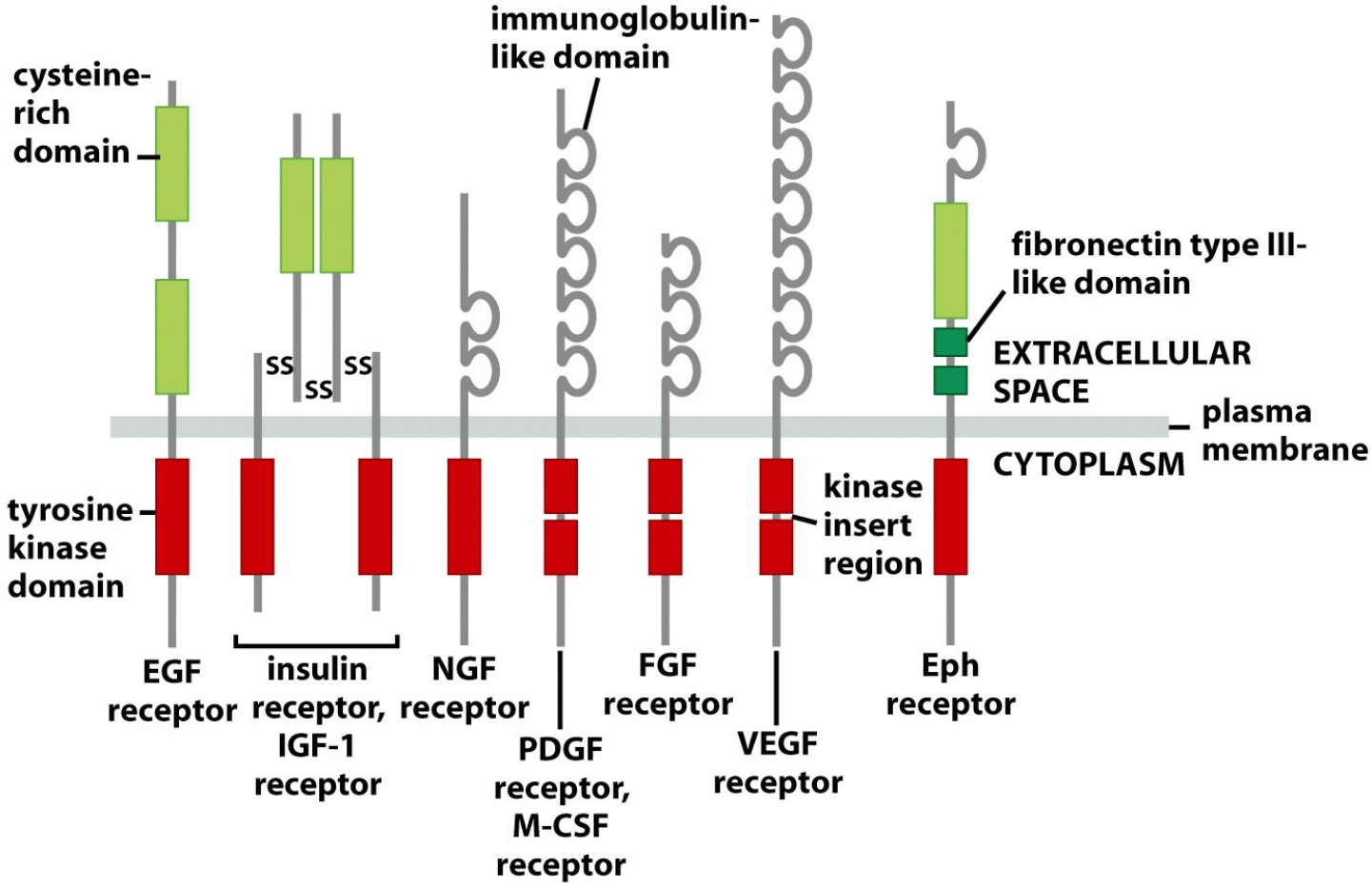
Fusion of genes for RTK with other genes



Constitutive dimerization of receptor as a result of **gene** fusion with **a gene that normally form dimers** or oligomers.

Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Structure and main classes of RTK



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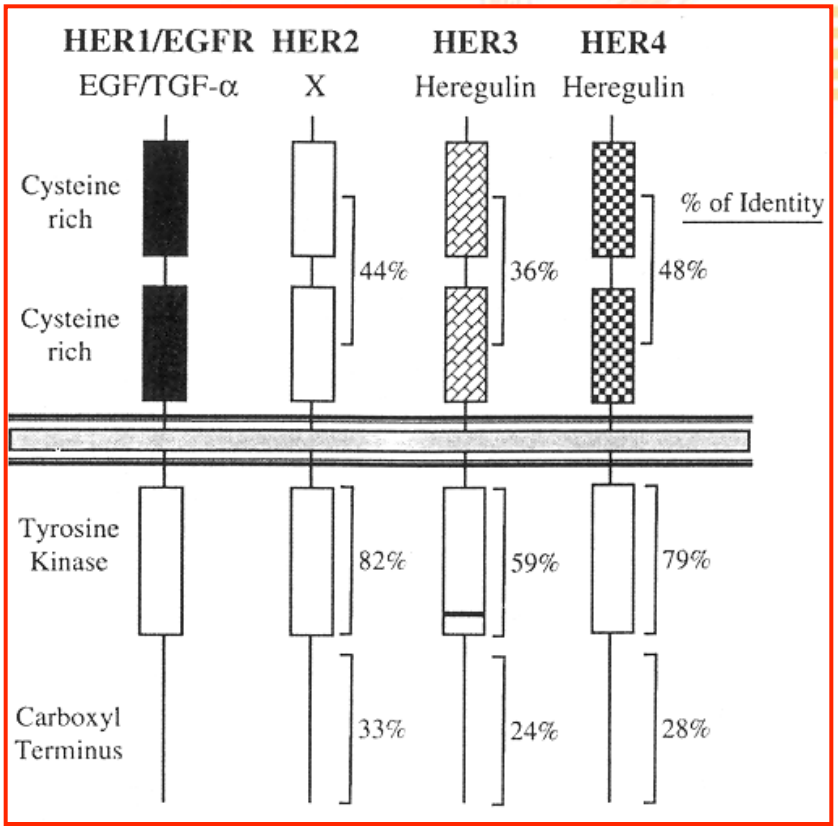
EGF family

Family of EGFR **receptors**:

erbB-1 (EGFR); *erbB-2* (HER-2/neu); *erbB-3* (HER-3); *erbB-4* (HER-4)

Growth factors, that binds to EGFR receptors: EGF, TGF-α, amphiregulin (AR), HB-EGF (heparin-binding EGF-like growth factor), cripto, betacellulin (BTC), epiregulin



EGF family

EGF (*epidermal growth factor*)

- 53 amino acids
- First identified and purified growth factor (Stanley Cohen – Nobel prize 1986)
- Produced as a precursor (1217 AA)
- Stimulates proliferation and differentiation of various cell types

TGF-α (*transforming growth factor α*)

- 50 AA, 40% homology with EGF, also mitogen
- First identified autocrine growth factor
- Produced as a membrane bound precursor, released by proteolytic cleavage in two steps
- Expressed by many cancer cell lines and in many tumors that express EGFR

EGFR family

EGFR (EGF receptor)

- Is encoded by **c-*erbB* (*ERBB*) *gene*** − homologous with v-*erbB* (carried by retrovirus *avian erythroblastosis virus*: viral counterpart has deleted extracellur domain)
- Sometimes denoted as erb-B receptors or HER (human EGF receptor)
- Function as homodimers and heterodimers

- Overexpression of EGFRs detected in breast, esophagus, stomach, ovarian, endometrial, cervical and other cancer; associated with poor prognosis in breast, esophagus and ovarian cancer
- Point mutations of ***EGFR1*** *(ERBB1)* in lung cancer : EGFR - target of anti-cancer therapy by small inhibitors of kinase domain (TKI), point mutations predict good response to gefitinib

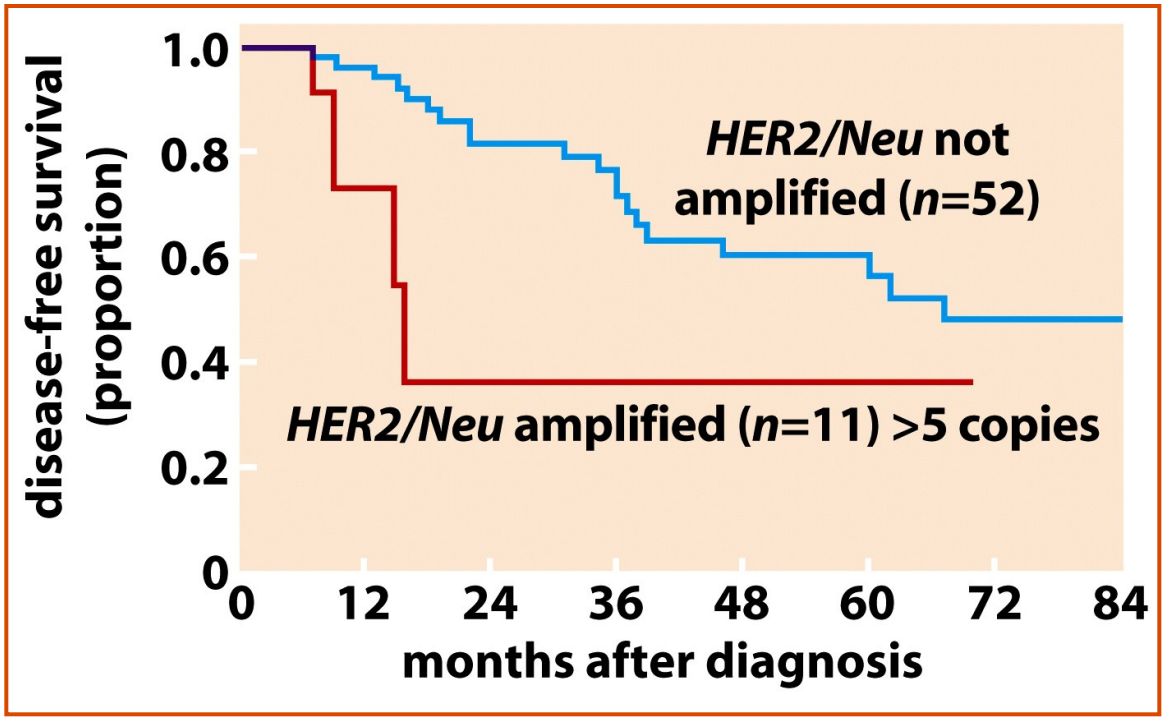
EGFR family

HER2/*Neu* encoded by gene ***ERBB2***

- amplification and/or overexpression of c-*erb*B2 (*ERBB2*) found in breast adenocarcinomas, stomach, salivary glands, ovarian and colorectal cancer
- In breast and ovarian cancer is amplification/overexpression of HER2/Neu associated with worse prognosis
- Amplification of *HER2*/Neu is prognostic and predictive marker in breast cancer: patients with HER2/Neu amplifications undergo more radical treatment and may receive **Herceptin** − monoclonal antibody specific for protein HER2/Neu

Amplification of *HER2*/neu in breast cancer

- Amplification of *HER2*/neu first described in breast cancer in **1987**
- Amplification resulting in minumum **5 copies of *HER2/Neu* gene per cell** is associated with poor prognosis



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EGFR-targeted anti-cancer therapy

A. Monoclonal antibodies

Herceptin (**Trastuzumab**) – anti ErbB2 (HER2): therapy of metastatic breast cancer with overexpression (amplification) of *HER2*/neu

Cetuximab (**Erbitux**) – anti EGFR: therapy of advanced CRC positive for *EGFR*

(Cetuximab binds EGFR with approx 50times higher affinity compared to EGF – mechanism of action is direct competition with ligand)

K-ras status needs to be determined! (Why?)

EGFR-targeted anti-cancer therapy

B. Small molecules – inhibitors of TK domain

Gefitinib (**Iressa**), **Erlotinib** (**Tarceva**) – block ATP binding site of EGFR: therapy of advanced non-small lung carcinomas; gefitinib is efficient only in a subgroup of patients with specific mutations of TK domain of EGFR

Lapatinib (**Tyverb®**)

– 4-anulinochinazolin

- dual inhibitor of HER1 and HER2
- inhibits intracellular tyrosinekinase domains of HER1 and HER2
- Used as a therapy of advanced metastatic breast cancer with overexpression of *HER2*

PDGF family

PDGF (*platelet-derived growth factor*) − efficient mitogen – stimulates **mesenchymal cells** (fibroblasts, adipocytes, endothelial cells, smooth muscle cells)

- homodimer (AA, BB) or heterodimer (AB) of two related chains A and B: B is encoded by c-*sis* proto-oncogene

- In 1983 the homology between B chain and **v-*sis* oncogene** transduced by Simian sarcoma virus: altered B chain is constitutively expressed that leads to the sustained stimulation of cells with PDGF receptor

- Elevated expression of PDGF A or B or both frequently detected in sarcomas, gliomas, lung, breast, esophagus, stomach and colorectal carcinomas

VEGF family

VEGF (*vascular endothelial growth factor*) / VPG (*vascular permeability factor*)

- Mitogenic effect on endothelial cells, important for **angiogenesis**
- VEGF/VPG has 18% sequence homology with PDGF A and B

FLT *(FLT1 =Fms-Related Tyrosine Kinase 1, VEGFR1)* encodes receptor for VEGF/VPG, binds VEGF with high affinity and mediates its biological function

other receptors for VEGF described: **KDR** (*VEGFR2*) and ***FLT4*** *(VEGFR3, modulates KDR signaling by forming heterodimers)*

receptor encoded by ***FLT3*** binds FLT3L not VEGF, expressed by haematopoetic stem cells, mutations in AML)

FGF family

- Large family −many polypeptides: FGF1 to FGF18, rather original names are used (not all of them stimulate fibroblasts for proliferation!!)
- **bFGF** (*FGF2*) a **aFGF** (*FGF1*) (*basic / acidic fibroblast growth factor*)
  - approx 17 kDa, they have 55% homology, similar function, name derived from different isoelectric points
  - Mitogens of mesenchymal, neuroectodermal and epiderimal origin, involved in **angiogenesis**
  - Autocrine growth factor in gliomas and meningiomas
  - Overexpression found in advanced astrocytomas

FGF family

FGF receptors - 4 genes: FGFR1 (*flg* nebo *fms*-like gen), FGFR2, FGFR3 and FGFR4 − in addition genes can be alternativelly spliced, resulting in complex group of receptors (almost 100 combinations) with different affinity to FGF factors

K-sam* −*FGFR2

- Overexpression correlates with invasiveness and poor prognosis of stomach cancer, amplifications in breast and lung cancer: tumors with over-active FGFR signaling treated with targeted therapeutics: ATP competitive TKI inhibitors dovitinib, ponatinib (also inhibits mutant FGFR2)

