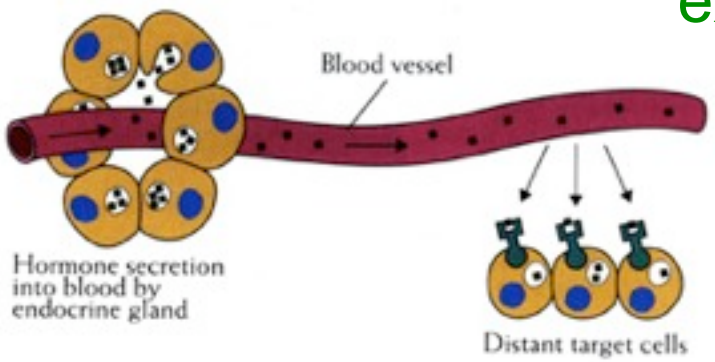
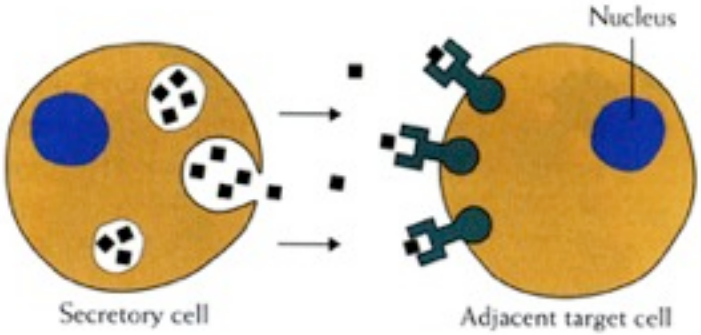
The three patterns of extracellular signaling in animals



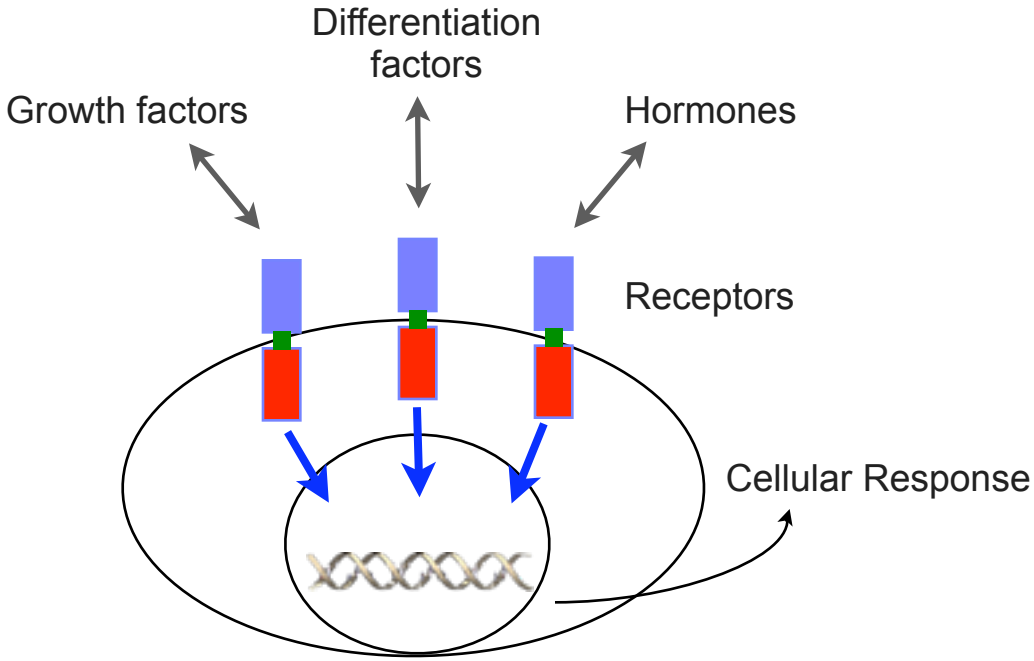
Endocrine



Paracrine

Autocrine

Cell surface receptors

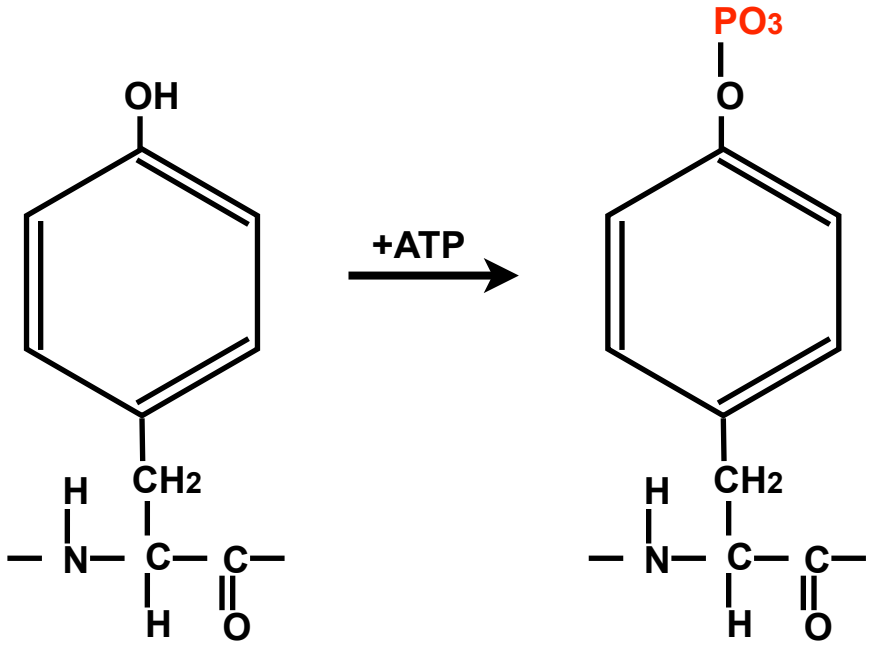


A large number of these receptors belong to the family of transmembrane tyrosine kinases

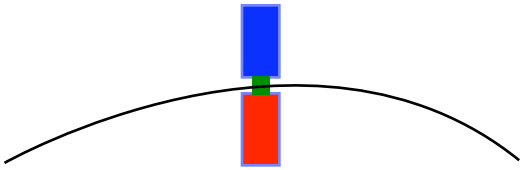
Many oncogenes, often altered versions of growth factor receptors, are also tyrosine kinases

Therefore, this class of enzymes plays an important role both in normal cell regulation and in oncogenic transformation

Protein-tyrosine kinases catalyze the addition of a phosphate group on the phenyl ring of tyrosine

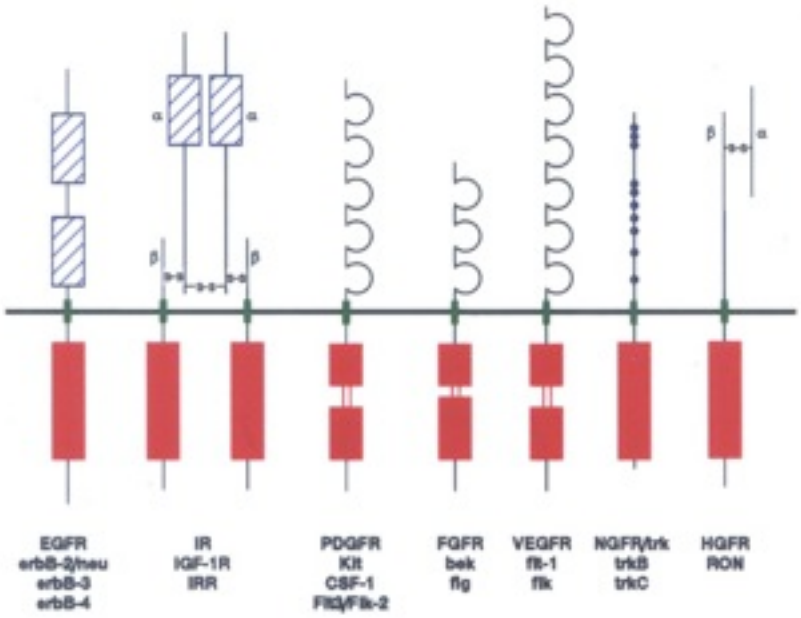


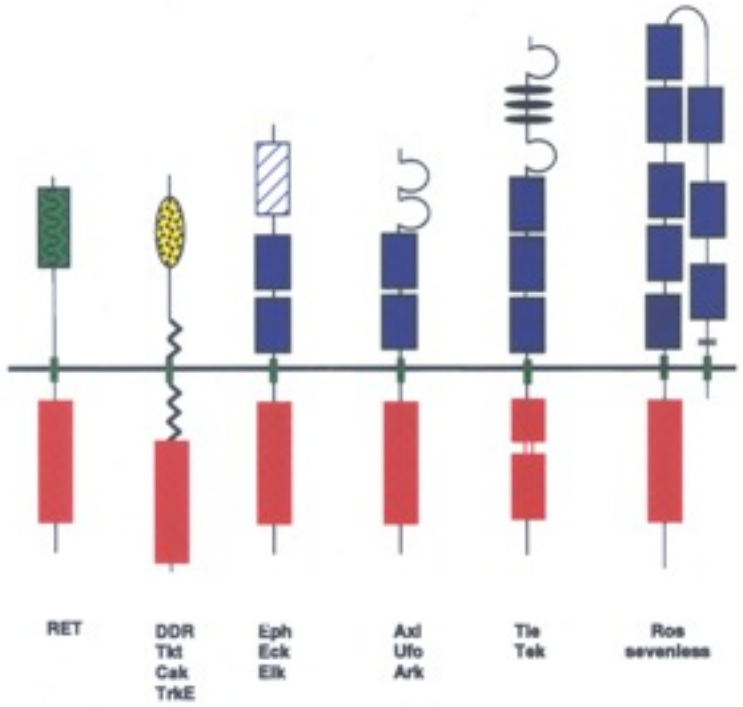
Common structural features of receptor-tyrosine kinases (RTK)

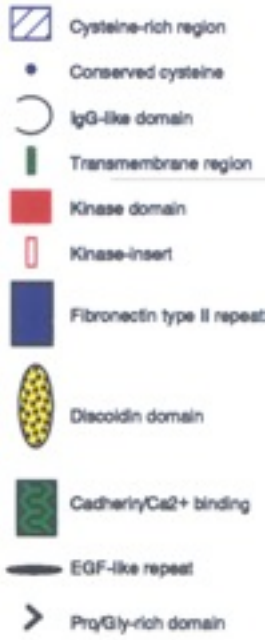


1. *Large extracellular ligand binding domain*
2. *Single transmembrane domain*
3. *Cytoplasmic catalytic domain and regulatory sequences*