HGF family

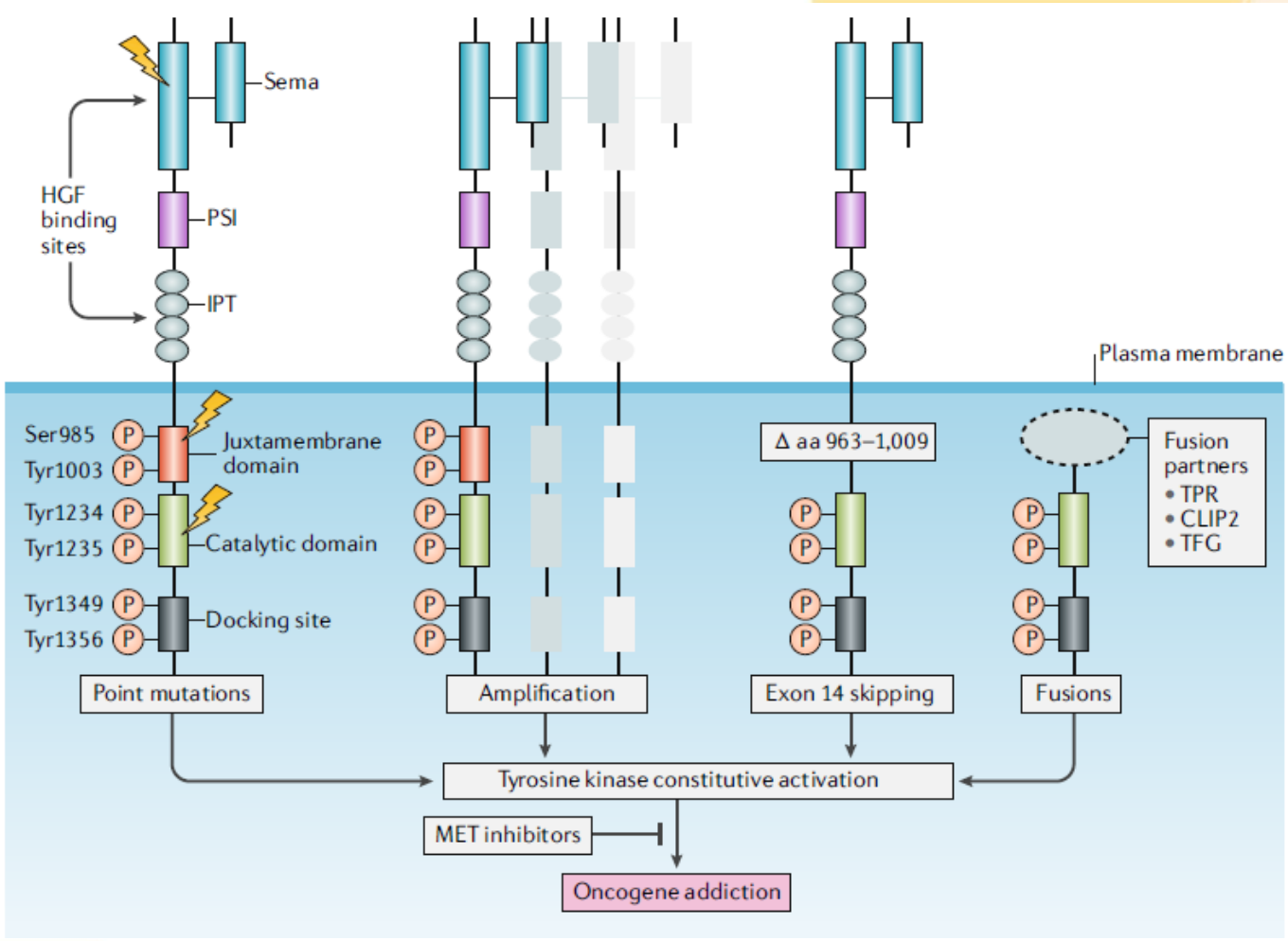
HGF (*hepatocyte growth factor*; hepatopoietin A)

- Stimulates proliferation of hepatocytes (not exclusively), involved in liver regeneration
- Is one of the **scatter factors** (SF): participates in loosening of the cell-cell interactions in epithelial tissues and blood vessels.

c-met (*MET*) encodes receptor for HGF

- Amplification of c-*met* (stomach carcinomas, melanomas)
- mutant oncogenic form of c-*met,* constitutively active (without binding of HGF): point mutations, shortened extracellular domain; gene fusions
- HGF produced by stromal cells (overexpression) and c-*met* is expressed on stromal and cancer cells: cooperation in growth and morphogenesis of some tumors

Oncogenic alteration of *MET*



Comoglio et al. *Nat Rev Cancer* 18: 341-357, 2018

Hereditary papillary renal cancer (HPRC)

- autosomal dominant syndrome; highly penetrant
- Caused by inherited mutations of **c-*met***; (locus 7q31)
- Papillary renal carcinoma represents cca 15 % of kidney cancer
- Predispose to multifocal, bilateral papillary renal tumors, not early metastatizing
- Not accompanied by congenital developmental defects
- Most germinal (as well as somatic) mutations are point missense mutations in TK domain of *MET*
- Met activation blocks **terminal differentiation** of renal tubular cells
- protooncogen c-*met* is mutated in more than 10 % of sporadic papillary renal cancers

RET

- orphan receptor
- potential ligands: GDNF (*glial cell line derived neutrotrophic factor*), neurturin, persephin, artemin
- Highest similarity with FGFR
- Expressed mainly in neural, neuroendocrine and nephrogenic tissues

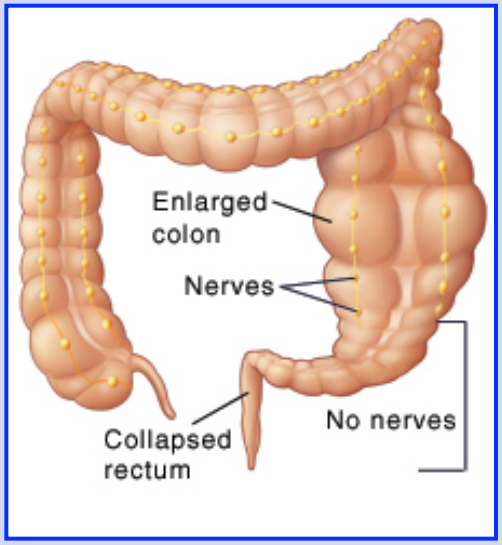
➢ 2 types of mutations described:

- **loss-of-function** mutations (inactivation of RET receptor) − associated with congenital disorder **Hirschprung's disease** (absence of some or all ganglion cells in myenteric and submucosal plexus of colon, causing chronic constipation)

- **gain-of-function** = oncogenic mutation − activation of RET

Hirschprung‘s disease

- 1886 – Harald Hirschprung described the disorder causing death of two infants
- Absence of inervation leads to insufficient peristaltics and chronic constipation
- appears in 18.6 per 100,000 of live births
- Combination of mutations in two genes *RET* (CH 10) and *EDNRB* (CH 13) accounts for most cases
- **RET receptor** assists cells of the neural crest in their movement through the digestive tract during the development of the embryo
- **EDNRB** connects these nerve cells to the digestive tract



Mutations in these two genes could directly lead to the absence of certain nerve fibers in the colon

Hirschprung‘s disease

- nearly 20% of affected children have associated developmental disorders and syndromes: often associated with Down syndrom (7%), deafness, medullary thyroid carcinoma, MEN2, neurofibromatosis, neuroblastoma
- Most cases are sporadic, familial burder increases risk 200x
- Multigenic disease with more than 10 involved genes, RET proto-oncogene is the most studied
- ***RET mutations***: half of hereditary and 7-35% of sporadic cases

Ježová M et al. *Cesk Patol* 55 (1): 53-59, 2019

RET

- **gain-of-function** = oncogenic mutations
  gain-of-function is caused by several types of mutations:

- **germline missense** mutations
  - Cause constitutive activation of RET
  - Linked with hereditary cancer syndromes with increased malignancy risk (autosomal-dominant inheritance):

    MEN type 2A and 2B (*multiple endocrine neoplasia*)

    and

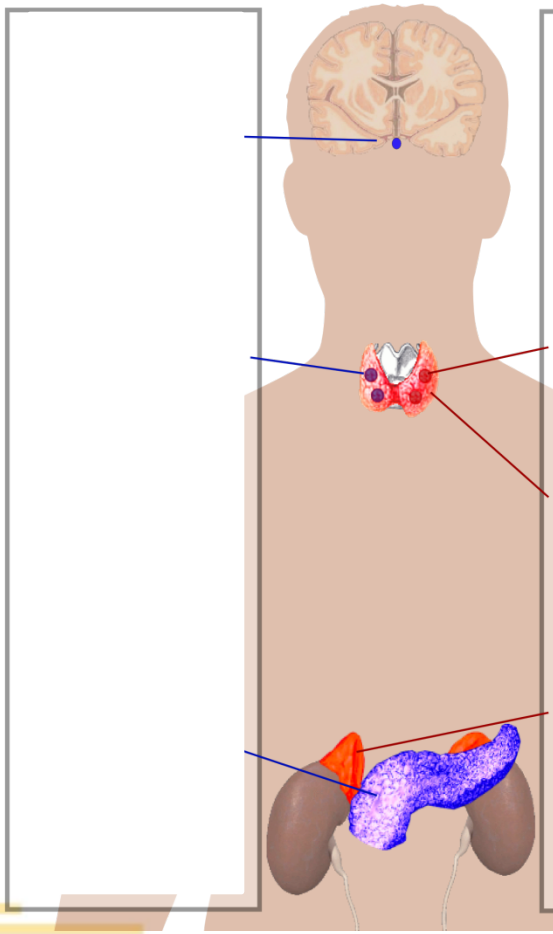
    FMTC (*familial medullary thyroid carcinoma*)

Multiple endocrine neoplasia type 2 (MEN2)

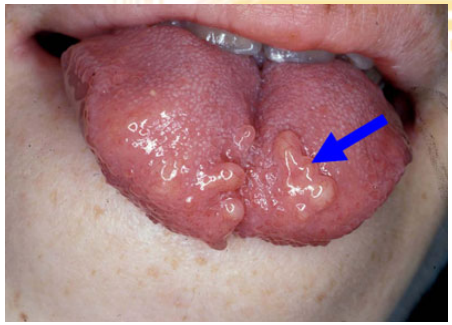
- Autosomal dominant inheritance associated with mutations of ***RET***
- gen locus: 10q11.2
- type A: high risk of medullary thyroid carcinomas, often combined with feochromocytomas or benign adenomas of the parathyroid glands

- type B: Additional features include mucosal neuromas of the lips and tongue.
- type A is more frequent compared to type B
- Medullary thyroid carcinomas occur at different age - in 70 years there is a 70% penetrance

Multiple endocrine neoplasia type 2 (MEN2)



MEN 2A	MEN 2B
	Mucosal neuromas
	Marfanoid body habitus
Parathyroid hyperplasia	
Medullary thyroid carcinoma	Medullary thyroid carcinoma
Pheo-chromo-cytoma	Pheo-chromo-cytoma



Pheochromocytomas

- tumor of the adrenal medulla (80%), approx 20% outside of adrenal gland (i.e. paraganglioma)
- 90% benign, approx 10% malignant-metastatic
- These neuroendocrine tumors are capable of producing and releasing massive amounts of catecholamines, metanephrines, without treatment it is a life-threatening disease („malignant“?)
- Mostly sporadic
- around 20% hereditary, in this case multifocal; occur as a part of cancer syndroms: MEN II (A, B), FMTC, VHL, NF1

Familial medullary thyroid carcinoma FMTC

- Less frequent disease compared to MEN, manifestation later in life and less aggressive
- Associated with specific ***RET*** mutations

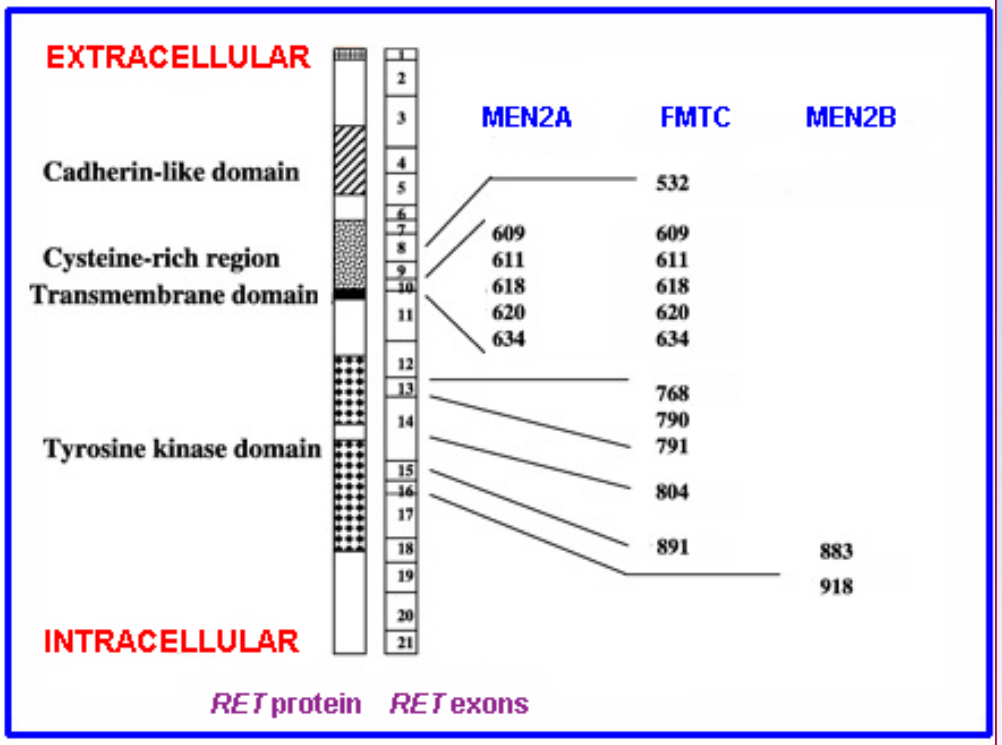
RET mutations in MEN2 vs. FMTC

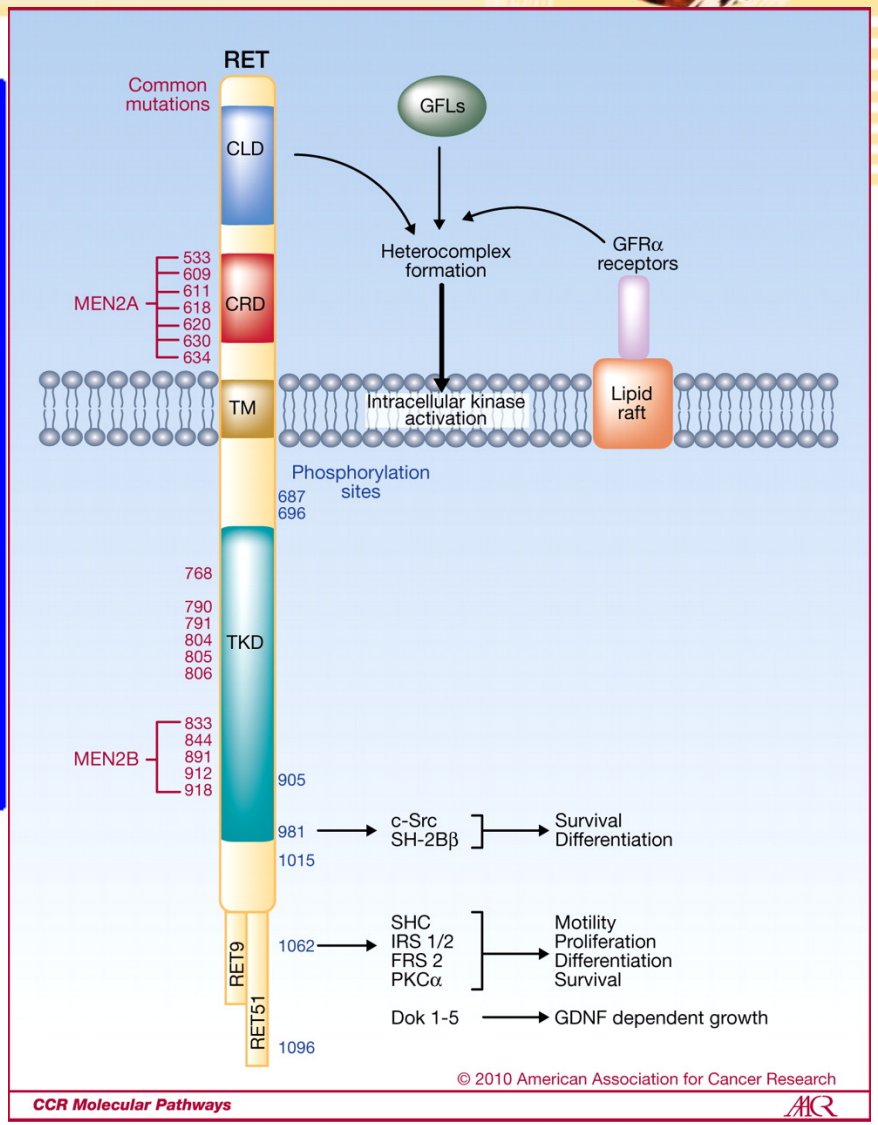
1. Mutations occur mostly in extracellular part of receptor in cystein rich domain: C609, 611, 618, 620, 634 (in MEN 2A and FMTC) causing permanent dimerization (by disulfidic bonds between cystein residues) thus activation of receptor without ligand binding

2. In some FMTC families mutations found in catalytic - kinase domain (codons 768, 804).

3. MEN 2B syndrom is associated with mutations in second part of kinase domain (codons 883, 918)

RET mutations in MEN2 vs. FMTC





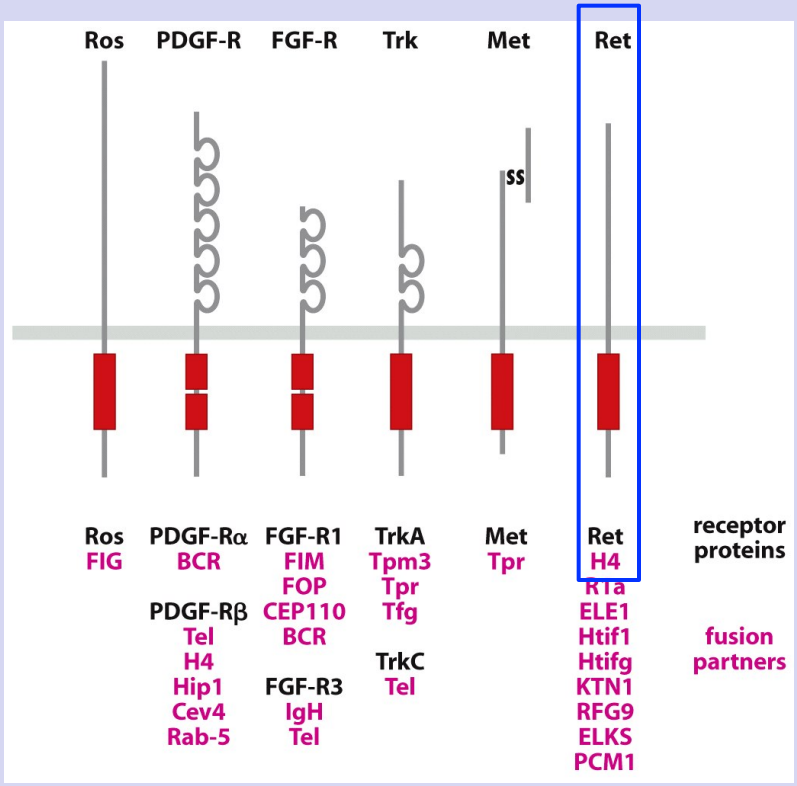
RET

- **gain-of-function**

- missense mutations

- **Translocations** resulting in deregulation of chimeric protein; RET discovered in 1985 as a proto-oncogene that underwent rearrangement during the transfection of DNA extracted from human T-cell lymphoma into NIH-3T3 cells (*RET – REarranged during Transfection*)
  - Translocation of 3´- terminus of *RET* with 5´- part of other gene occurs in thyroid papillary carcinomas: chimeric protein contains tyrosine kinase domain of *RET* and polypeptide that allows permanent dimerization

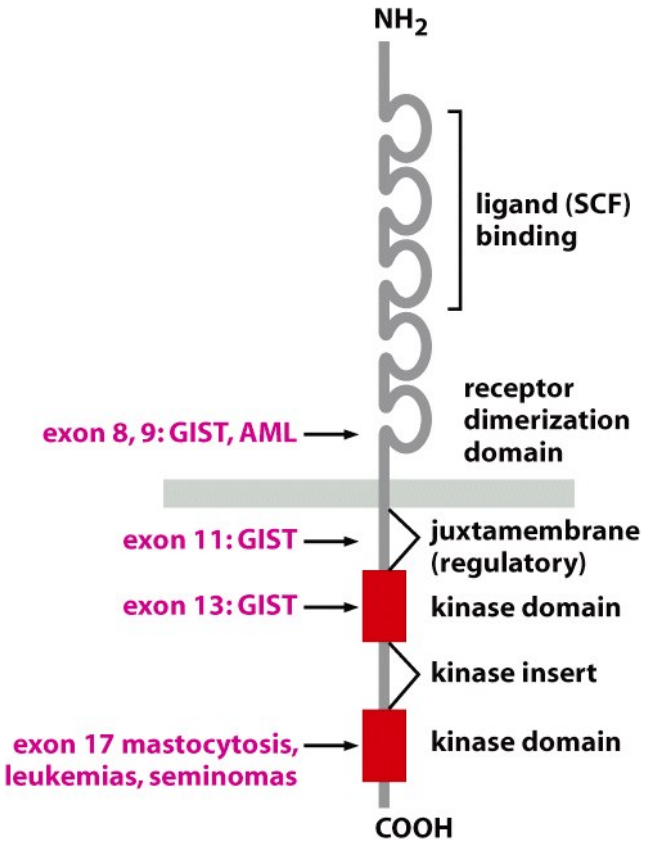
RET



- *radiation-induced human papillary thyroid cancer (PTC) is associated with chromosomal inversions that involve the genetic loci H4 and RET on chromosome 10*

RTK: Kit

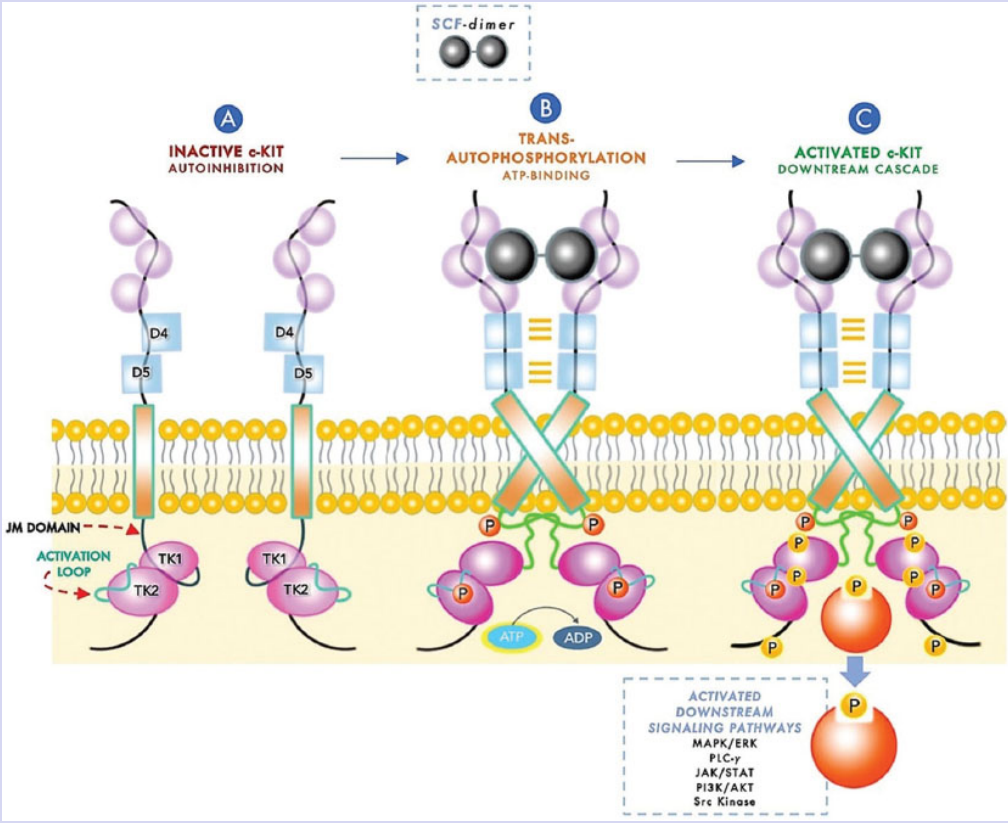
- Originally identified as an oncoprotein endoced by feline sarcoma retrovirus
- Ligand for this receptor is SCF (*stem cell factor*); stimulates some blood cells but also non-haematopoietic cells (e.g. melanocytes)
- Mutant forms of Kit transduce the signal constitutively, without ligation, typical mutations occur in juxtamembrane cytoplasmatic domain



Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Activation of Kit in cancer cells

- juxtamembrane (JM) domain negatively regulates Kit receptor
- Binding of ligand leads to transphosphorylation of tyrosine residues of JM domain. That opens JM domain to interact with N-terminal part of catalytical domain resulting in phosphorylation (activation) of kinase domain
- Mutations in JM domain result dimerization of Kit receptor, but receptor activity may be inhibited by TKI (unlike receptors with mutated catalytic domain)



Sheikh E et al. Bosn J Basic Med Sci, 22(5):683-698, 2022

Mutation of c-*kit*

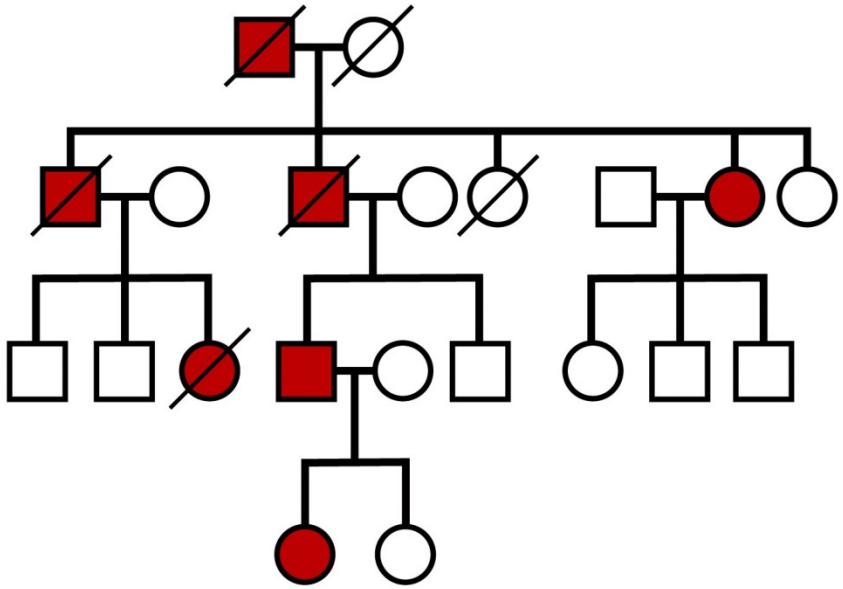
- GISTs (gastrointestinal stromal tumors) originate in mesenchymal cells of lower gastointestinal tract: normally function to ensure peristaltic contractions of smooth muscle cells (experimental mutations of Kit in these cells prevent this function in mice)

- **activating** mutation of c-*kit* found in almost all GISTs, frequently found in leukemias as well; often point mutations of JM domain, sometimes deletions of JM domain (worse prognosis) and mutations in catalytic domain.

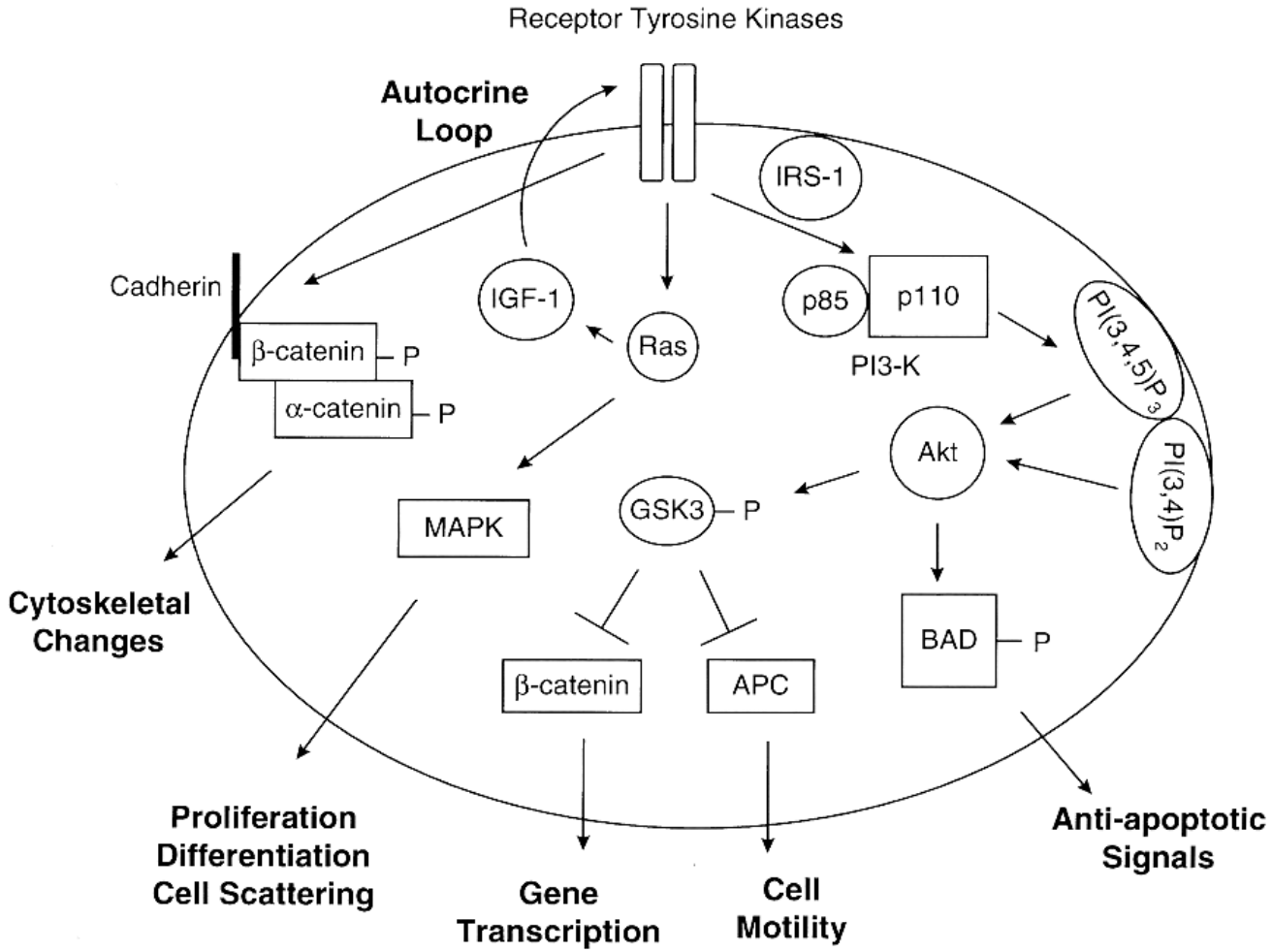
- (hereditary inactivating Kit mutations − piebaldisms − absence of melanocytes in certain areas of skin and hair)

Familial gastrointestinal stromal tumors (GISTs)