Receptor Tyrosine Kinases

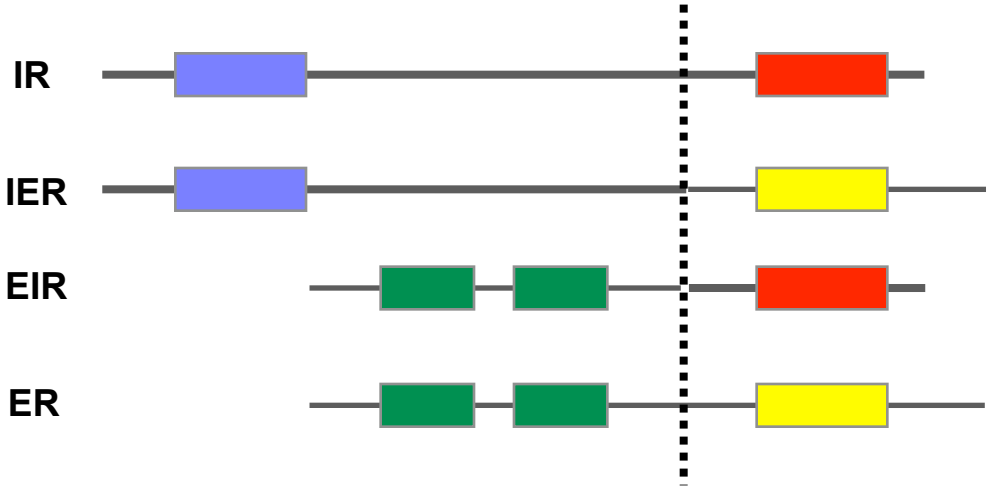






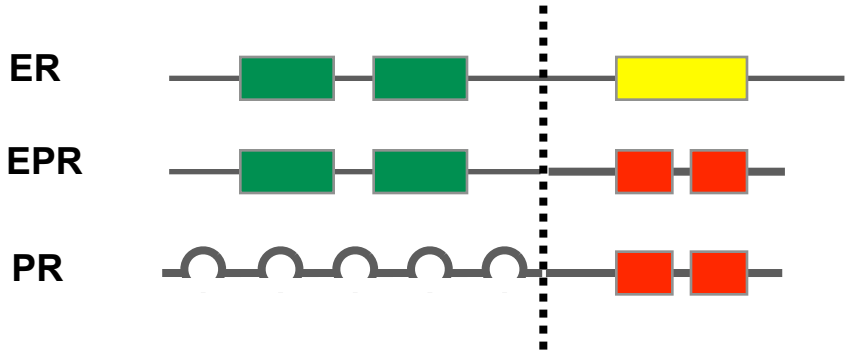
Receptor chimeras demonstrate the structural and functional independence of domains

EGF Receptor and Insulin Receptor



All chimeric receptors are activated by ligand binding

EGF Receptor and PDGF Receptor



How can the ligand binding site on the receptors by defined?

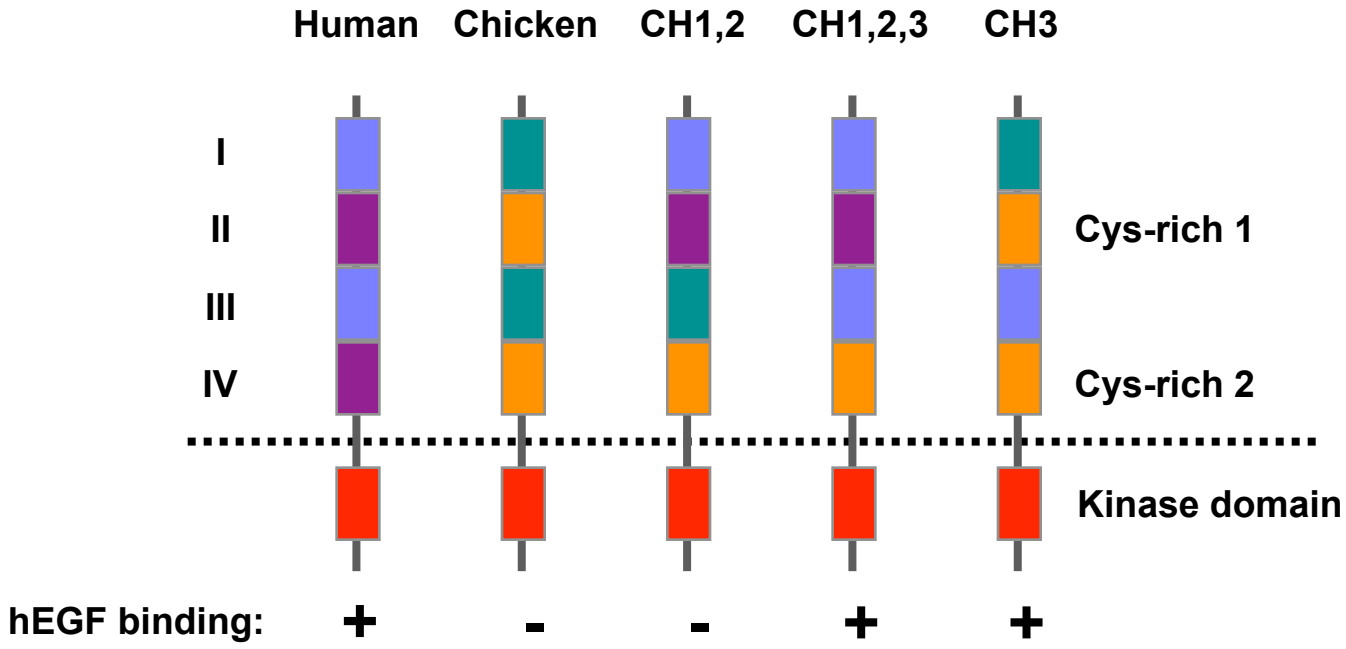
Example: The Epidermal Growth Factor Receptor

Two ligands exist for the EGFR: EGF and TGFα

Human EGFR: Binds with high affinity human EGF and human TGFα

Chicken EGFR: Binds human EGF with >250-fold lower affinity and TGFα with high affinity

Experiment: Swap sub-domains between the human and chicken receptor and test for EGF binding



Sub-domain III of the EGFR contains the major EGF-binding domain

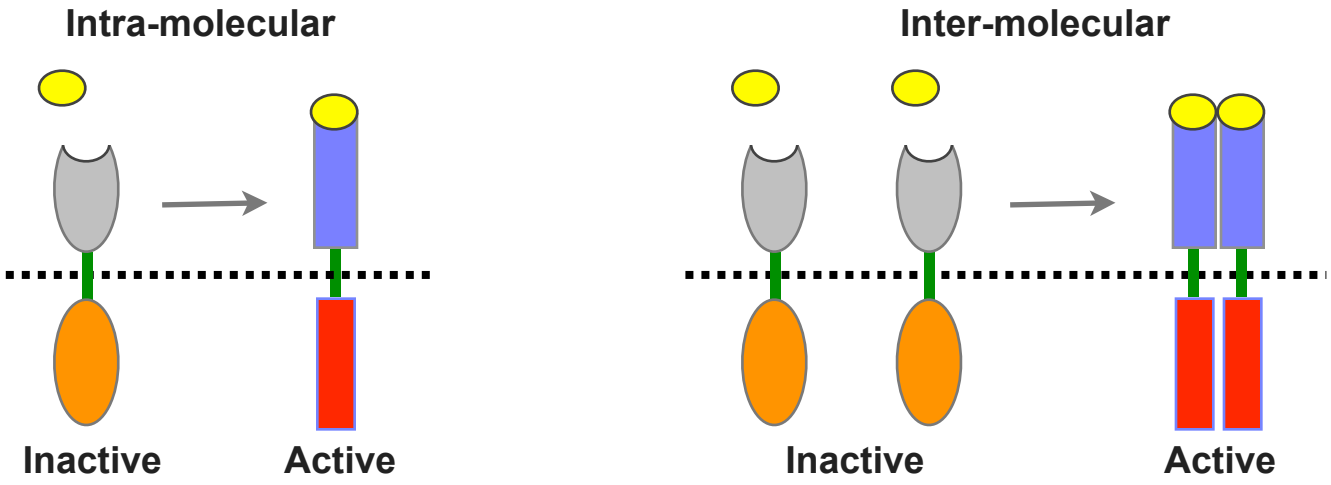
Does ligand binding result in a conformational change in the extracellular domain of the receptor?

Experiment:

1. Express and purify soluble, recombinant domain in large amounts
2. Obtain Circular Dichroism (CD) spectra in the near and far UV, +/- ligand
3. Differences in the spectra indicate a conformational change in the receptor upon ligand binding

How is the conformational change transmitted through the membrane?

Two models:



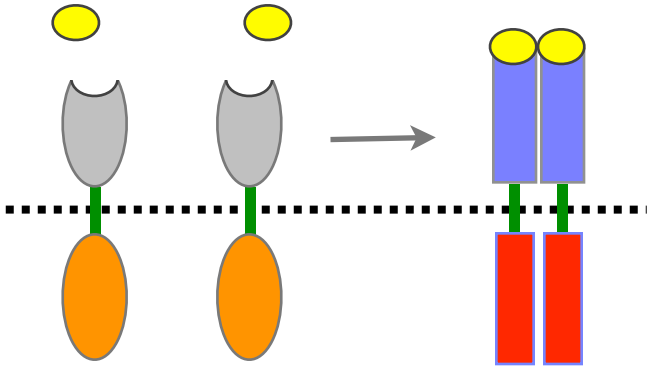
Transmission of a conformational change through a single transmembrane helix would be energetically unfavoured.

Most evidence supports the inter-molecular model

Receptor activation by dimerization: Possible models

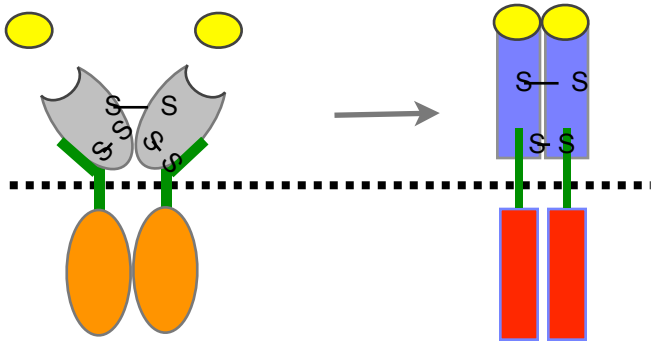
Examples:

EGFR



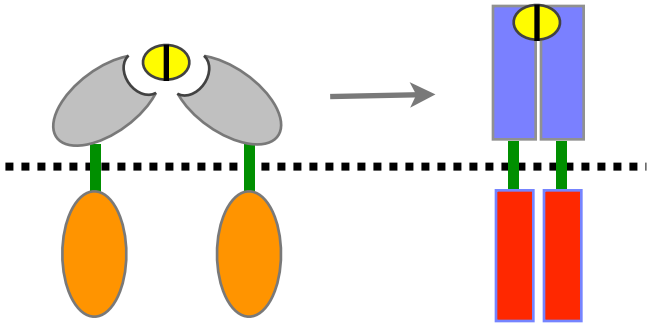
Conformational change in extracellular domain leads to dimerization

Insulin Receptor



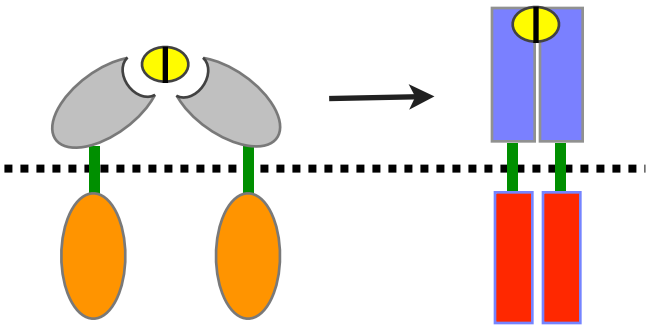
Intra-complex conformational change

PDGFR



Dimeric ligand leads to receptor dimer formation

Receptor activation by dimerization: The case of PDGFR



Platelet-Derived Growth Factor (PDGF) is a homo- or hetero-dimer of two chains, A and B.