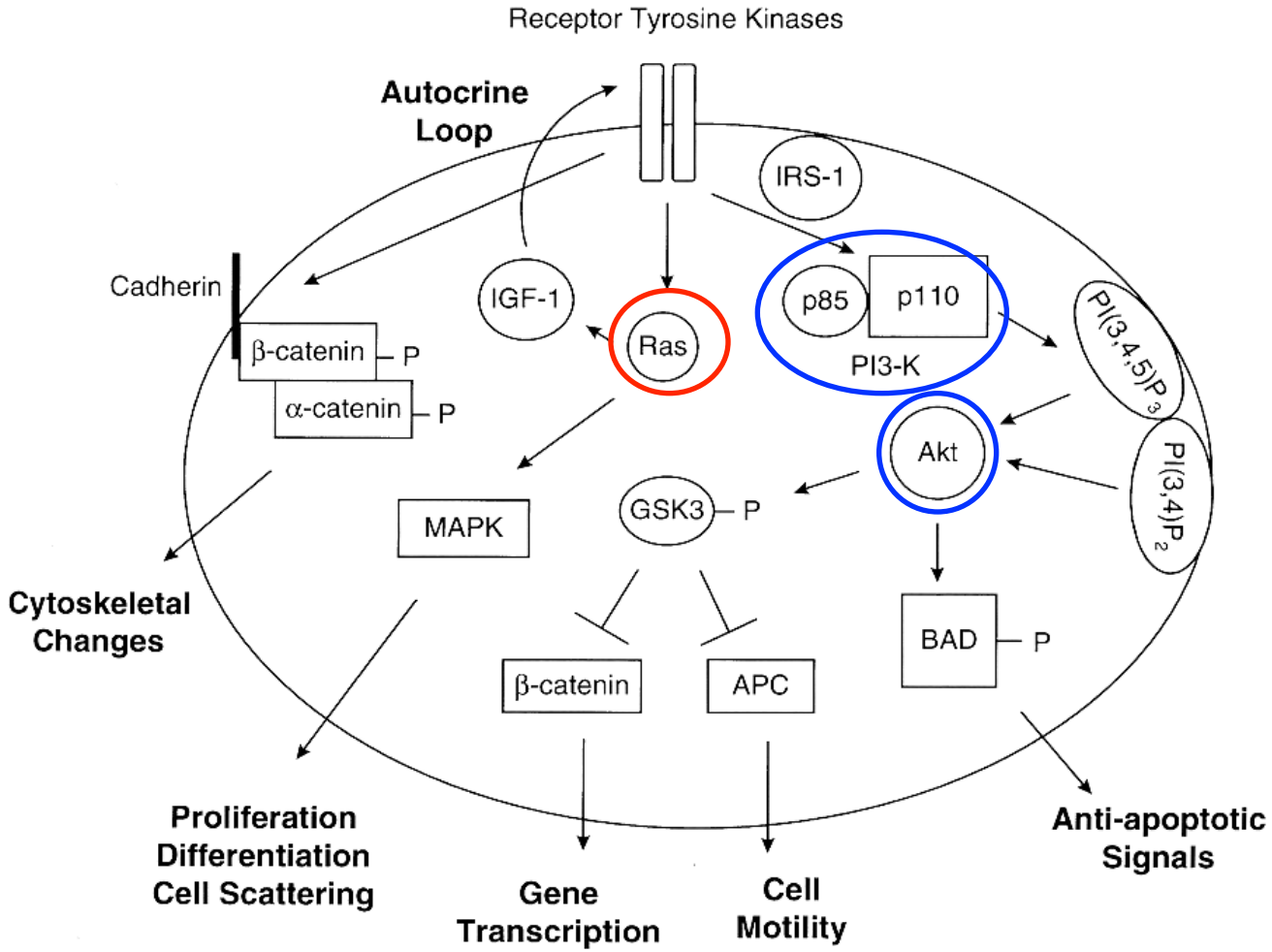
- hereditary GISTs associated with activating c-***kit*** mutations in germinal lineage
- Set of germinal mutations is similar to somatic mutations occuring in sporadic GISTs
- Gen occupies locus 4q11-12
- Example of a family with germinal Kit mutation:
- Deletion of 1 AA in JM domain
- Manifestation of the mutation late in life, thereby present in 4 generations



Signaling pathways activated by RTK



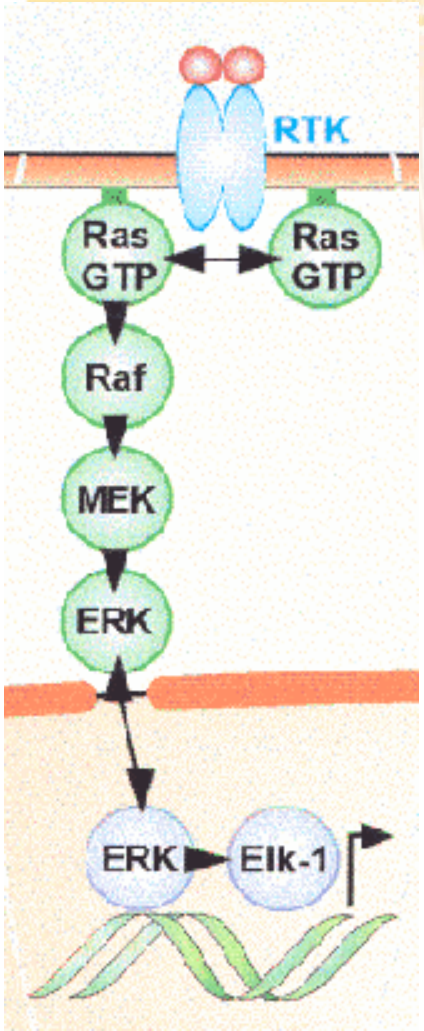
Signaling pathways activated by RTK



Ras proteins

ras/Ras − first identified oncogene

Originally thought to function as mediator of **Raf-1** binding to plasmatic membrane



Proteiny Ras

Current knowledge show that:

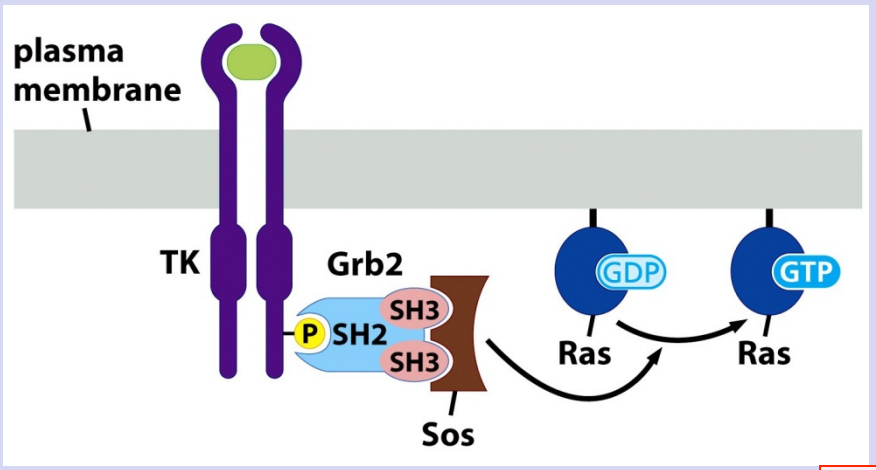
- Raf-1 activation is a **complex multistep** process. Direct interaction between Ras and Raf-1 is mediated by several binding sites in Ras and at least 2 binding sites of Raf-1

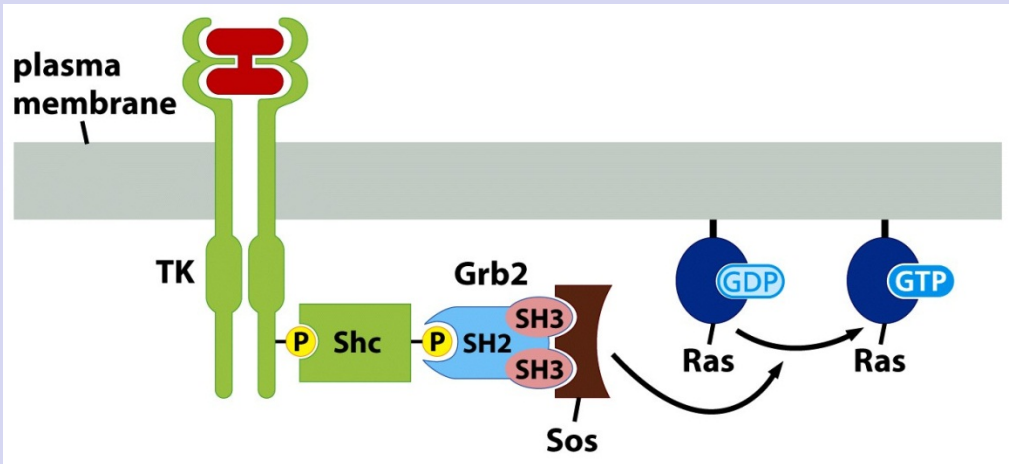
- Raf-1 is not the only substrate (*downstream* effector) of Ras proteins.

- **Ras superfamily** consists of 35/65 different members (RAW/ 2000). All have some sequence homology, but differ in signaling capacity.

Signal transmission from RTK to Ras

- Even transmission process is more complex than originally estimated
- activated RTKs recruit the adaptor protein GRB2 that binds phosphotyrosine consensus sequences via its SH2 domain. Via its SH3 domains, GRB2 then recruits the guanine nucleotide exchange factor SOS to the plasma membrane, where it activates the membrane-localized small G protein RAS by exchanging GTP for GDP





Weinberg RA. The Biology of Cancer. *Garland Science* 2007

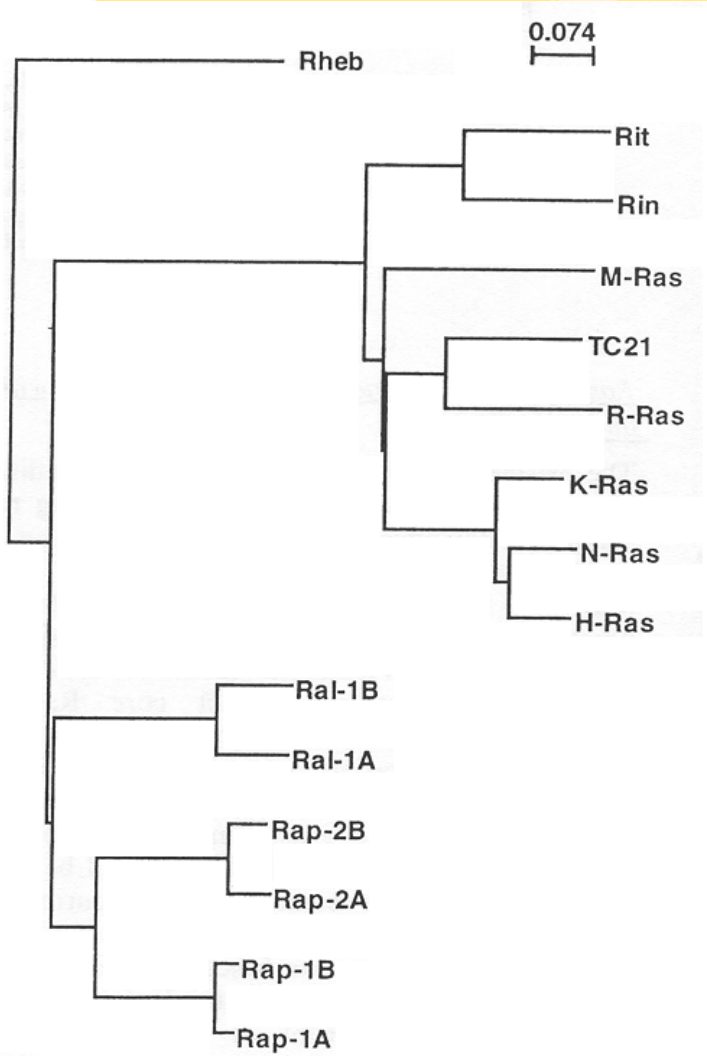
Ras proteins

Ras superfamily has at least 65 members:

Families: Ras, **Rho**, Rab, Ran, Rad, Arf

Ras family has subfamilies: Rap, **Ral**, R-Ras, Rheb, H-Ras, Ras

➢Together called **small G proteins**



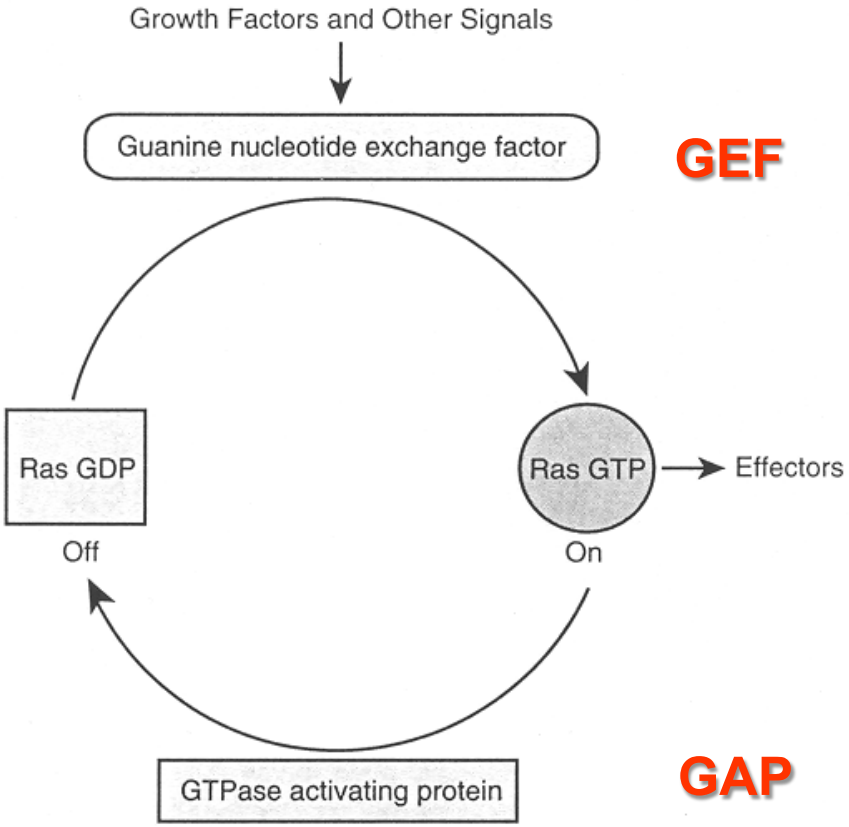
Ras proteins

Proteins **H-Ras**, **N-Ras**, **K-Ras4A** and **K-Ras4B** have 188/189 AA. They reside at the cytosolic side of plasma membrane and function there as molecular switches during signal transduction to cytoplasm.

Ras proteins are GTPases: are inactive in GDP-bound form and active if GDP is exchanged for GTP. Capacity of Ras proteins to exchange of GTP/GDP and hydrolyse GTP is small, thereby other proteins participate:

- **Guanine nucleotide exchange factors (GEFs)**, facilitate formation of active GTP-bound Ras protein, help to GDP to dissociate from small GTPase (e.g. SOS1/2, RasGRF/ mCDC25,...)
- **GAPs**: GTPase-activating proteins, act antagonistically to inactivate GTPases by increasing their intrinsic rate of GTP hydrolysis (p120 GAP, NF1/neurofibromin,...)

GTPase cycle of Ras proteins



GEF

GEFs help to dissociate GDP from Ras − active Ras

GAP

GAPs facilitate GTP hydrolysis − formation of inactive Ras

Ras proteins

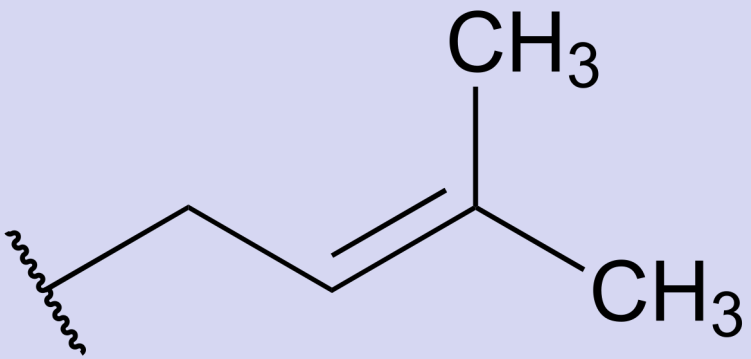
Posttranslational modifications:

Interaction with inner part of cytoplasmatic membrane (required for Ras activation) is mediated by posttranslational modifications of CAAX motif at C-terminus of Ras

Cys186 of motif CAAX is farnesylated

Intermezzo: Prenylation

- **Prenylation** – the covalent attachment of a lipid consisting of either three (farnesyl) or four (geranylgeranyl) isoprene units (intermediates of cholesterol biosynthesis) to a free thiol of a cysteine side chain at or near the C-terminus of a protein.

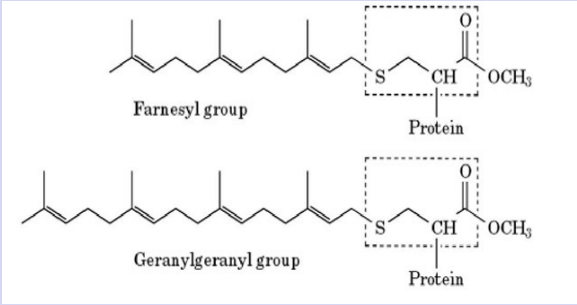


Prenyl group

Intermezzo: Prenylation

farnesylation – attachment of farnesyl group (15 C)

geranylgeranylation – attachment of geranylgeranyl group (20 C)



- Catalysed by 3 prenyltransferases: farnesyltransferase (FT), geranylgeranyltransferases I and II (GGTI and GGTII).

- There is more than 300 proteins in humans with Cystein at C-terminus, but it is unknown how many are prenylated
- Farnesylation confirmed for: H-, K- and N-Ras, nuclear lamina, CENPE (kinetochors); geranylgeranylation: Rho A and C, Rac1, cdc-42;
- RhoB – farnesylated and geranylgeranylated;
- K-Ras – geranylgeranylovated, if FT is inhibited

Ras proteins

Posttranslational modifications:

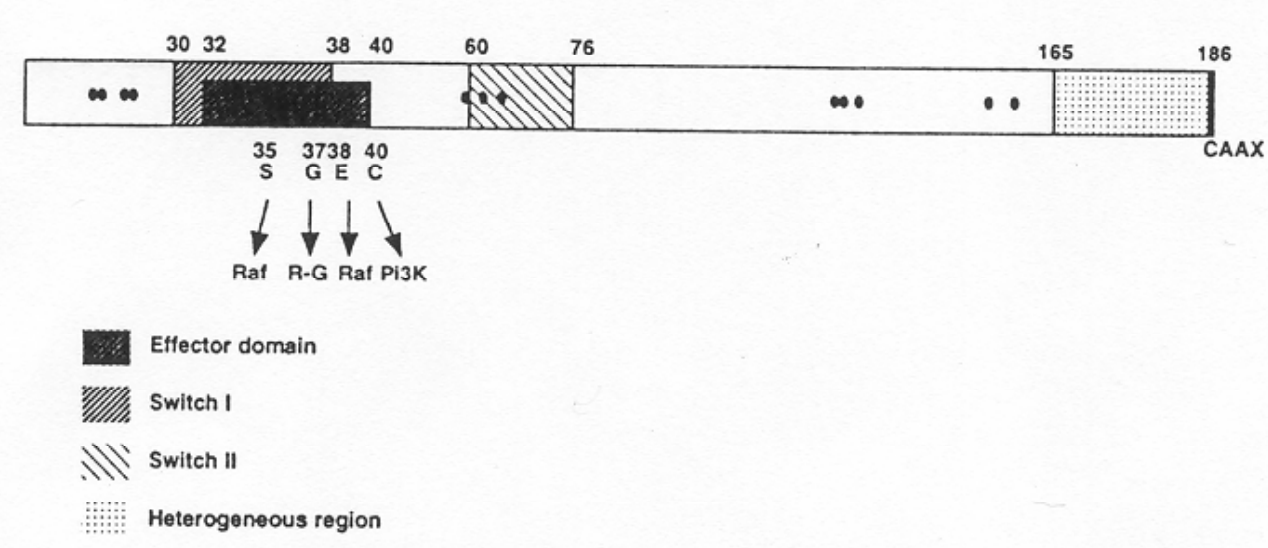
Interaction with inner part of cytoplasmatic membrane (required for Ras activation) is mediated by posttranslational modifications of CAAX motif at C-terminus of Ras:

- Cys186 of CAAX motif is farnesylated
- proteolytic cleavage of AAX sequence
- terminal Cys186 is carboxymethylated

- protein with these modification is more hydrophobic and has higher affinity to cell membrane

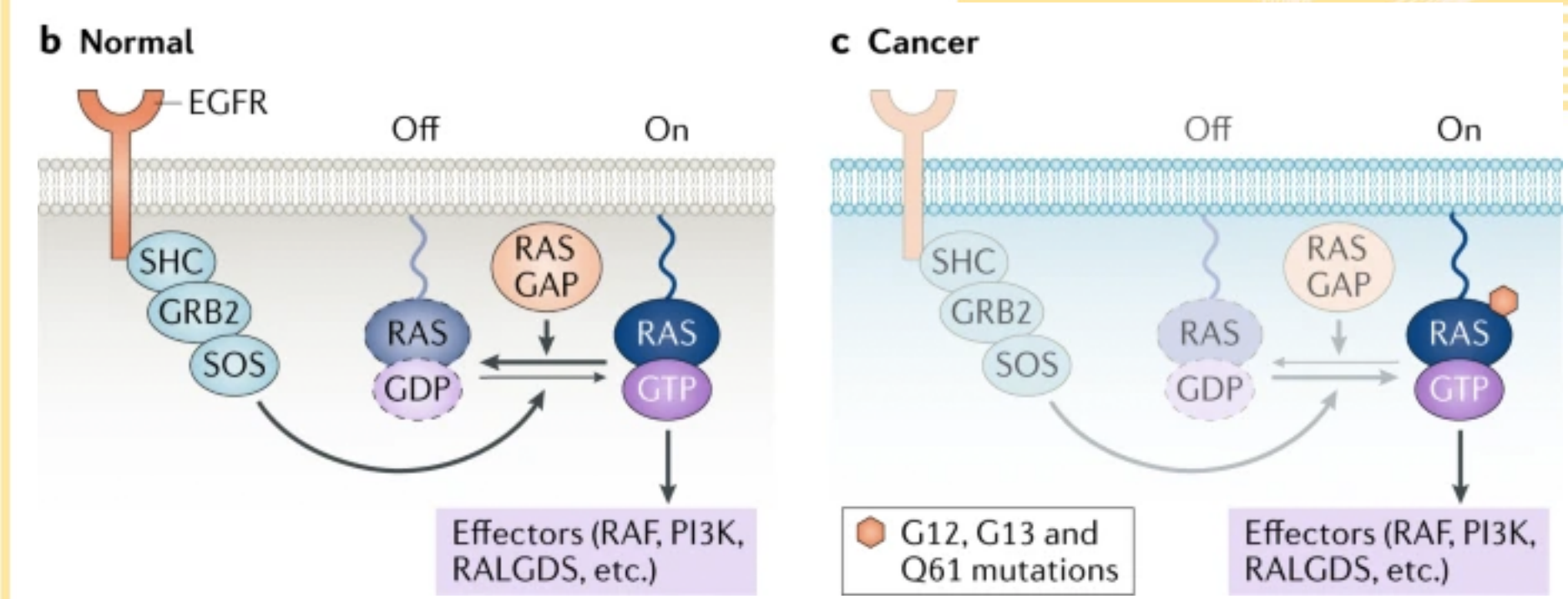
For oncogenic activation of Ras is crucial **farnesylation**!

Stucture of Ras proteins



- Heterogenous domain: Ras proteins are highly homologous in first 164AA, the C-terminus (25AA) is variable
- Effector domain (AA 32-40) mediates interaction with effector molecules
- Switch I (30-38) major binding site for GAPs
- Switch II (60-76) binding of GEFs

Oncogenic activation of Ras



Siqi et al. Nat Rev Cancer, 2018 Dec;18(12):767-777.

Oncogenic activation of Ras

Oncogenic mutation of *Ras* detected in almost 35% of human cancer.

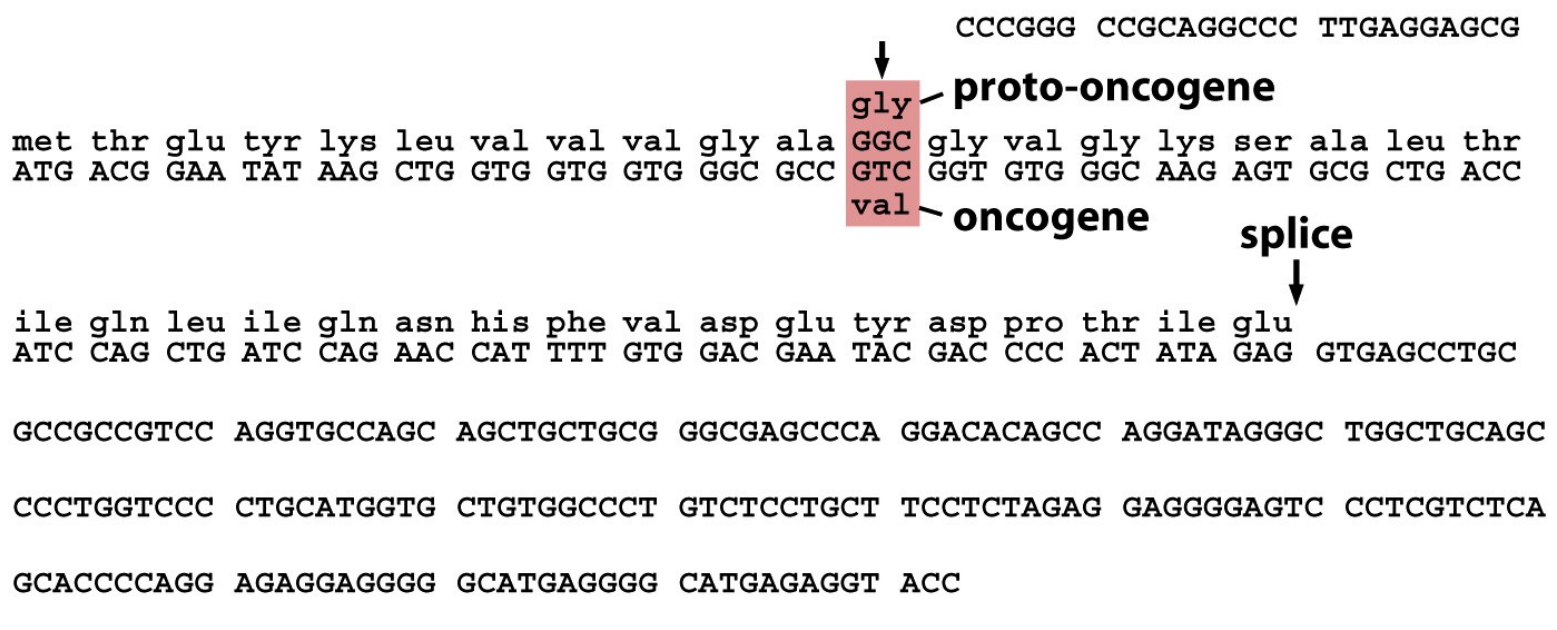
- Mutation of **H-*ras*** mostly in skin cancer and head and neck squamous carcinoma
- Mutation of **K-*ras*** mainly in adenocarcinomas, colorectal carcinomas and pancreatic cancer
- Mutation of **N-*ras*** frequent in haematopoetic cancers, mostly in AML and MDS

Oncogenic mutations modify Ras activity:

- In codons: **12**, **13**, **59**, **61**, **63** − altered proteins have impaired GTPase activity and are resistant to GAPs
- In codons: **16**, **19**, **116**, **117**, **119**, **144**, **146** − resulting proteins have facilitated exchange of GDP for GTP
- Some tumors overexpress Ras

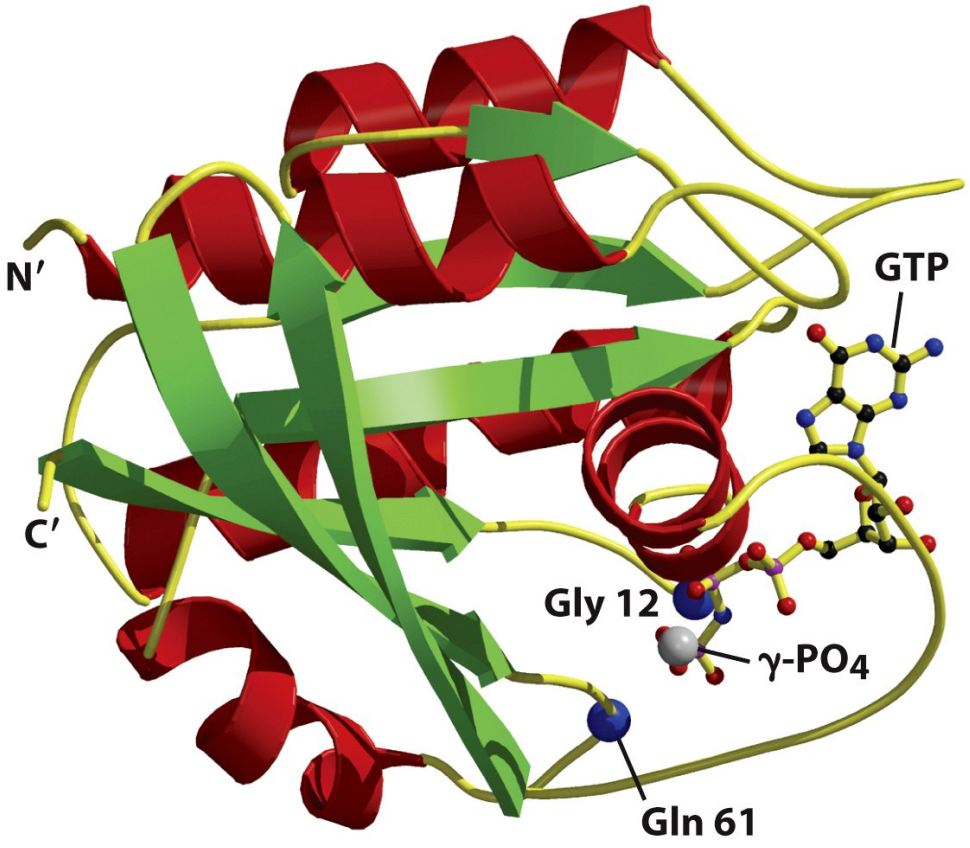
Oncogenic activation of Ras

1982: Ras mutation in codon 12 identified (at the same time in 3 laboratories!). It is the first point mutation causally associated with cancer development.



Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Oncogenic activation of Ras



Glycine **12** and glutamine **61** are critical for binding of GFP to Ras

That is why these codons are mostly mutated in cancer

Weinberg RA. The Biology of Cancer. *Garland Science* 2007

Inhibition of Ras proteins

- for oncogenic features of Ras is crucial farnesylation!