Three forms: PDGF AA, PDGF AB, PDGF BB

Two closely related PDGF Receptors exist: PDGFRα and PDGFRβ

PDGFRα binds the A or B chain of PDGF

PDGFRβ binds only the B chain

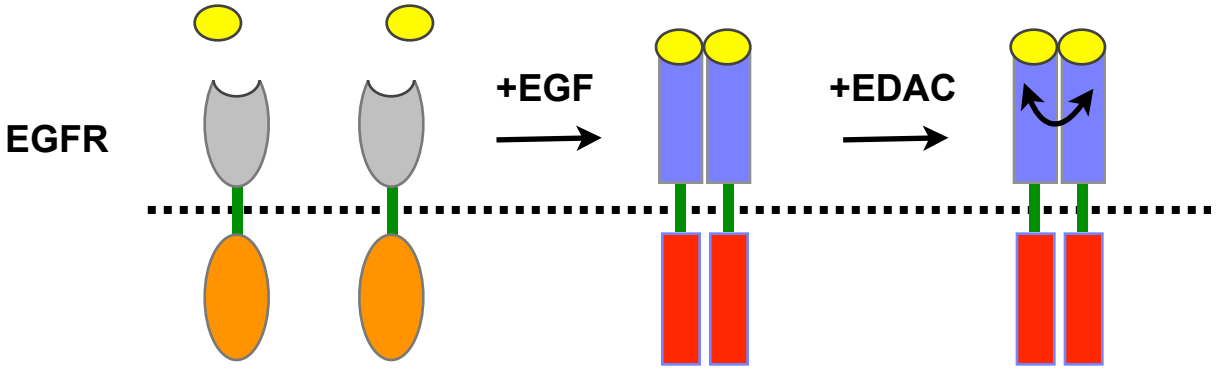
Possible combinations:

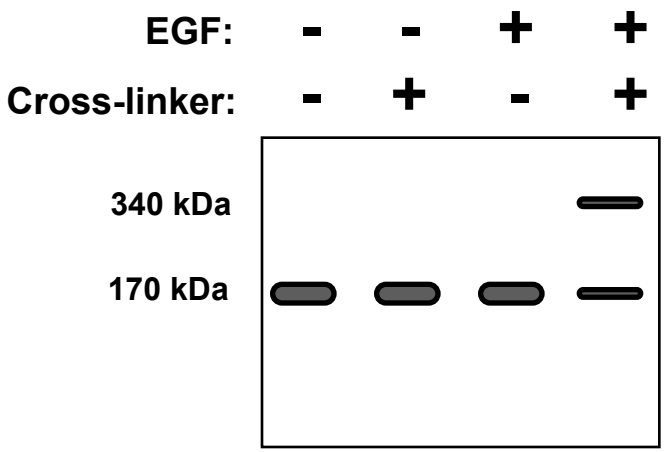
Receptor dimer	Ligand
αα	AA, AB, BB
αβ	AB, BB
ββ	BB

How can we observe ligand-induced receptor dimerization?

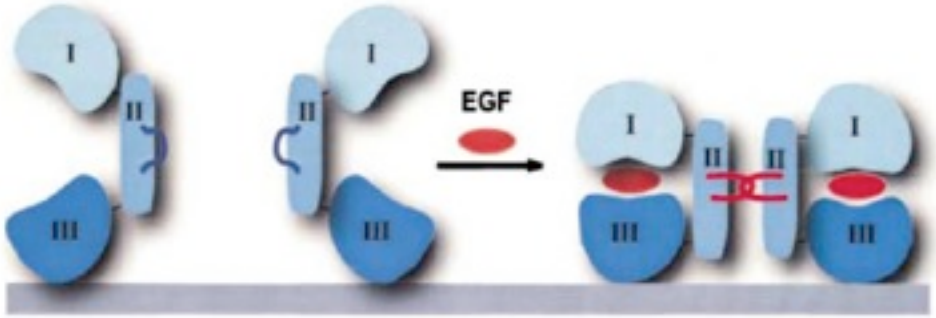
Example: EGFR

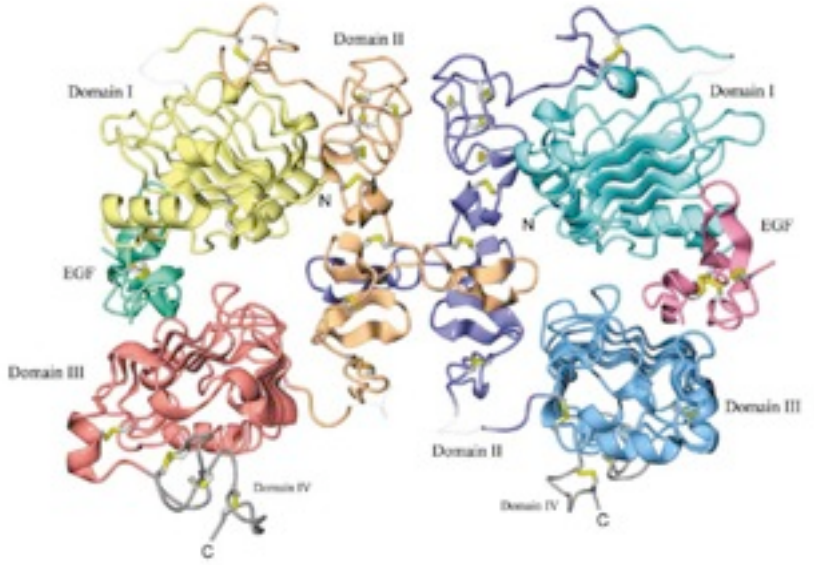
1. Incubate +/- EGF
2. Add a bivalent cross-linking agent
3. Separate proteins in SDS-PAGE
4. Visualize receptors with antibodies

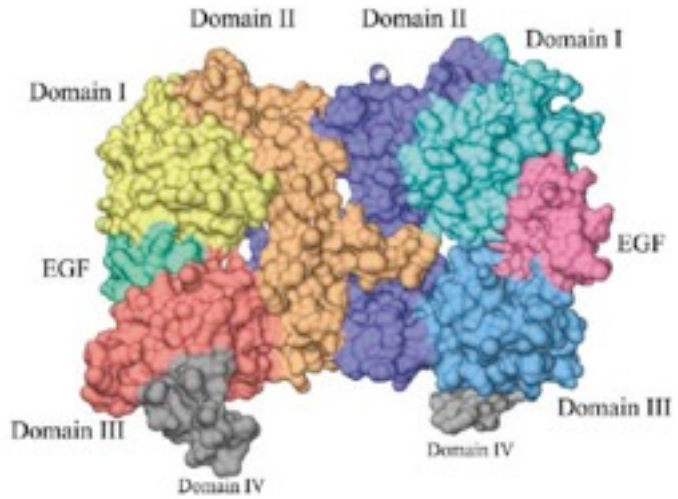




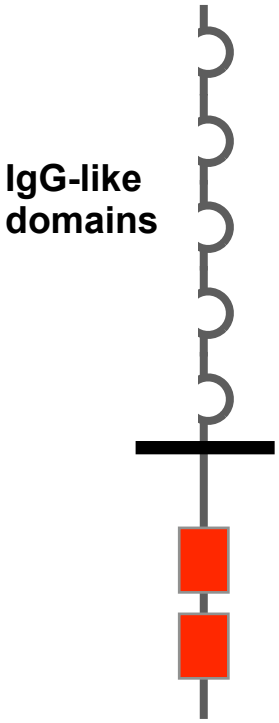
The crystal structure of the EGF-EGFR complex reveals the mechanism of ligand-induced receptor dimerization

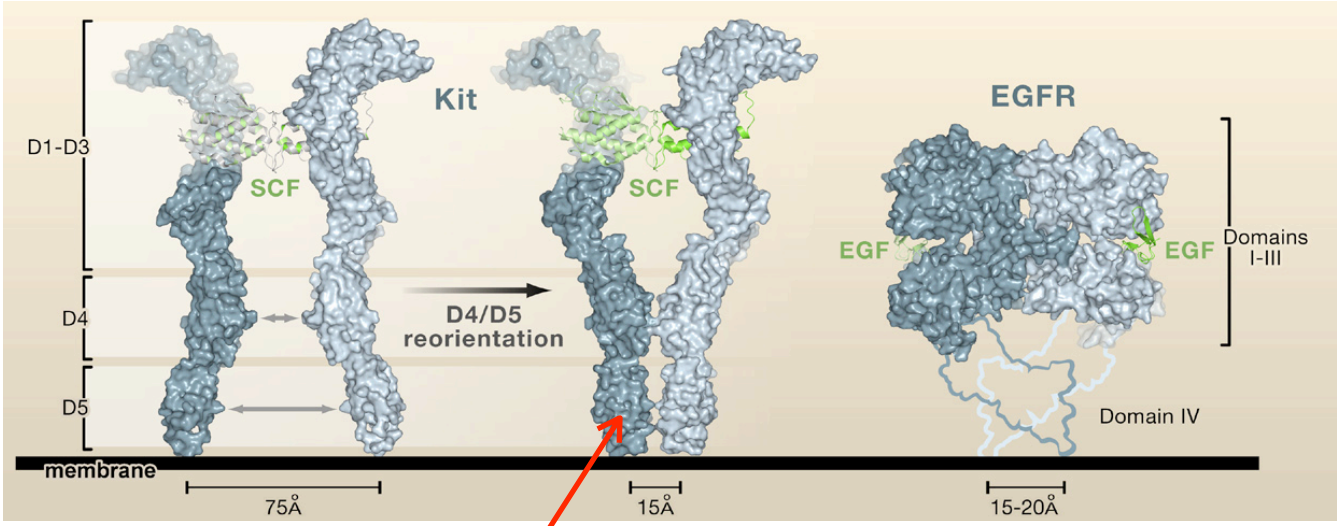






The crystal structure of the SCF-Kit complex reveals the mechanism of ligand-induced receptor dimerization