➢ **Inhibitors of farnesyl transferases (FTIs)**

(e.g. CAAX peptides − cell-permeant, specific and efficient, low cytotoxicity)

- By inhibiting farnesylation they prevent post-translational modifications of Ras and thereby its activity
- Potential anti-cancer therapeutics, but:
  - Ras proteins have many functions in cells and FTIs blocks all of them (side effects)
  - Ras proteins may be also geranylgeranylated (and FTIs do not inhibit geranylgeranyltransferases)
  - Ras proteins are not the only targets of FTIs (Rho)

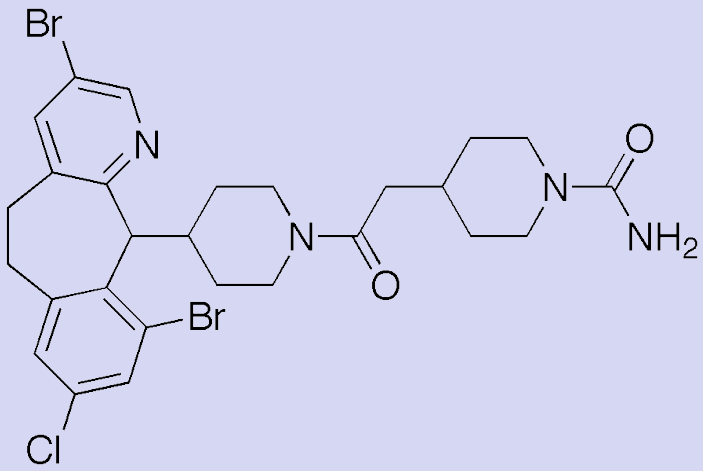
FT inhibitors (FTIs)

At least 6 inhibitors tested in clinical trials:

1. **BMS-214662** (Bristol-Myers Squibb, Princeton, NJ)
2. **L778123** (Merck and Co., Inc., Whitehouse Station, NJ)
3. **lonafarnib** (experimental name SCH66336; Sarasar™; Schering-Plough Corporation, Kenilworth, NJ)
4. **FTI-277** (Calbiochem, EMD Bio-Sciences, San Diego)
5. **L744832** (Biomol International L.P., Plymouth Meeting, PA)
6. **tipifarnib** (experimental name R115777; Zarnestra®; Ortho Biotech Products, L.P., Bridgewater, NJ); most advanced testing

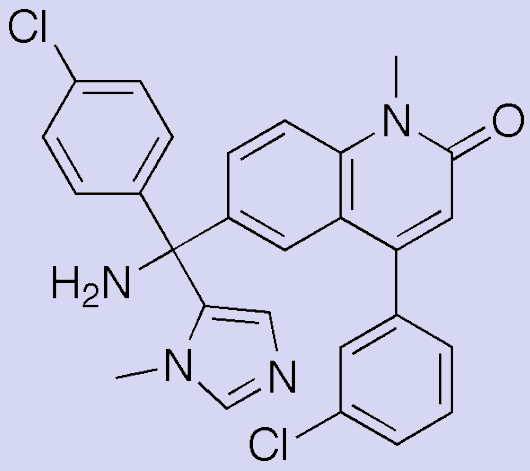
Lonafarnib: FT inhbitors

- synthetic tricyclic halogenated carboxamide with antineoplastic properties
- used primarily for cancer treatment
- A coctail of lonafarnib and two other drugs proved beneficial for patients with progeria.



Tipifarnib: FT inhibitors

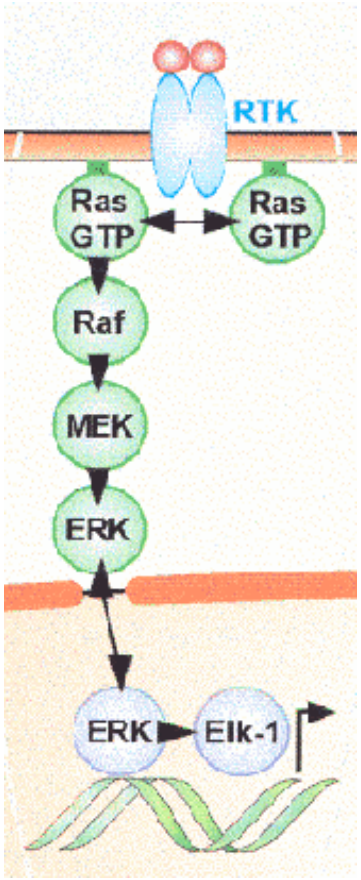
- Clinical trials: administration of Tipifarnib to older patients with **AML** (aged 65 and more) – not approved by FDA, not efficient anti-cancer activity (2005)
- Further tested for efficiency in breast cancer, neurofibromatosis, and other malignacies

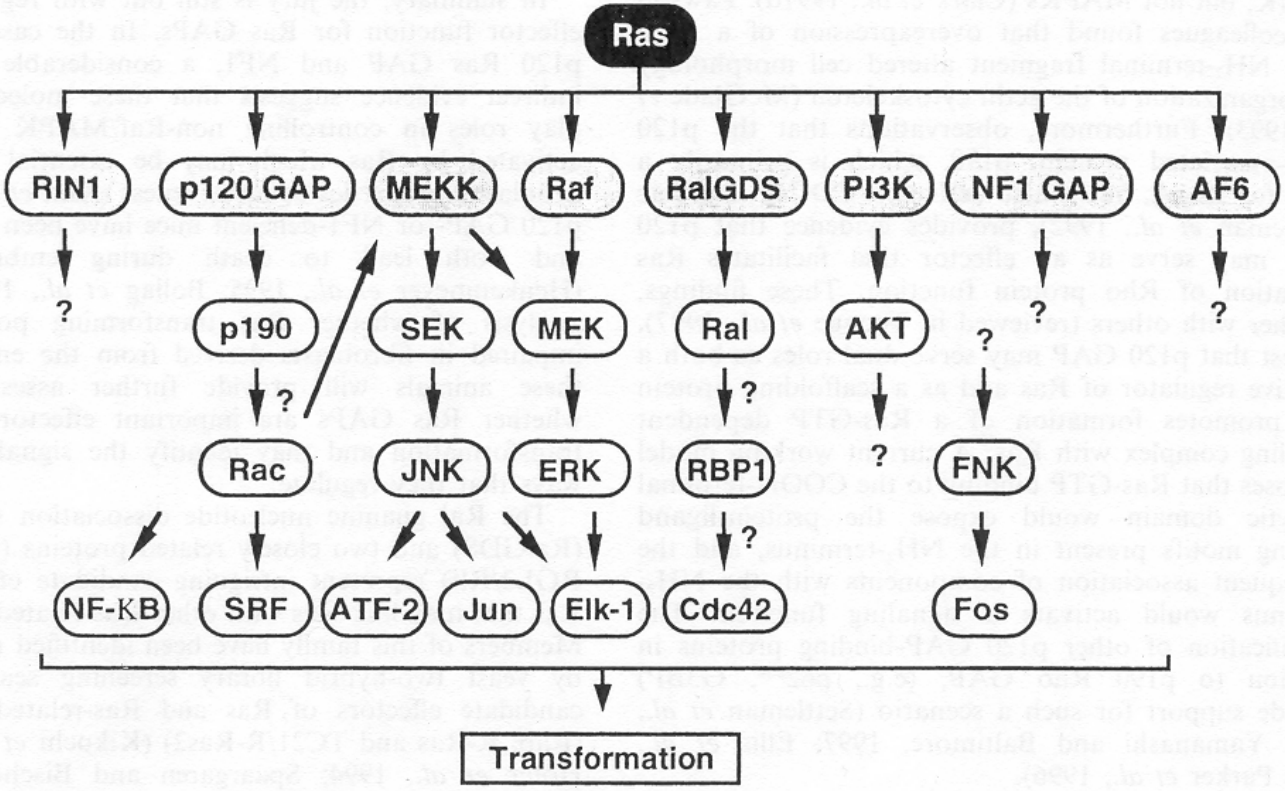


FT inhibitors (FTIs)

- Not satisfactory outcome in clinical trials so far
- Side effects
- Not clear whether they function primarily via Ras proteins

Ras proteins have many effectors





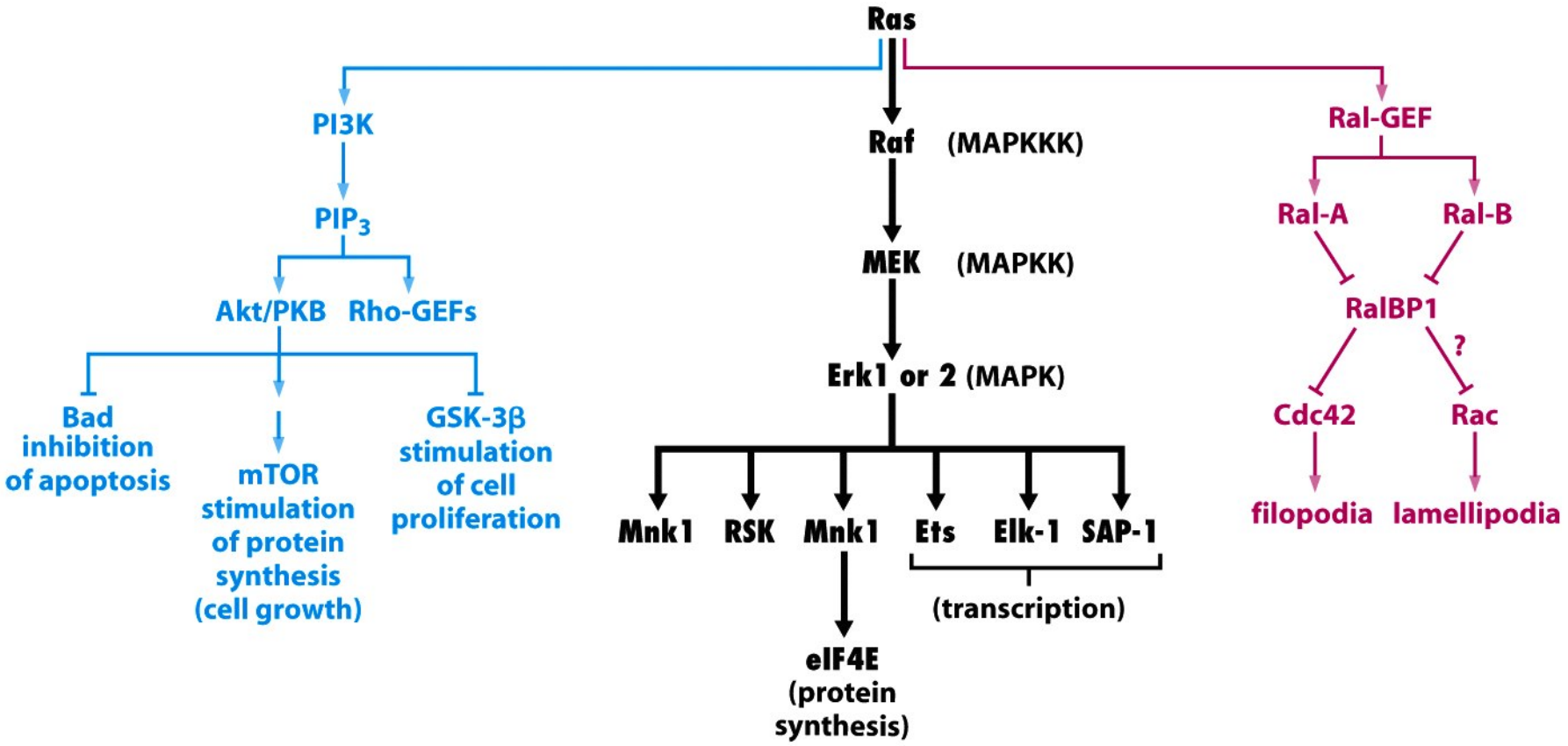
Ras triggers 3 key effector pathways

1. **Raf** pathway (activation of genes stimulating cell growth/ division, anchorage-independent growth, loss of contact inhibiton)

2. **PI3K** pathway (anti-apoptotic effect)

3. **Ral** pathway (facilitates invasion and metastatic dissemination of tumors)

1. Ras ⇒ Raf


Ras
PI3K
PIP3
Akt/PKB
Rho-GEFs
Bad
inhibition
of apoptosis
mTOR
stimulation
of protein
synthesis
(cell growth)
GSK-3β
stimulation
of cell
proliferation
Raf (MAPKKK)
MEK (MAPKK)
Erk1 or 2 (MAPK)
Mnk1
RSK
Mnk1
Ets
Elk-1
SAP-1
(transcription)
eIF4E
(protein
synthesis)
Ral-GEF
Ral-A
Ral-B
RalBP1
?
Cdc42
Rac
filopodia
lamellipodia

Raf proteins

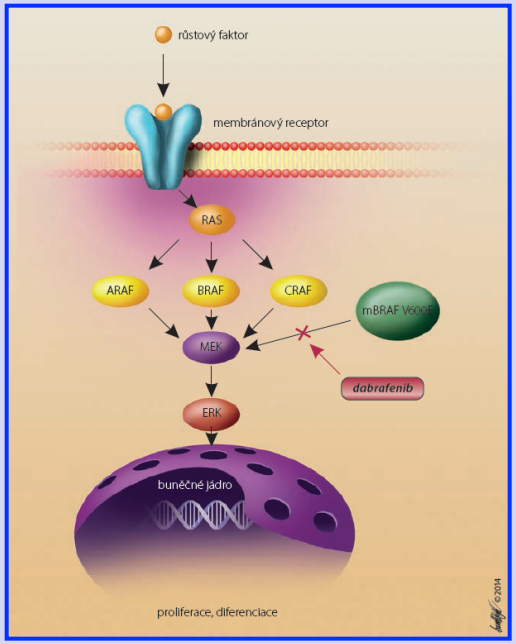
- **Raf**: cytosolic proteins, recruited to the cell membrane by activated Ras – and activated itself
- active Raf stimulates **MEK** (*MAP-kinase extracellular signal-regulated kinase*) by serine phosphorylation
- active MEK is dual kinase: phosphorylates both tyrosine and serine/ threonine of target molecules: including MAP kinases **p44 ERK1** a **p42 ERK2** (*extracellular signal-regulated kinases*)
- ERKs have many substrates in cytosol (**Rsk**, **SOS**, **MEK**,...) and (upon nuclear translocation) also nuclear (**Ets**, **Elk-1**, **SAP-1**,...)
- **Jun** and **Fos** belong among the target TFs of this pathway

- almost 60-70% melanomas have mutated *B-Raf* „instead of“ *Ras*

BRAF

- *BRAF* gene (7q34) has 18 exons, mRNA is 2478 bp long
- Mutations of *BRAF* are associated with many different cancers: : colorectal carcinoma, malignant melanoma, Non-hodgkins lymfoma, thyroid papillary carcinoma, non-small cell lung carcinoma, lung adenocarcinoma

- **80%** of mutations is caused by T1799A transversion that leads to amino acid exchange **V600E**
- This mutation is transforming, because it simulates T599 phosphorylation and/or S602 phosphorylation in activating domain of BRAF; mutated BRAF is thus in permanently active mode, independent on Ras signaling



Krajsová I. Dabrafenib v léčbě metastazujícího melanomu. *Remedia* 24 (3): 215-219, **2013**

RASopathy

- RAS/MAPK Syndromes
- Group of neuro-cardio-facio-cutaneous syndromes (NCFC)
- Specific group of developmental disorders caused by germinal mutations of genes encoding proteins of RAS/MAPK signaling cascade
- phenotypically similar group of syndromes: symptoms- e.g. characteristic facial features, cardiac defects, cutaneous abnormalities, neurocognitive delay and a predisposition to malignancies
- Noonan syndrome, neurofibromatosis type 1, Legius syndrome, Costello syndrome a další
- Each syndrome exhibits unique phenotypic features, however, there are numerous overlapping phenotypic features (early diagnosis is difficult)
- Overall RASopathies are one of the largest group of malformation syndromes affecting 1 in 1000 subjects

RASopathy

- somatic mutations in RAS/MAPK pathway lead frequently to malignant diseases. That is why much higher rate of cancer development was expected in RASopathies: risk is only 3,5x higher than in normal population; the most prevalent are haematologic malignancies

- Some activating somatic mutations are the same as germinal mutations, but RASopathy-associated mutations are less activating: e.g. The most frequent somatic oncogenic mutation of ***BRAF*** (**V600E**) does not occur in cranio-facio-cutaneous syndrome (typically caused by *BRAF* mutations)

➢ It is assumed that strongly activating oncogenic mutations **are** lethal in germ cells

Svobodová E. Molekulární diagnostika syndromu Noonanové a přidružených RASopatií pomocí NGS. *DP PřF*, **2018**

RASopathies: overview of syndromes

Syndrome	Phenotype	Mendelian inheritance in man	Gene(s) mutated	Reference
Neurofibromatosis type 1 (NF-1)	Café-au-lait spots; intertriginous freckling; neurofibromas and plexiform neurofibromas; Iris Lisch nodules; osseous dysplasia; optic pathway glioma; mild neurocognitive impairment	16220	*NF1*	[5]
Noonan syndrome (NS)	Facial dysmorphism (improves with age: high forehead, hypertelorism, downslanting palpebral fissures, epicanthal folds, ptosis, low-set and posteriorly rotated ears); feeding difficulties in infancy (75%); congenital heart defect (in 80%: pulmonic stenosis, hypertrophic cardiomyopathy, atrial septal defect); cryptorchism (60-80% of males); bleeding tendency (60%); spinal abnormalities (30%); intellectual impairment (20%); lymphatic abnormalities (20%); sensorineural hearing loss (10%).	605275	*PTPN11*, *KRAS*, *SOS1*, *RAF1*, *NRAS* *PTPN11*[6]	Clinical picture[6,7] *SOS*[9,10] *KRAS*[11] *RAF*[12,13] *NRAS*[14]
NS-like syndromes	*CBL-syndrome:* In comparison to NS: less often congenital heart disease; in addition to NS: delayed brain myelination, hypoplasia of the cerebellar vermis, café-au-lait spots.	613563	*CBL*	[15,16]
	NS-like disorder with loose anagen hair. NS-like facial features and hairless and darkly pigmented skin with eczema or ichthyosis.	607721	*SHOC2*	[17]
Costello syndrome (CS)	Coarse facial features, benign nasal and perianal cutaneous papilloma; premature aging and hair loss, loose skin; developmental delay, moderate intellectual disability; congenital heart defect (most commonly pulmonic stenosis); predisposition to solid tumors (rhabdomyosarcoma, neuroblastoma, bladder carcinoma)	218040	*HRAS*	[18,19]
Cardio-facio-cutaneous (CFC) syndrome	Shares features with NS and CS; ectodermal abnormalities with sparse curly hair and sparse or absent eyelashes.	115150	*BRAF*, *MEK1*, *MEK2*, *KRAS*	[20,21]
Legius syndrome (NF-1-like syndrome)	Shares many phenotypical features with NF-1, Lisch nodules and central nervous tumors are, however, absent.	611431	*SPRED1*	[22]

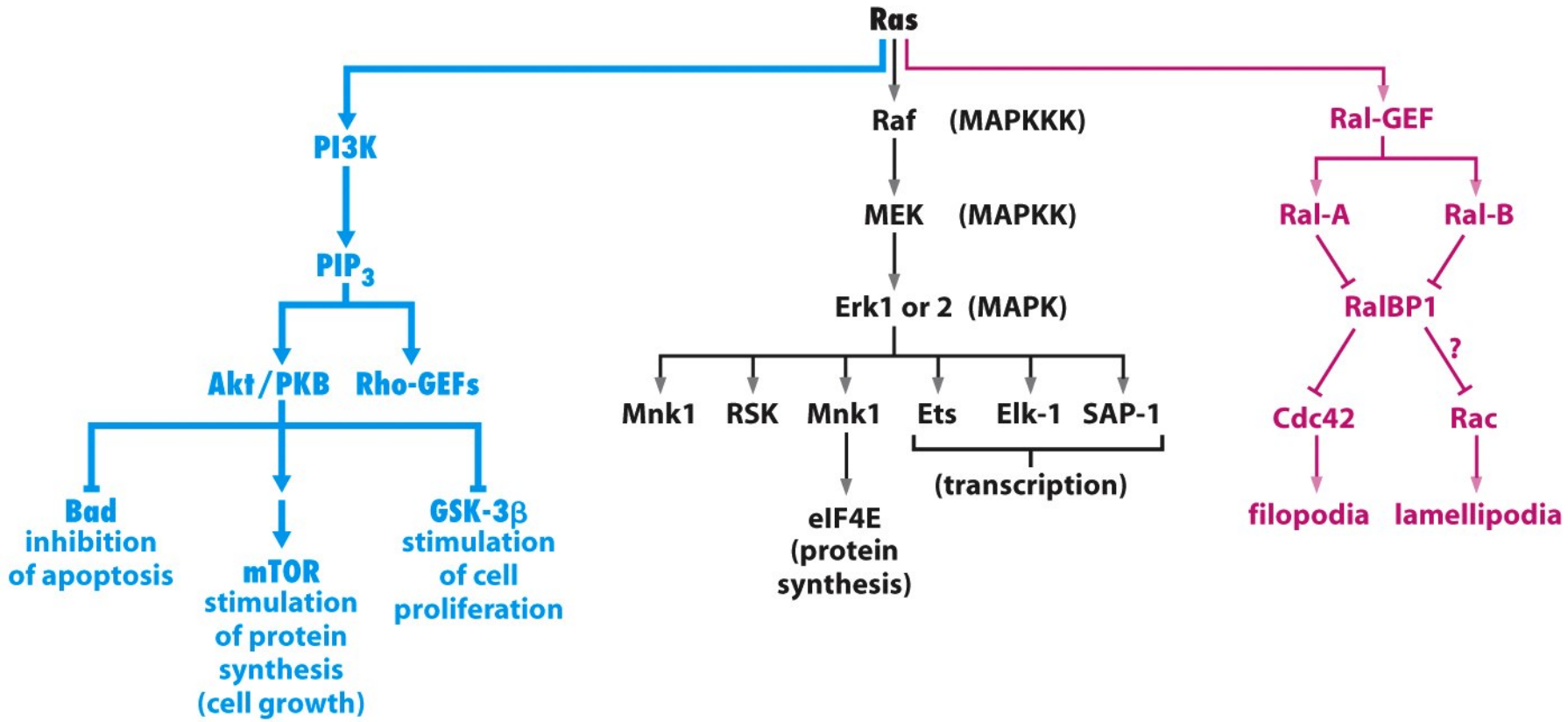
Niemeyer CM, Haematologica. 2014 Nov;99(11):1653-62

Germinal mutations of proto-oncogenes?!

Why tolerated??

- Expression limited to a certain tissue (cell type) (*RET*, c-*kit*)
- Expression of the proto-oncogene occur later during organogenesis thus enabling normal embryogenesis (c-*met*)
- „mildly“ activating mutation (*ras*opathy, c-*kit*)
- specific types of mutations (*cdk 4*)

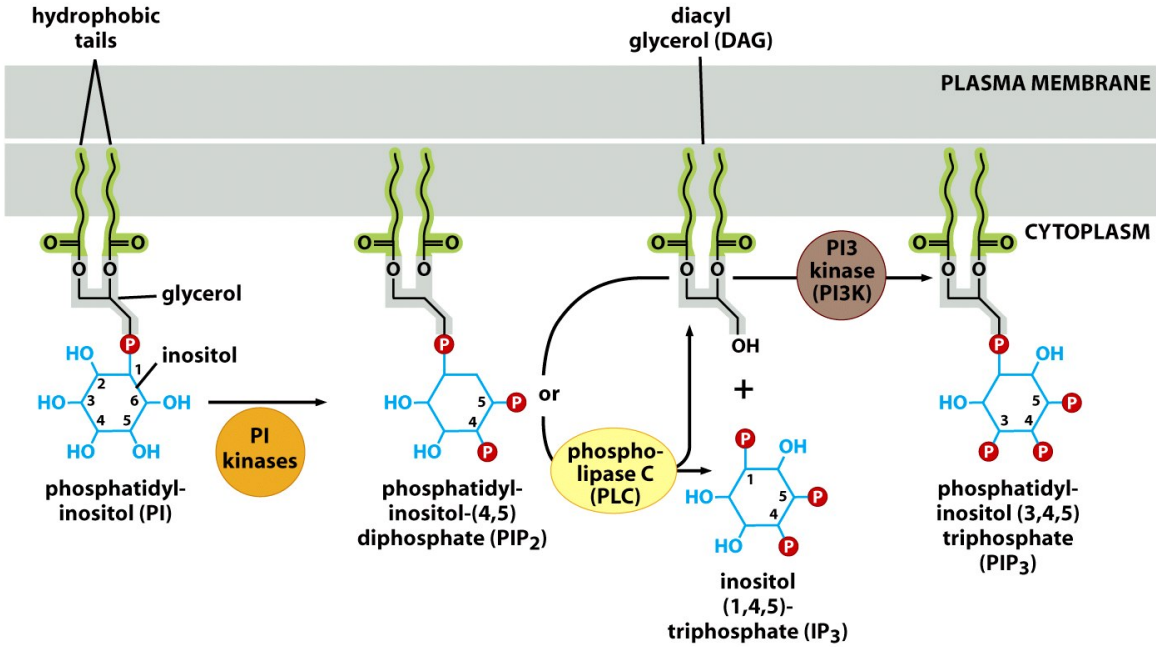
2. Ras ⇒ PI3K


Ras
PI3K
PIP3
Akt/PKB
Rho-GEFs
Bad
inhibition
of apoptosis
mTOR
stimulation
of protein
synthesis
(cell growth)
GSK-3β
stimulation
of cell
proliferation
Raf (MAPKKK)
MEK (MAPKK)
Erk1 or 2 (MAPK)
Mnk1
RSK
Mnk1
Ets
Elk-1
SAP-1
(transcription)
eIF4E
(protein
synthesis)
Ral-GEF
Ral-A
Ral-B
RalBP1
?
Cdc42
Rac
filopodia
lamellipodia

PI3K

Phosphatidyl inositol 3 – kinase - **lipid** kinase

- Composed of 2 subunits: regulatory p85 and catalytical p110
- catalyze the phosphorylation of the D3 position of the inositol ring of phosphoinositides to produce phosphatidylinositol 3-phosphate (PtdIns3P = PIP3) −secondary messenger triggering downstream signaling



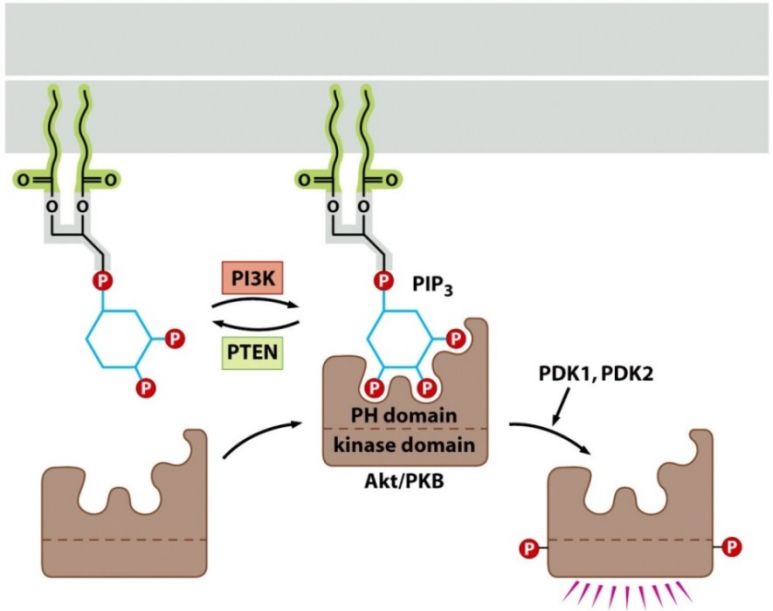
PI3K a PIP_3

- PIP_3 level is tightly regulated. PIP_3 is produced by **PI3K** and converted back to PIP_2 by phosphatases: PTEN, SHIP1, SHIP2
- **PTEN** = PIP_3 lipid phoshatase; tumor suppressor (breast cancer, glioblastoma, Cowden syndrome)
- PI3K may be activated e.g. by PDGF, NGF, IGF-1, interleukin-3,

- PI3K produces PIP_3 and PIP_3 interacts with **Akt**
- Thereby Akt is recruited to the cell membrane, phosphorylated and thus activated

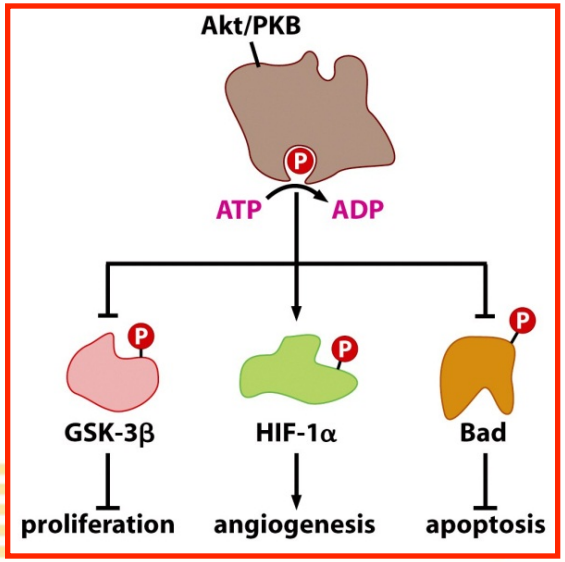
➢ Two mechanisms leading to the Akt hyperactivation :

1. Aktivation of PI3K
2. Inactivation of PTEN



AKT/PKB

- One of the most common activated protein kinases in cancer
- Hyperactivation of Akt is linked with (1) resistence to apoptosis, (2) enhanced proliferation and (3) motility
- serine/threonine kinases of PKB family (mammalian cells express 3 highly homologous (80%) isoforms of Akt encoded by 3 different genes - *AKT1*, *AKT2* and *AKT3* (alias PKBα, PKBβ and PKBγ)



Biological effect	Substrate of Akt/PKB	Functional consequence
Anti-apoptotic		
	Bad (pro-apoptotic)[a]	inhibition
	caspase-9 (pro-apoptotic)[b]	inhibition
	IκB kinase (anti-apoptotic)[c]	activation
	FOXO1 TF (pro-apoptotic)[d]	inhibition
	Mdm2 (anti-apoptotic)[e]	activation
Proliferative		
	GSK-3β (anti-proliferative)[f]	inhibition
	FOXO4 (anti-proliferative)[g]	inhibition
	$p21^{Cip1}$ (anti-proliferative)[h]	inhibition
Growth		
	Tsc2 (anti-growth)[i]	inhibition

Activated AKT ...

- May phoshorylate more than 9000 proteins
- induces antiapoptotic signals, prevents release of cytochromu C from mitochondria, inactivates TF (Forkhead that transactivates **FasL)**, inactivates pro-apoptotic factor **Bad** and **procaspase-9**
- Activates cell cycle: regulates **cyclin D stability**, blocks transport of **$p21^{WAF1}$** and **$p27^{KIP1}$** into nucleus
- stimulates activity of telomerase via **TERT**
- induces translocation of **MDM2** into nucleus (thereby p53 degradation)
- inactivates **GSK3** which leads to increase of β-catenin levels
- inactivates **TSC2** (*tuberous sclerosis complex 2*) that functions as **mTOR** inhibitor; mTOR is associated with proliferation rate

- AKT often changes **subcellular localization** = regulates (**compartmentalisation**) of its substrates