Activating, oncogenic mutations of Kit (gastrointestinal tumors) map to the D5 dimer interface, promoting ligand-independent dimerization

The transmembrane domain

- Defined as a stretch of 20-25 hydrophobic amino acids
- Present in all receptor-tyrosine kinases
- Long enough to cross the membrane only once

EGFR: - I A T G M V G A L L L L L V V A L G I G L F M -

PDGFR: - V V V I S E A I L A L V V L T V I S L I I L I M L -

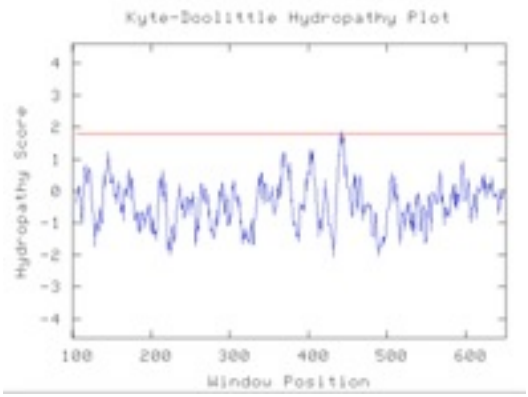
Insulin R: - I I I G P L I F V F L F S V V I G S I Y L F L -

Usually followed by a stretch of positively charged residues (Arg), which acts as a stop-transfer signal

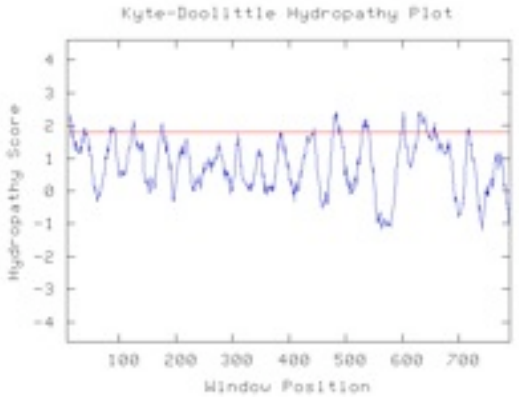
Kyte & Doolittle Hydrophobicity Scale:

Ile	Val	Leu	Phe	Cys	Met	Ala	Gly	Thr	Ser	Trp	Tyr	Pro	His	Gln	Asn	Glu	Asp	Lys	Arg
4.5	4.2	3.8	2.8	2.5	1.9	1.8	-0.4	-0.7	-0.8	-0.9	-1.3	-1.6	-3.2	-3.5	-3.5	-3.5	-3.5	-3.9	-4.5

EGF receptor



7-TM receptor

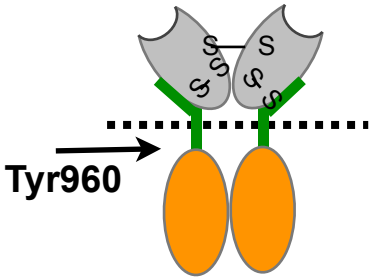


The juxtamembrane region

Contains residues that may play a key role in signal transduction
It is also involved in auto-inhibition of receptor tyrosine kinases

Example: The Insulin Receptor

The TM domain ends at aa941
The tyrosine at position 960 has been mutated to phenylalanine and the wt and mutant receptors expressed in cells



WT: Insulin stimulates:
- Receptor autophosphorylation
- Substrate phosphorylation **
- Glycogen synthase activation
- Uptake of amino acids
- DNA synthesis

***IRS1 is a major tyrosine-phosphorylated substrate in insulin-stimulated cells*

Tyr960 > Phe mutant:
- Receptor autophosphorylation
- Intact kinase activity, but...
- No IRS1 phosphorylation
- No biological effects

Conclusion:
- Tyr960 is important of the phosphorylation of cellular substrates involved in insulin-mediated signaling
- Tyrosine kinase activity is not sufficient of a biological response
- Is it necessary??

Is the tyrosine kinase activity necessary for the action of growth factor receptors?

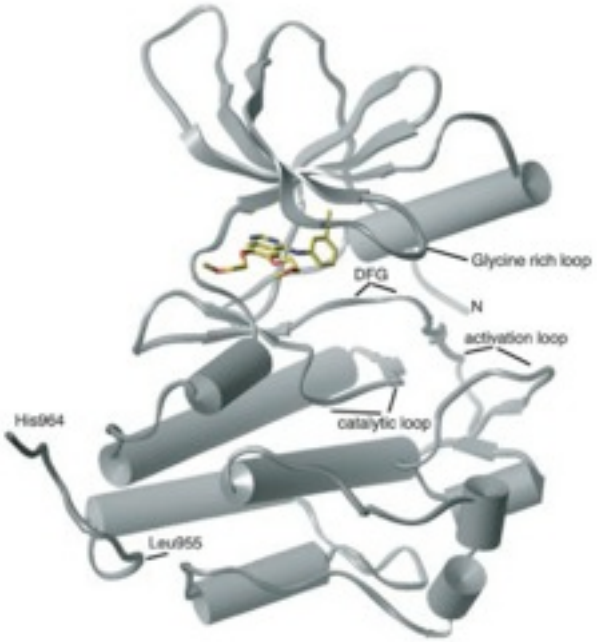
Experiment: Site-directed mutagenesis of the ATP-binding site

A typical ATP-binding site includes a Gly-X-Gly-X-X-Gly motif, followed by a highly conserved lysine residue approx. 14-23 aa downstream, which is directly involved in the phospho-transfer reaction

Mutation of this lysine to an arginine or other aa results in complete inactivation of the kinase domain

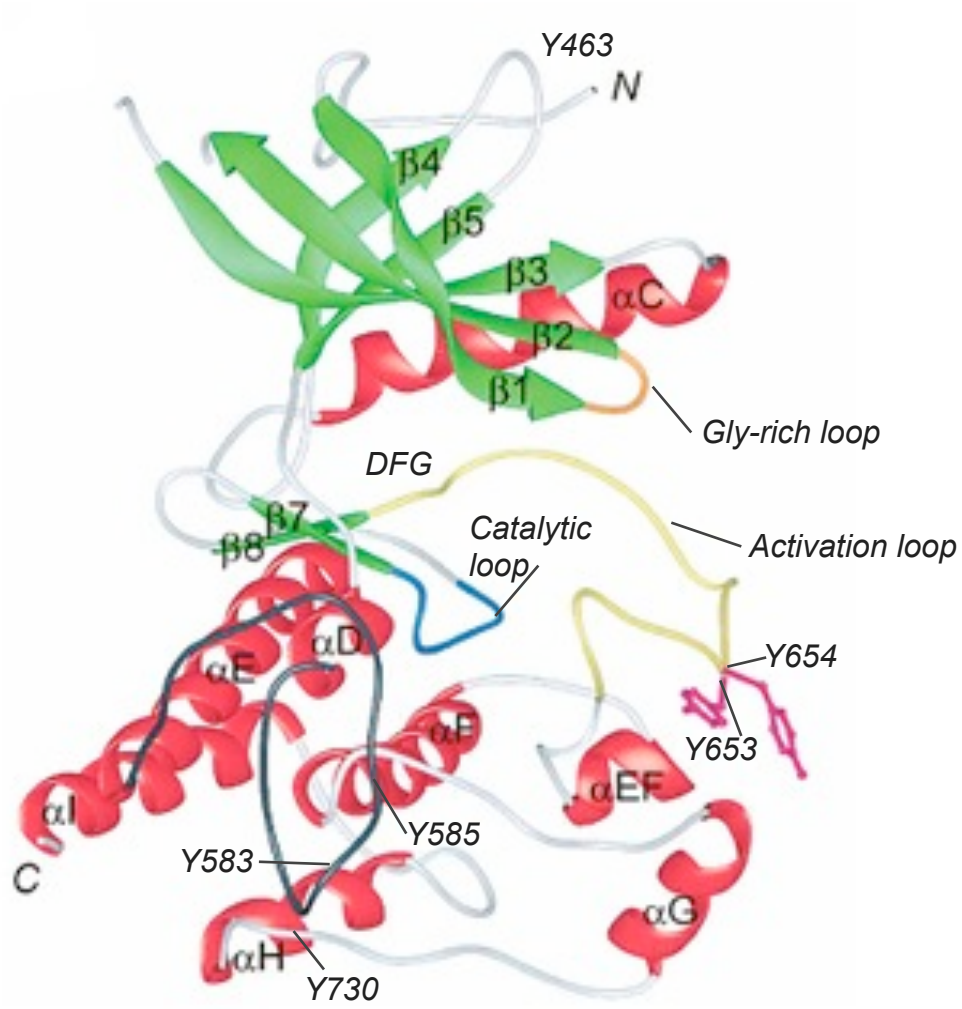
Expression of wt or mutant receptors in receptor-negative cells: they both bind ligand with the same affinity, but the mutant receptor cannot mediate any subsequent signaling event

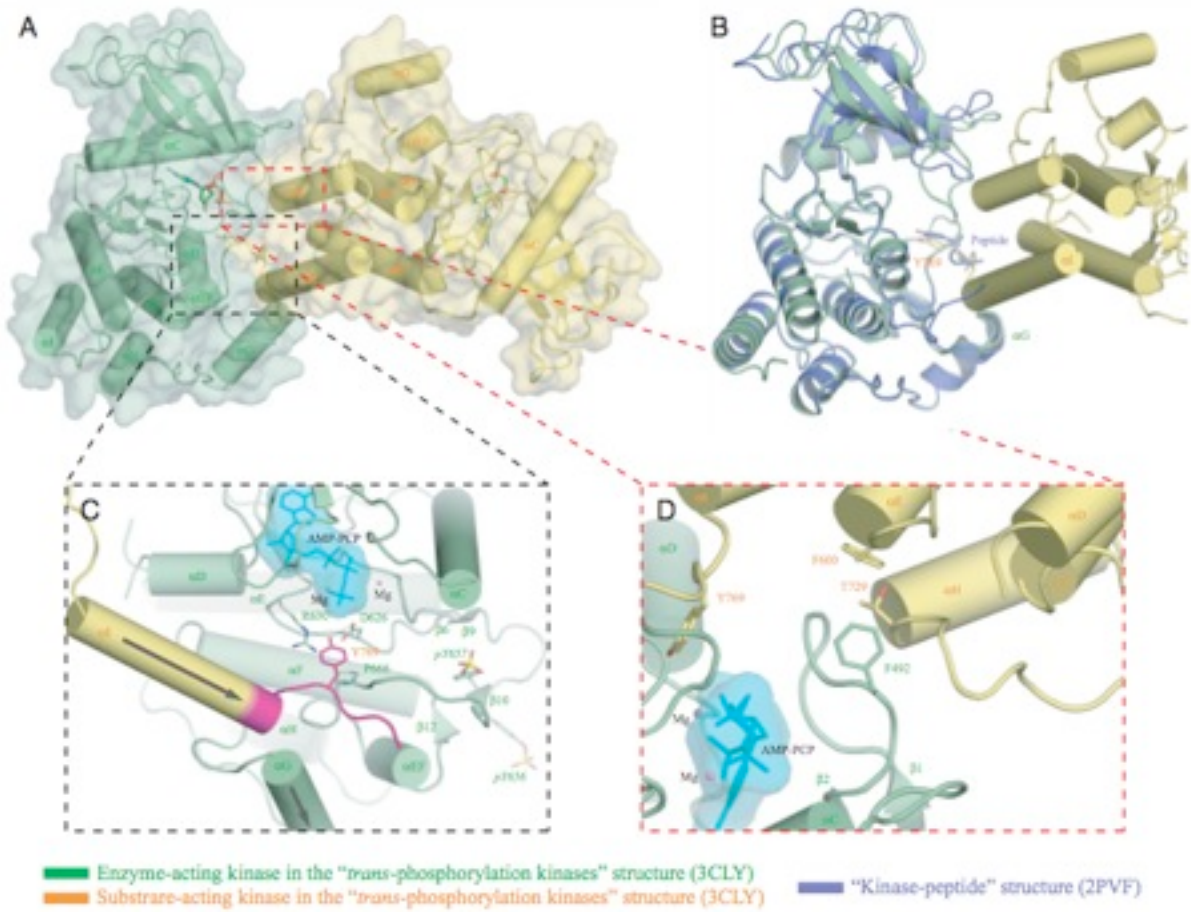
Demonstrated for the EGF, PDGF, insulin and other receptors as well as several cytoplasmic protein-tyrosine kinases