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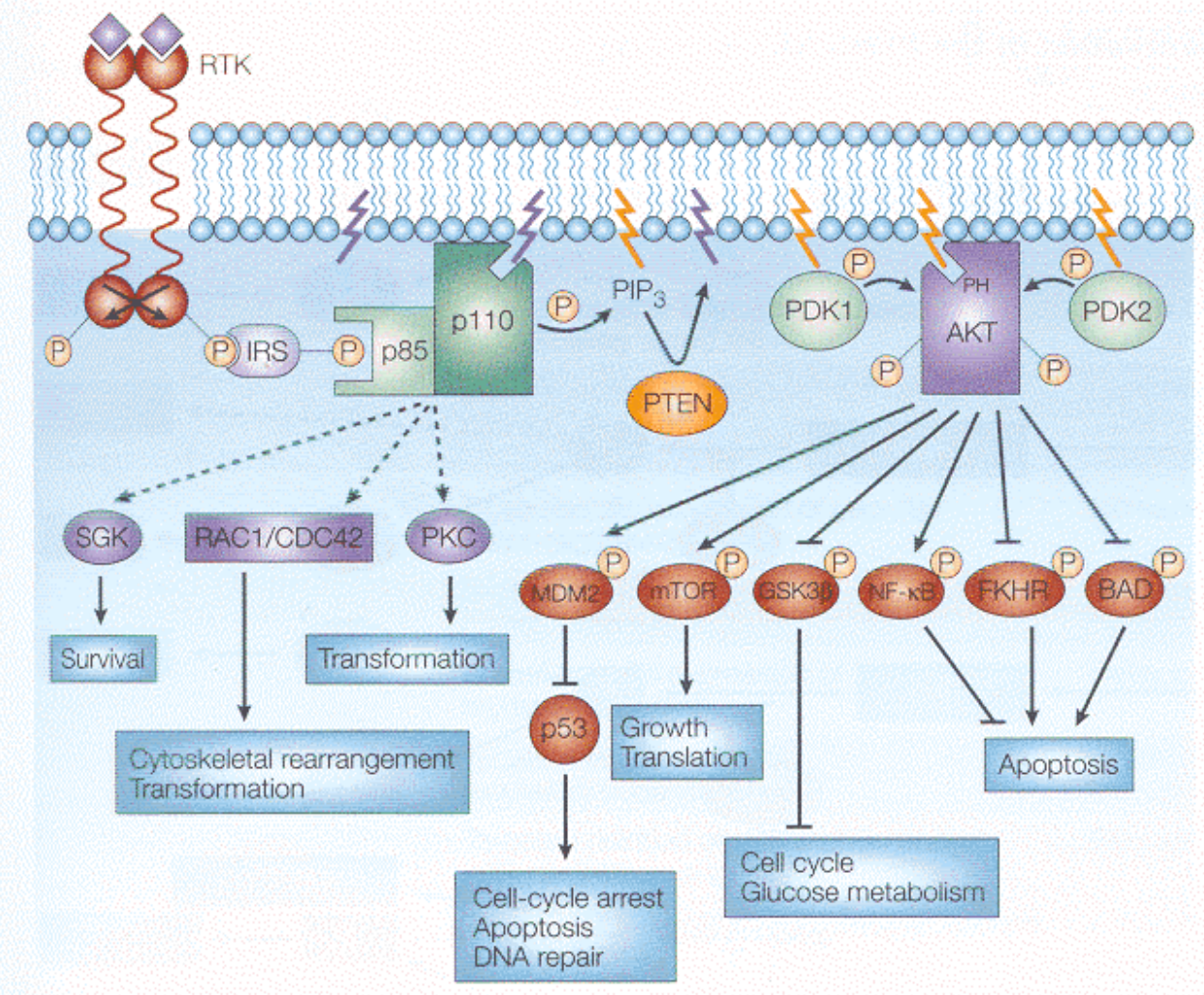
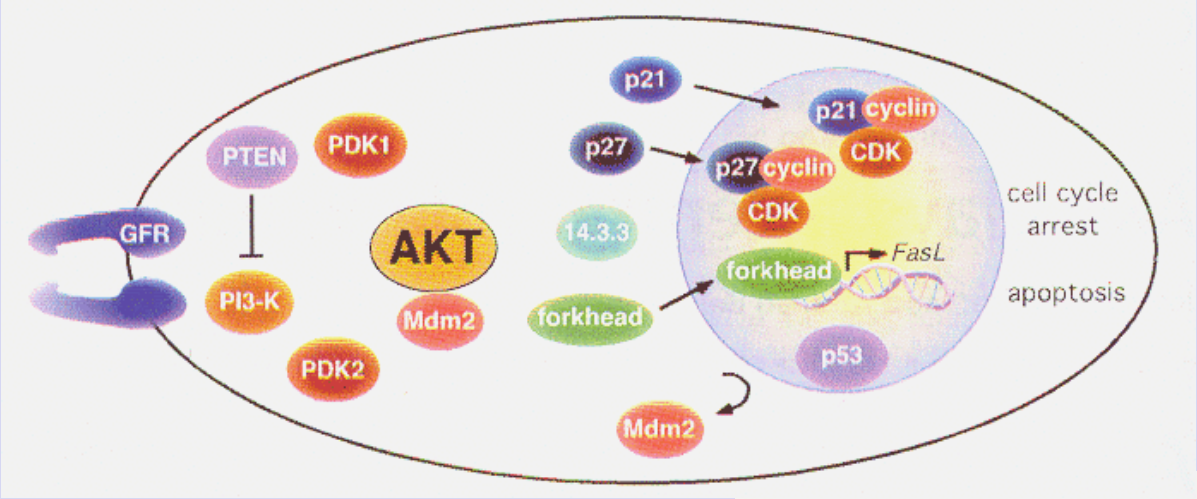
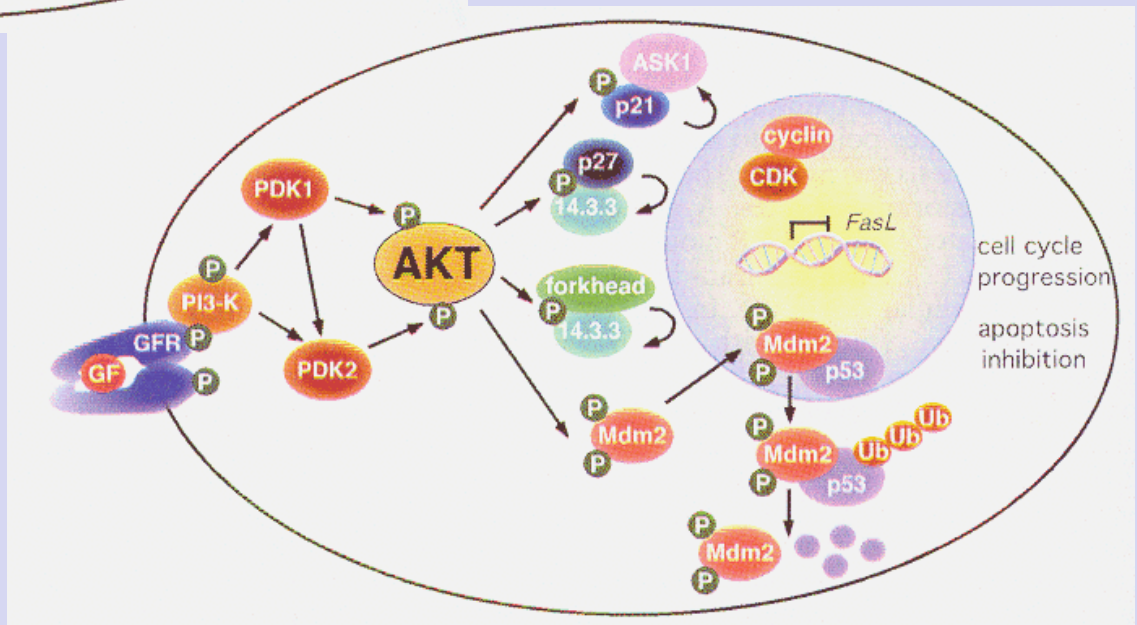


AKT regulates compartmentalisation of its substrates





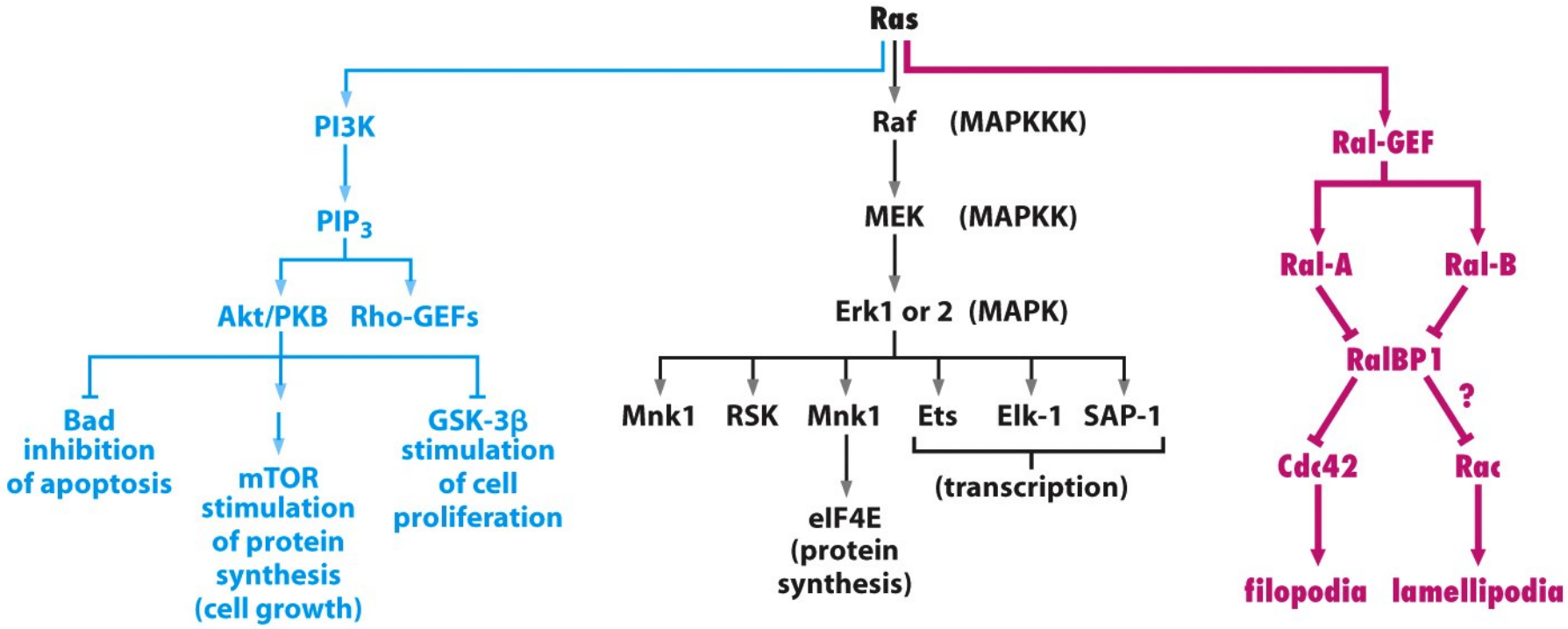
AKT in cancerogenesis

- amplification and overexpression of *AKT2* in 10-20% ovarian and pancreatic cancer, increased AKT2 activity in 40% of ovarian cancer
- Amplification of *AKT1* found in stomach cancers
- Increased activity of AKT1 in prostate and breast cancer − associated with worse prognosis (increased activity in breast carcinomas may be caused by *HER2*/neu amplification)
- inactivating mutations or deletions of *PTEN* (prostate and endometrial cancer, glioblastomas, melanomas)
- Amplifications or overexpression of catalytical subunit p110 of PI3K
- activating mutations of *ras* (around 1/3 of all epithelial tumors)
- Enhanced activity of RTKs, mainly ErbB2/ErbB3 (ErbB3 has docking site for PI3K)

PI3K pathway alterations in human cancers

Cancer type	Type of alteration
Glioblastoma (25–50%)	*PTEN* mutation
Ovarian carcinoma	*PTEN* mutation; *AKT2* amplification; *PI3K* amplification; PI3K *p85α* mutation
Breast carcinoma	increased Akt1 activity; *AKT2* amplification; *PTEN* mutation
Endometrial carcinoma (35%)	*PTEN* mutation; *PTEN* methylation[a]
Hepatocellular carcinoma	*PTEN* mutation
Melanoma	*PTEN* mutation; *PTEN* methylation[a]
Lung carcinoma	*PTEN* mutation
Renal cell carcinoma	*PTEN* mutation
Thyroid carcinoma	*PTEN* mutation; Akt/PKB overexpression
Lymphoid	*PTEN* mutation
Prostate carcinoma (40–50%)	*PTEN* mutation
Colon carcinoma (>30%)	Akt/PKB overexpression; *PI3K* mutation

3. Ras ⇒ Ral


Ras
PI3K
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Raf (MAPKKK)
MEK (MAPKK)
Erk1 or 2 (MAPK)
Mnk1
RSK
Mnk1
Ets
Elk-1
SAP-1
(transcription)
eIF4E
(protein
synthesis)
Ral-GEF
Ral-A
Ral-B
RalBP1
?
Cdc42
Rac
filopodia
lamellipodia

3. Ras ⇒ Ral

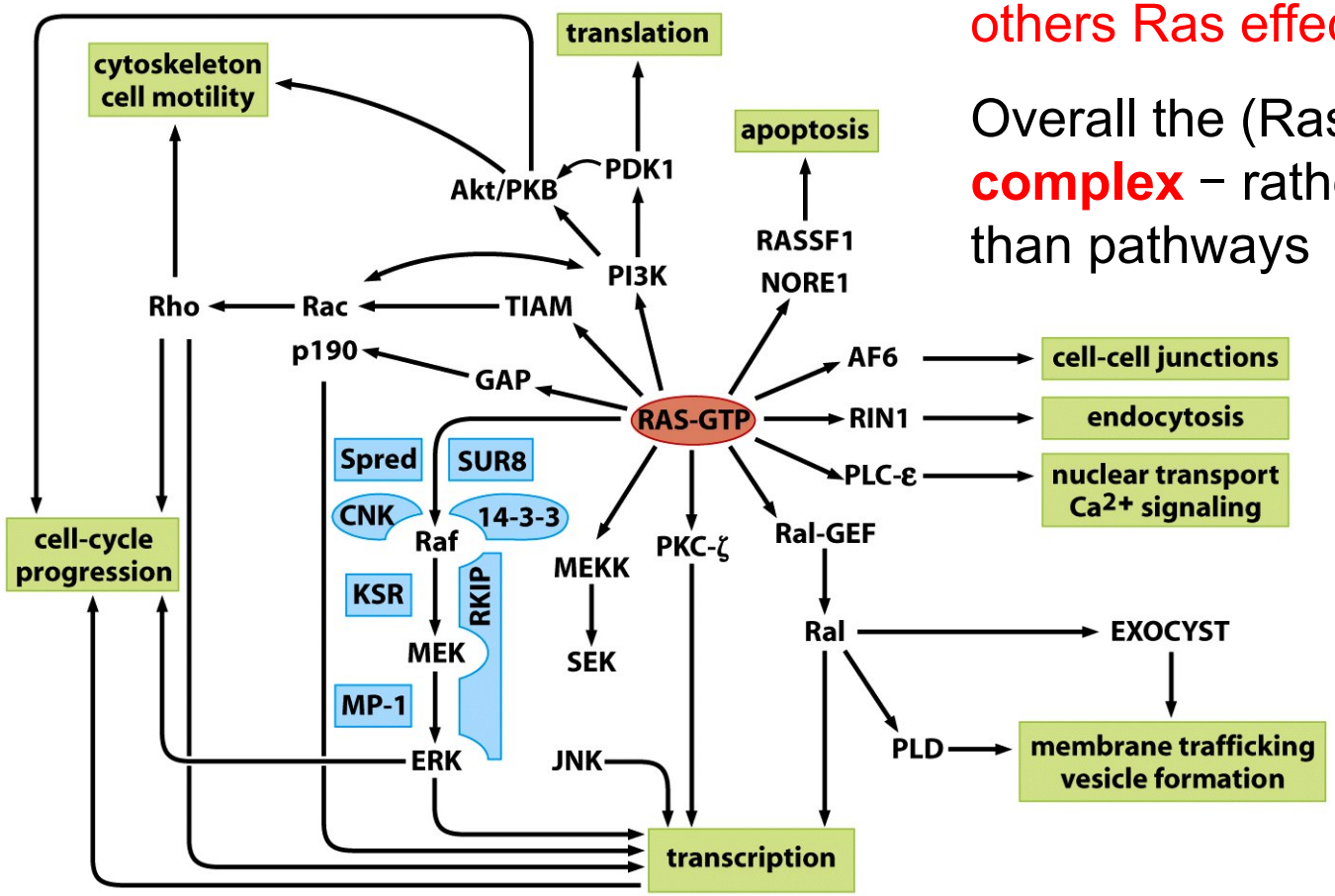
- Third of the most important Ras effectors: **Ral A** and **Ral B**; belong to Ras family, have 58% sequence homology with Ras
- Signaling from Ras to Ral goes through **Ral-GEF:** activated Ras directly binds to Ral-GEF followed by:

  a) recruitment of Ral-GEF to the cytosolic side of cell membrane

  b) conformation change of Ral-GEF → stimulation of GEF activity → activation of Ral-A and Ral-B
- Activated Ral-A and Ral-B activate their target molecules (Rho − actin dynamics)

- Outcome of this activation is increased invasion and metastatic activity of cancer cells

Ras effector pathways



Besides the three described pathways there is more than 8 others Ras effectors.

Overall the (Ras) signaling is more **complex** − rather signaling nets than pathways

Thank you for attention!