Structure of the EFGR kinase domain

Structure of the FGF receptor kinase domain





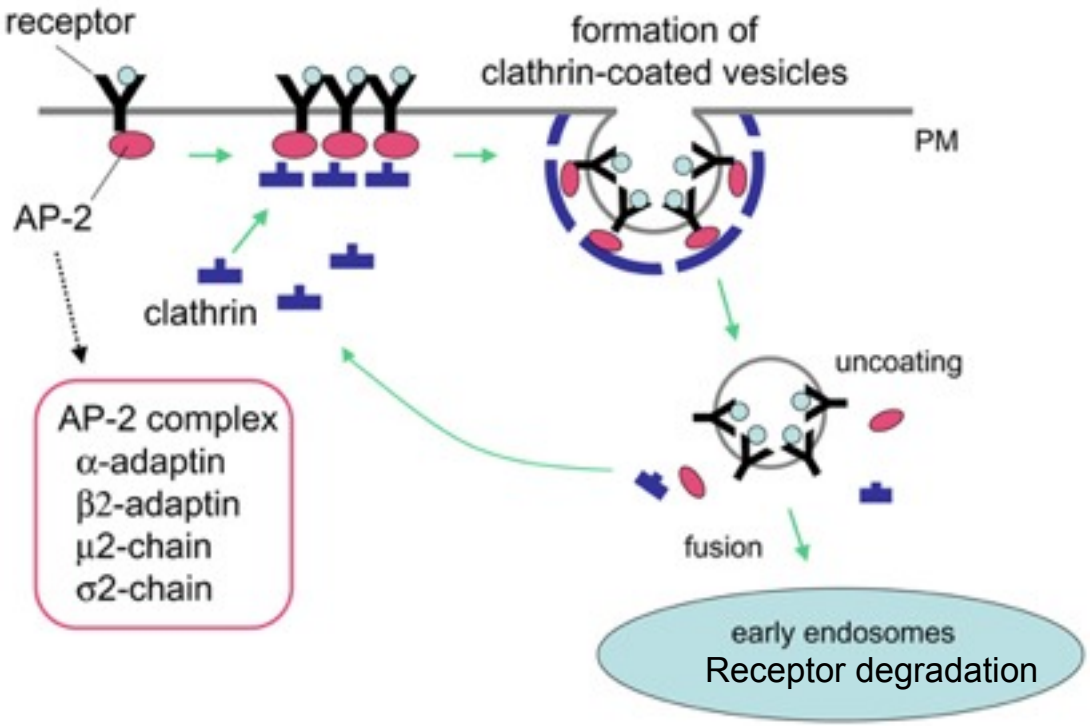
A crystallographic snapshot of tyrosine trans-phosphorylation in action

Huaibin Chen[a], Chong-Feng Xu[a,b], Jinghong Ma[a], Anna V. Eliseenkova[a], Wanqing Li[c], Pamela M. Pollock[d], Nelly Pitteloud[e], W. Todd Miller[c], Thomas A. Neubert[a,b], and Moosa Mohammadi[a,1]

19660 –19665 ⋄ PNAS ⋄ December 16, 2008 ⋄ vol. 105 ⋄ no. 50

Attenuation of the signal

1. Ligand-induced receptor internalization



How do we know that this is an attenuation mechanism and not a signal-transduction mechanism?

Experiment: Truncation of the AP2-binding site and expression of the mutant EGF receptors in receptor-negative cells:

- The non-internalizing receptor is mitogenic at lower EGF concentrations than the wt
- Morphological transformation is observed at low EGF concentrations

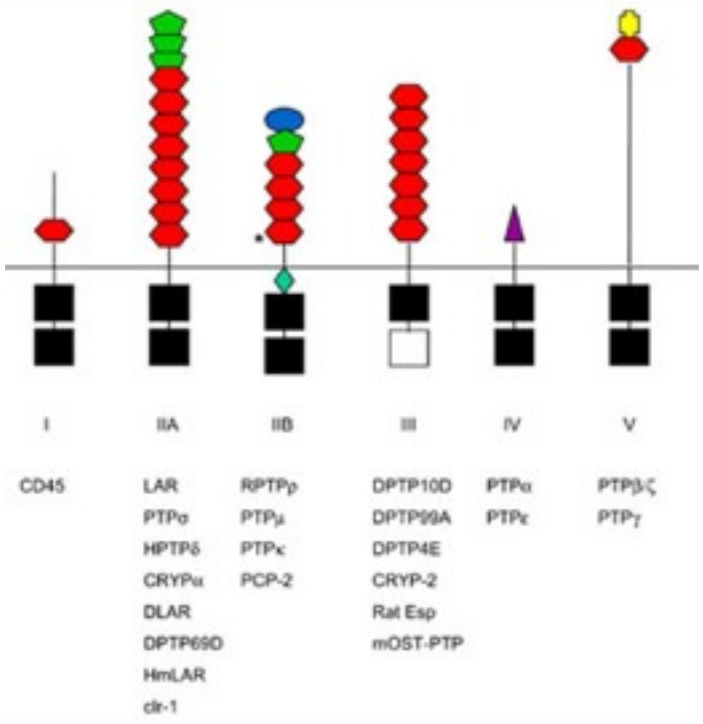
Attenuation of the signal

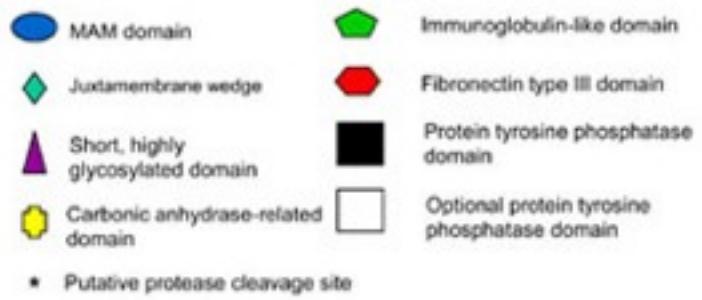
2. Protein Tyrosine Phosphatases

PTPs remove phosphate from receptors and substrates
A dynamic equilibrium exists between tyrosine phosphorylation and de-phosphorylation, allowing accurate regulation of signaling

Receptor PTPs

Many phosphatases have structural features of trans-membrane receptors.
Ligand binding could either enhance their catalytic activity or suppress a high basal level.





Genetic damage in cancer cells

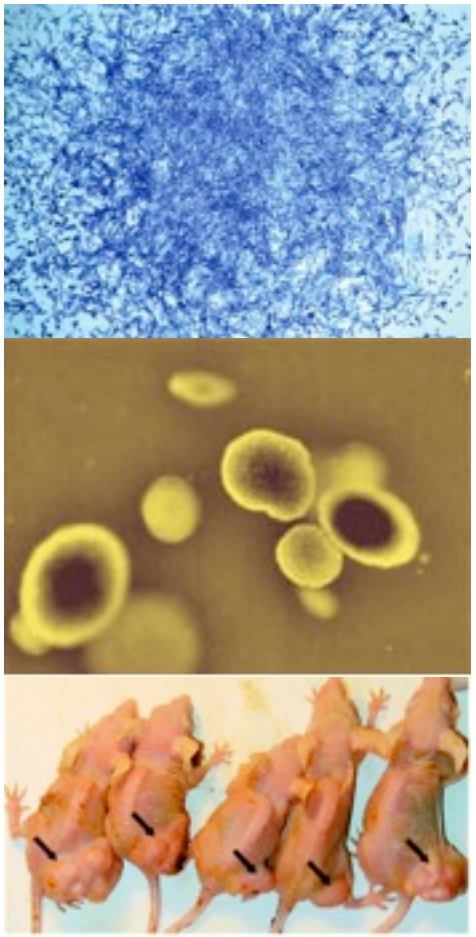
Dominant: Gain of function mutations - e.g. oncogenes

Recessive: Loss of function mutations - e.g. tumor-suppressor genes (p53, Rb,etc)

Properties of transformed cells in culture:

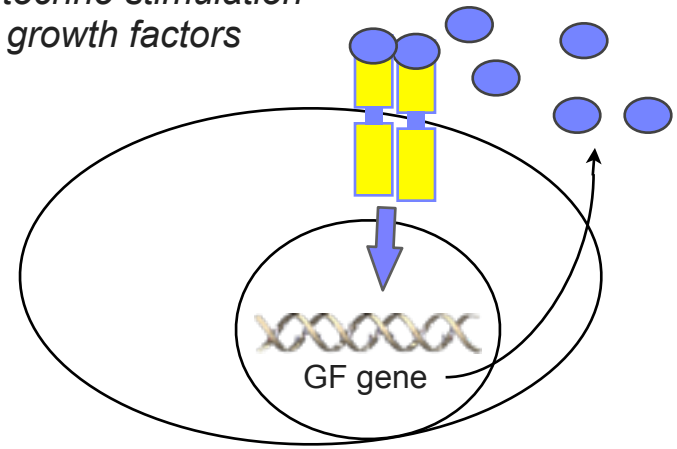
Reduced need for growth supplements (serum)
Loss of capacity to go into quiescence
Altered morphology
Loss of contact inhibition
Loss of anchorage dependance (growth in agar)
Form tumors in nude mice

Many cell lines derived from naturally occurring tumors have these properties

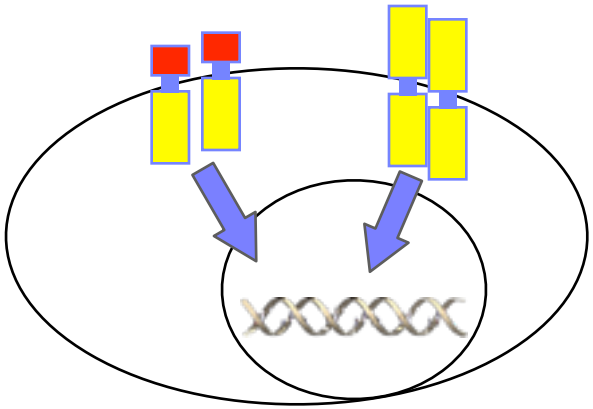


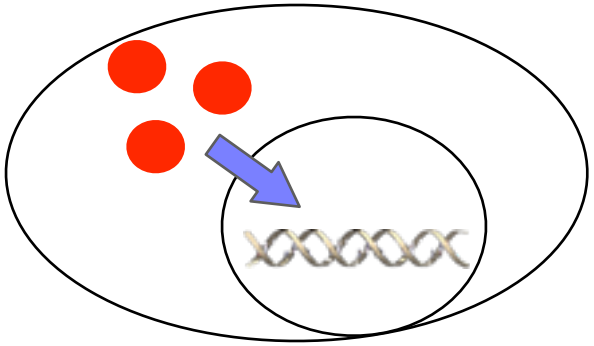
Four ways by which oncogenes can subvert growth regulation

Autocrine stimulation by growth factors

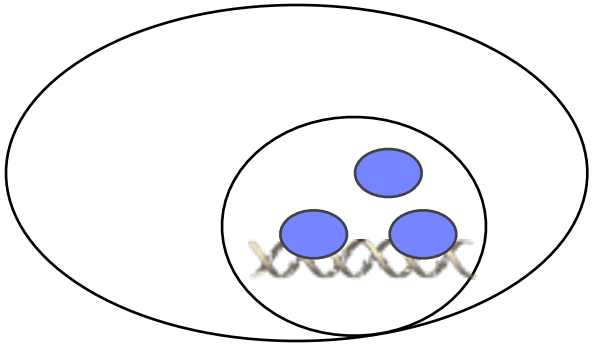


Mutant or over-expressed receptors



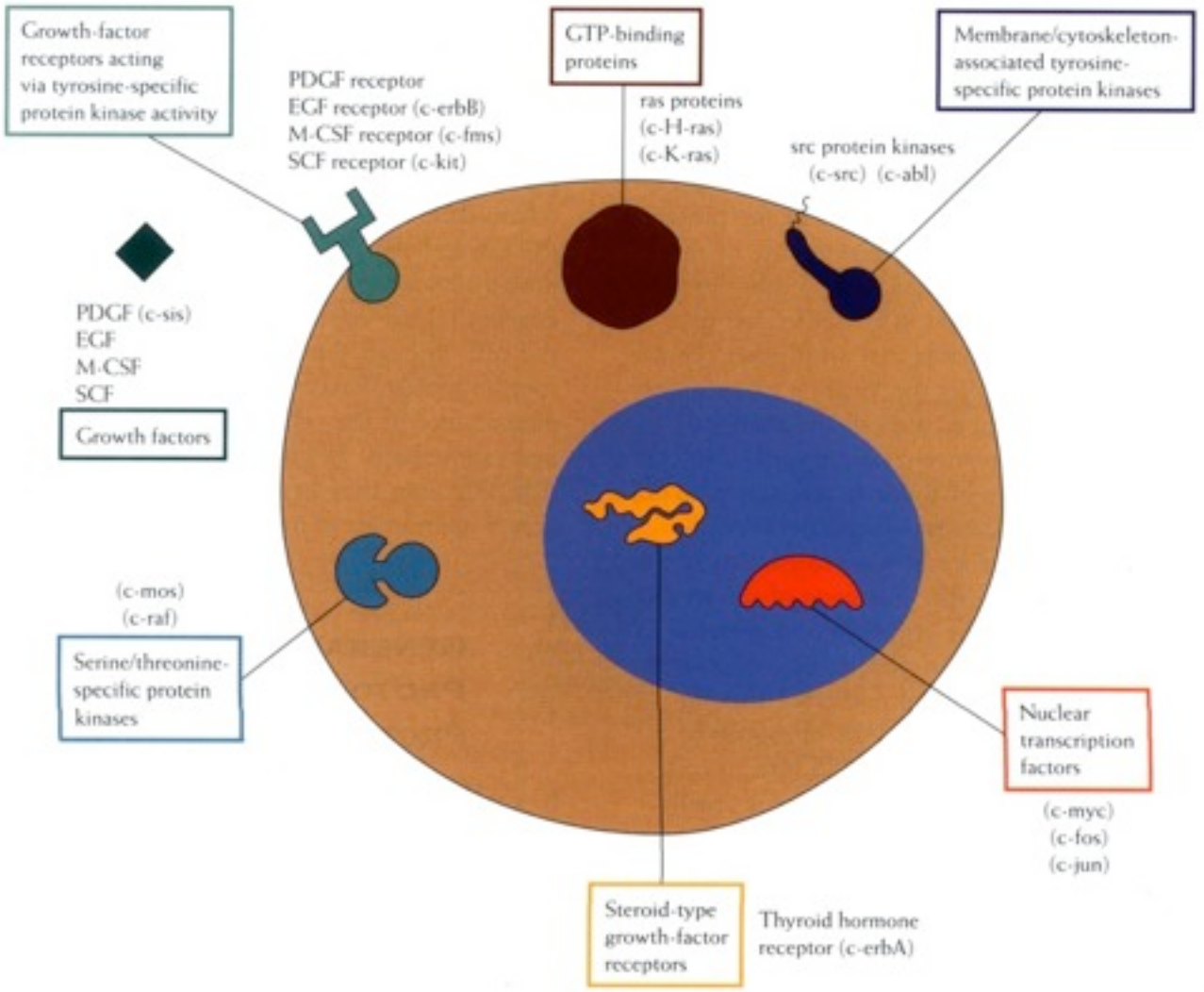


Abnormal signal transducers



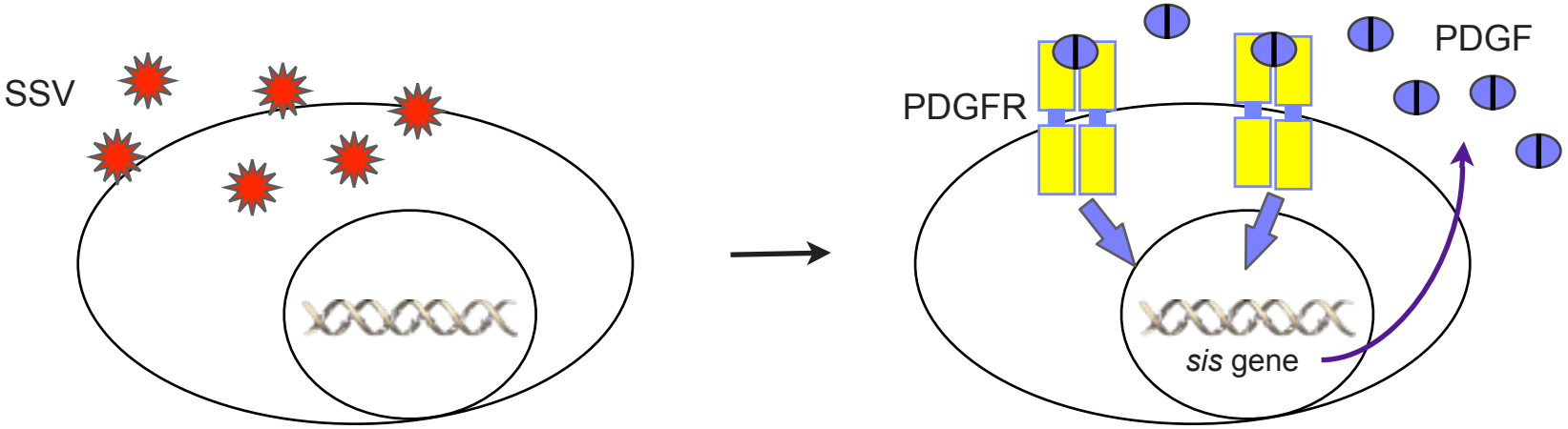
Unregulated transcription factors

Oncogenic subversion of signaling molecules



Growth factors and their receptors as oncogenes

1. Growth Factors: The product of the sis oncogene of the Simian Sarcoma Virus is PDGF



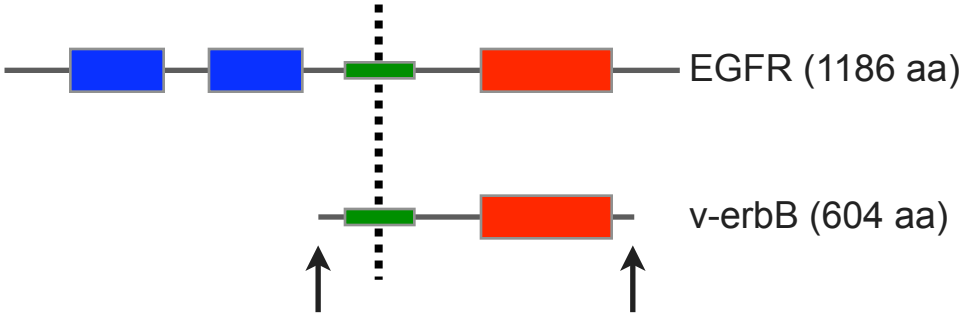
Viral infection and integration in host DNA

Constitutive secretion of PDGF (BB type)
and activation of the PDGF receptor

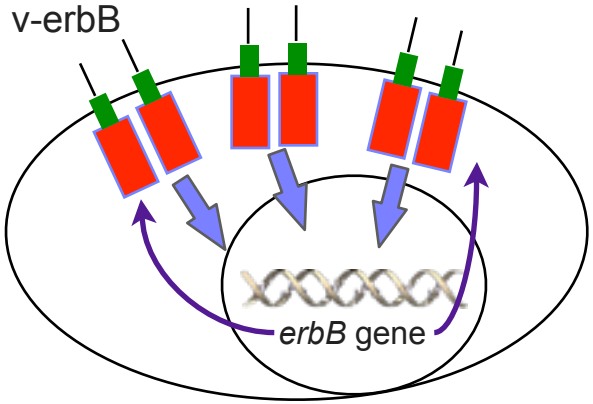
Autocrine stimulation contributes
to cell transformation

Growth factors and their receptors as oncogenes

2. Growth Factors Receptors: The product of the erbB oncogene of the Avian Erythroblastosis Virus is a truncated EGF receptor



Loss of the ligand binding domain
and of 32 aa at the C-terminus

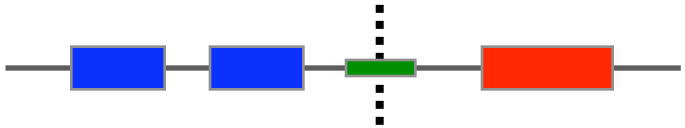


Constitutive activation in the
absence of ligand

Similar extracellular domain truncations found in human gliomas

Oncogenic activation of receptors by point mutation

Example: The neu oncogene (rat: neu - human: c-erbB2 or HER2)



Close similarity to EGFR

First identified as the activated oncogene present in chemically-induced rat neuroblastomas.

Comparison with the normal, non-oncogenic counterpart revealed only one amino acid difference in the trans-membrane domain

Normal neu: - T F I I A T V **V** G V L L F L I L V V V V G I L I -

Oncogenic neu: - T F I I A T V **E** G V L L F L I L V V V V G I L I -

657 Val 664 > Glu 680

The mutation results in ligand-independent dimer formation and kinase activation, resembling a ligand-stimulated receptor

Normal neu: - T F I I A T V **V** G V L L F L I L V V V V G I L I -

Oncogenic neu: - T F I I A T V **E** G V L L F L I L V V V V G I L I -

Val 664 > Glu

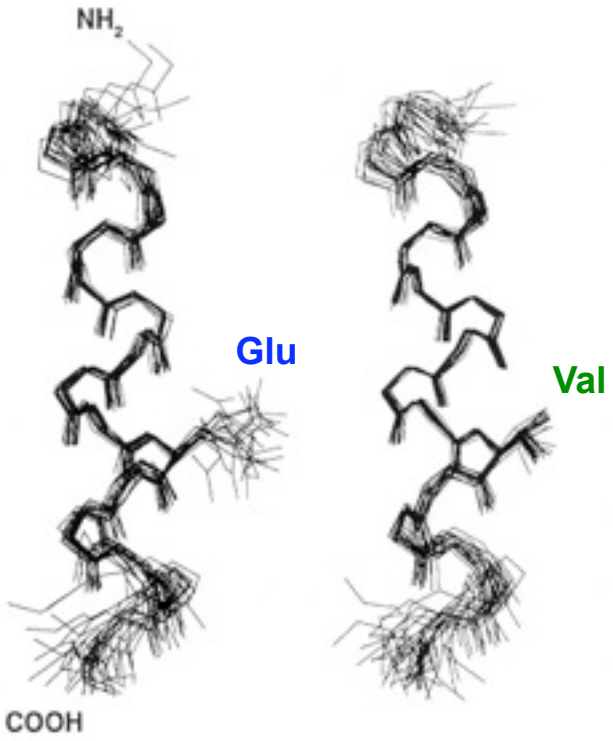
- *If the same mutation is introduced in the normal gene, it becomes oncogenic*
- *Only Val > Glu or Val > Gln have this effect*
- *If Glu is introduced in positions 663 or 665, there is no effect*
- *Mutation of residues 661, 663 or 665 abolishes the effects of the Val > Glu mutant*

	661	662	663	664	665
Transforming:	- Ala -	Thr -	Val -	Glu -	Gly -
Non-transforming:	Leu		Gly		Val

The Val > Glu activation involves highly specific interactions in the transmembrane domain

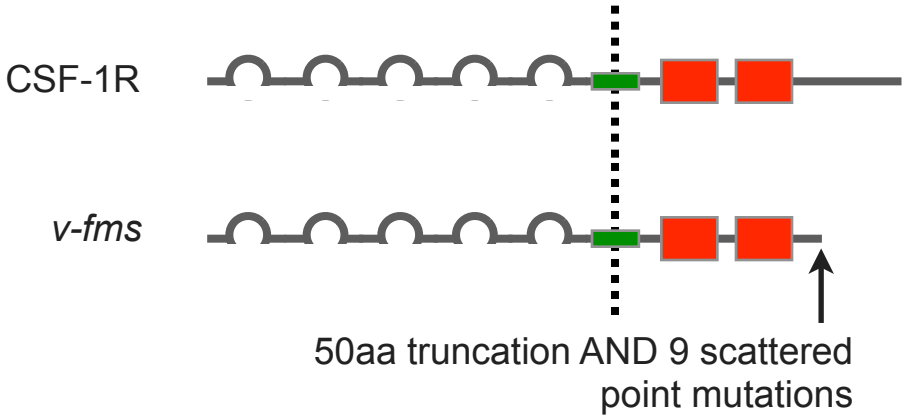
NMR structures: No conformational change

In the membrane environment, the Glu side-chain will be protonated and may form a hydrogen bond with a neighboring helix (possibly with a carbonyl oxygen), stabilizing dimerization



Oncogenic activation of receptors by truncation and point mutation

Example: The v-fms oncogene product of the feline sarcoma virus is a mutant CSF-1 receptor



What is the relative contribution of these structural alterations?

- *Truncate C-terminus of CSF-1R (c-fms) and transfect cells: Transformation, but only in the presence of ligand*
- *Introduce point mutations: Leu 301 > Ser and Ala 374 > Ser: Transformation in the absence of ligand*

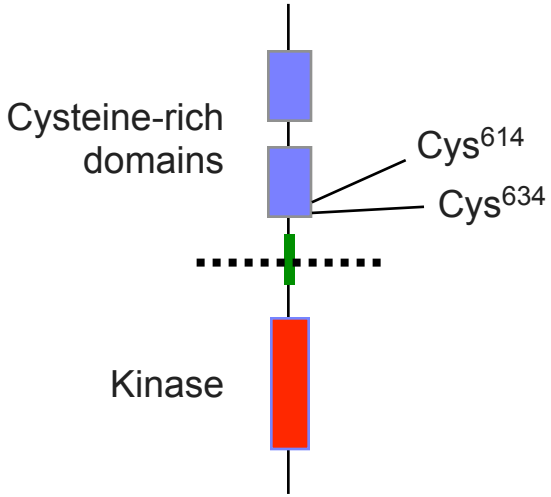
Multiple Endocrine Neoplasia 2A: Germ-line mutations of RET

MEN2A: Dominantly inherited cancer - Thyroid carcinoma and pheochromocytoma

Gene mapped with linkage techniques to the same region as *ret*

In 95% of cases: Mutation of Cys^{634} to Gly, Tyr, Ser or Phe

In 5% of cases: Mutation of Cys^{614} to Gly



Mutation and heterozygous expression give rise to MEN2A:

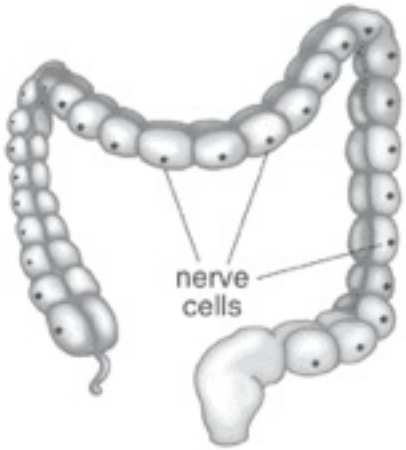
Dominant oncogenic activation

Ret

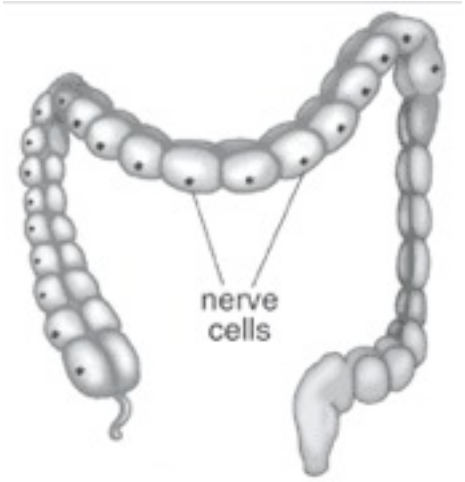
Activating mutations result in tumors

Loss-of-function mutations result in developmental anomalies

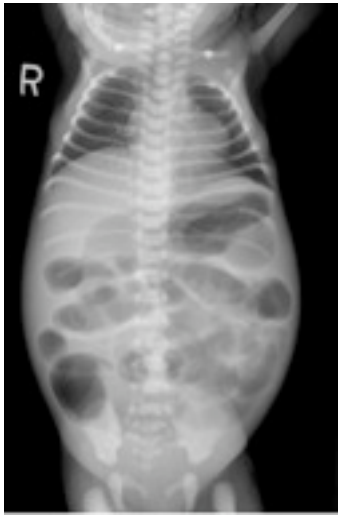
Hirschprung's disease: absence of ganglion cells in the mesenteric plexus of the colon



Normal



Hirschprung's disease



Affects 1 in 6000 people

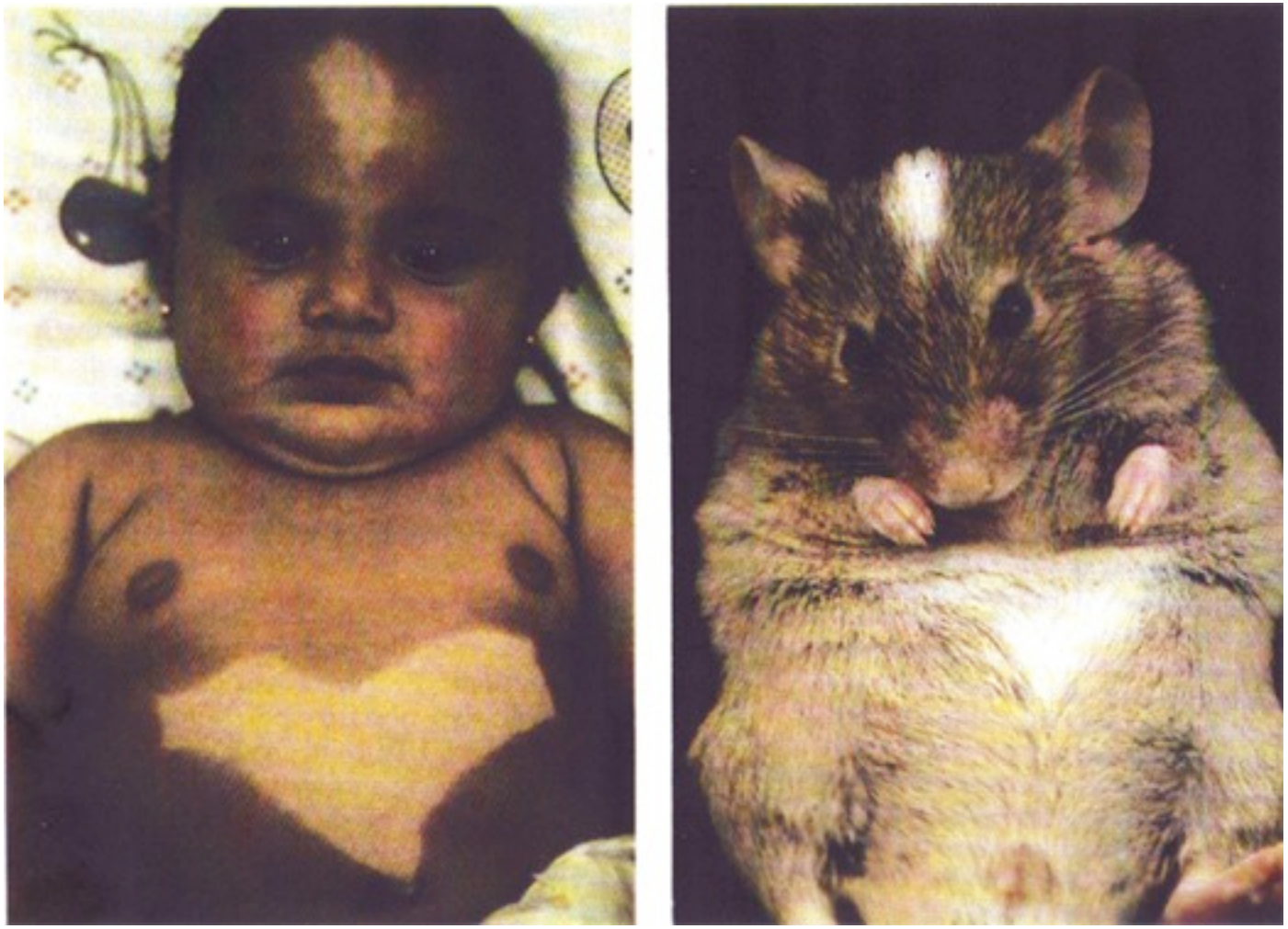
Dominant inheritance of:

- *Deletions*
- *Premature stop-codons*
- *Amino acid changes*

→ *Ret is inactive during normal development*

Of mice and men....

Transgenic mice as models for human disease



Similar phenotypes in humans and mice, caused by mutation of the *kit* gene

Targeted disruption of RTK genes (knock-out mice)

Ret:
- *No kidneys or severe dysgenesis*
- *No enteric neurons*

Met: ***(HGFR)***
- *No migration of precursors of the limb-bud myoblasts*
- *The same in diaphragm and tip of the tongue*
- *Reduced liver and placenta size*

Tie-1:
- *Defect in structural integrity of vascular endothelial cells*
- *Mice die after birth - edema and hemorrhage*

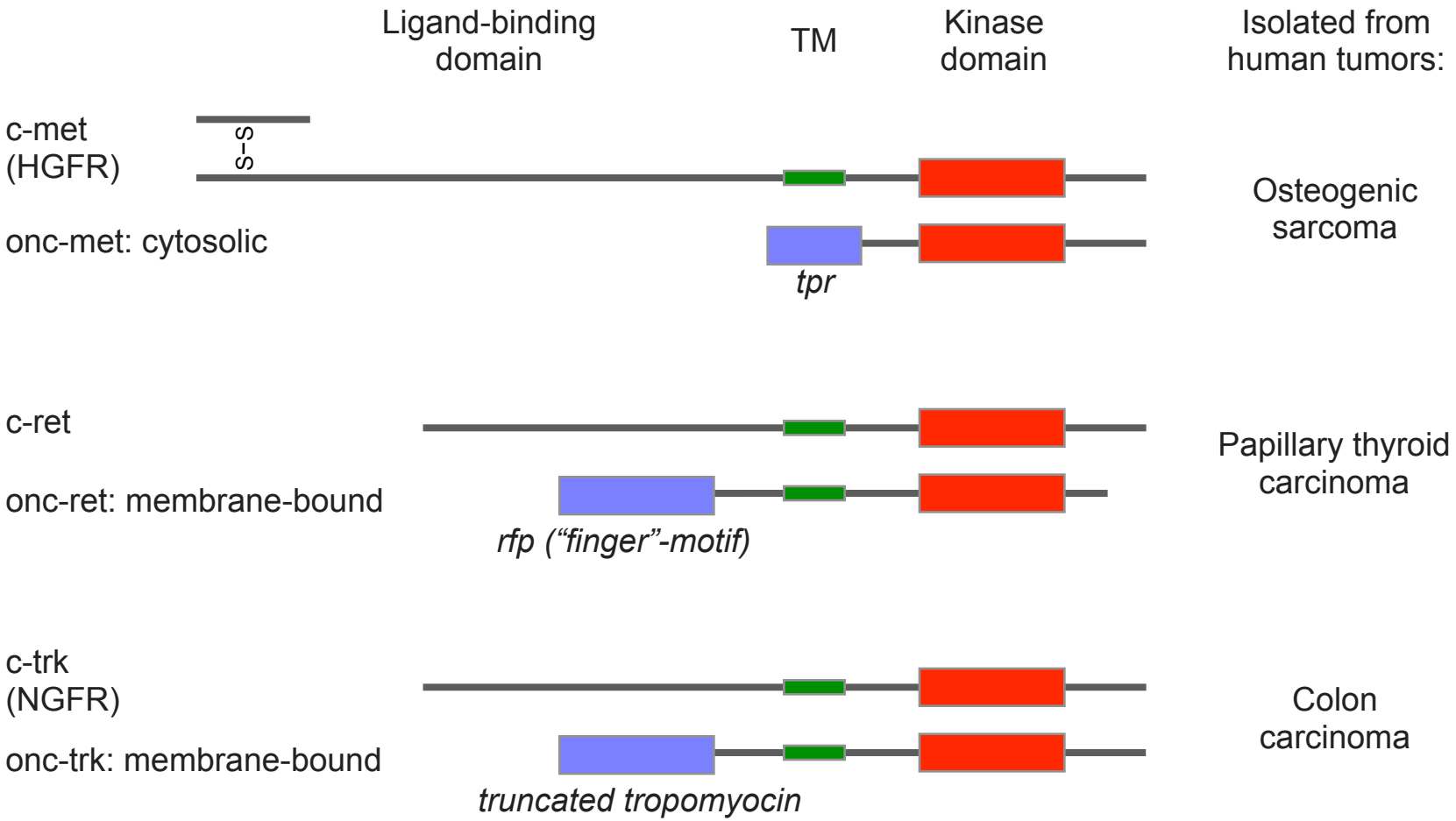
Tie-2:
- *Defects in vascular network formation (angiogenesis)*
- *Mice die at E10.5 - Growth retardation*

Flt-1:
- *Defects in organization of embryonic vasculature*
- *Defective cell-cell or cell-matrix interactions during vascular development*
- *Not essential for endothelial cell differentiation*

Flk-1:
- *Early defects in development of hematopoietic and endothelial cells*
- *Mice die at E9*

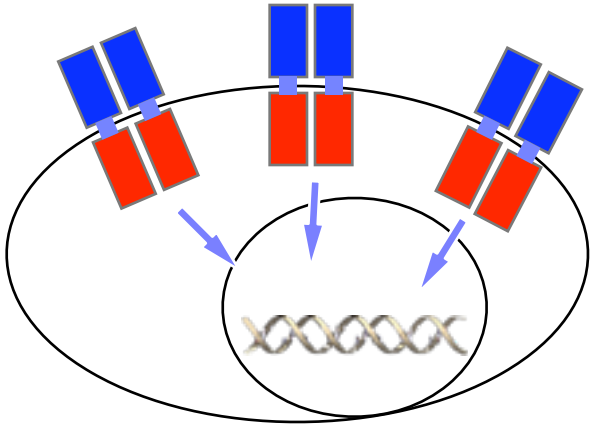
In all cases: Correlation between site of receptor expression and phenotype

Oncogenic activation by DNA rearrangement

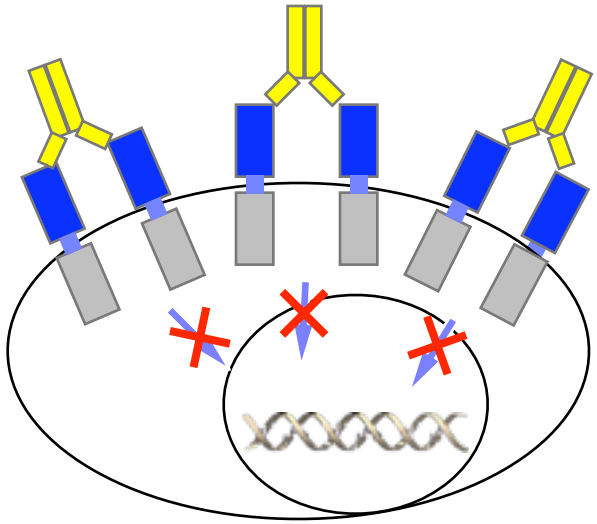


HER-2 over-expression in breast cancer

Correlation with tumor aggressiveness and poor prognosis



Constitutive activation of
HER2 by over-expression



Herceptin

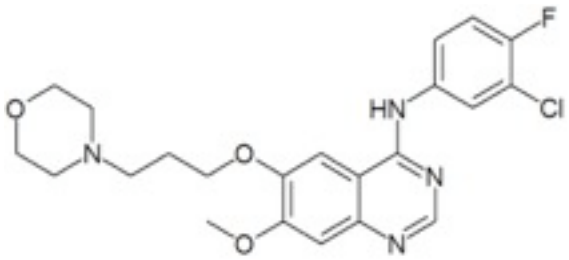
Anti-HER2 antibodies
block the signal

EGFR over-expression or mutation in human cancers

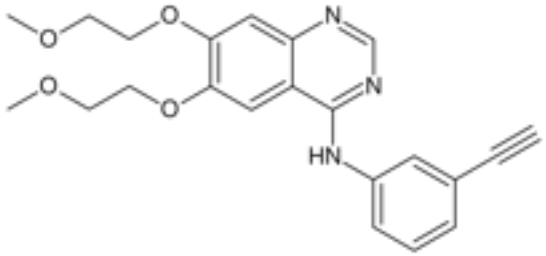
Over-expression through gene amplification or increased transcription

Found in lung, bladder, brain and many other tumors

Development of selective kinase inhibitors (ATP-binding site):

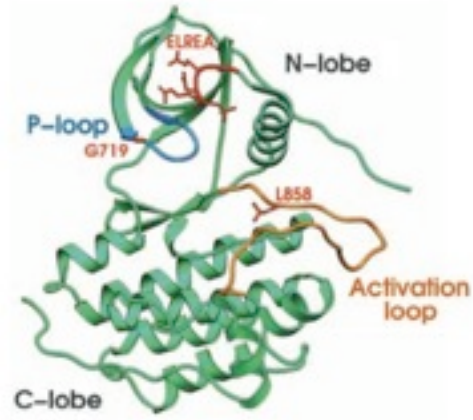


Gefitinib



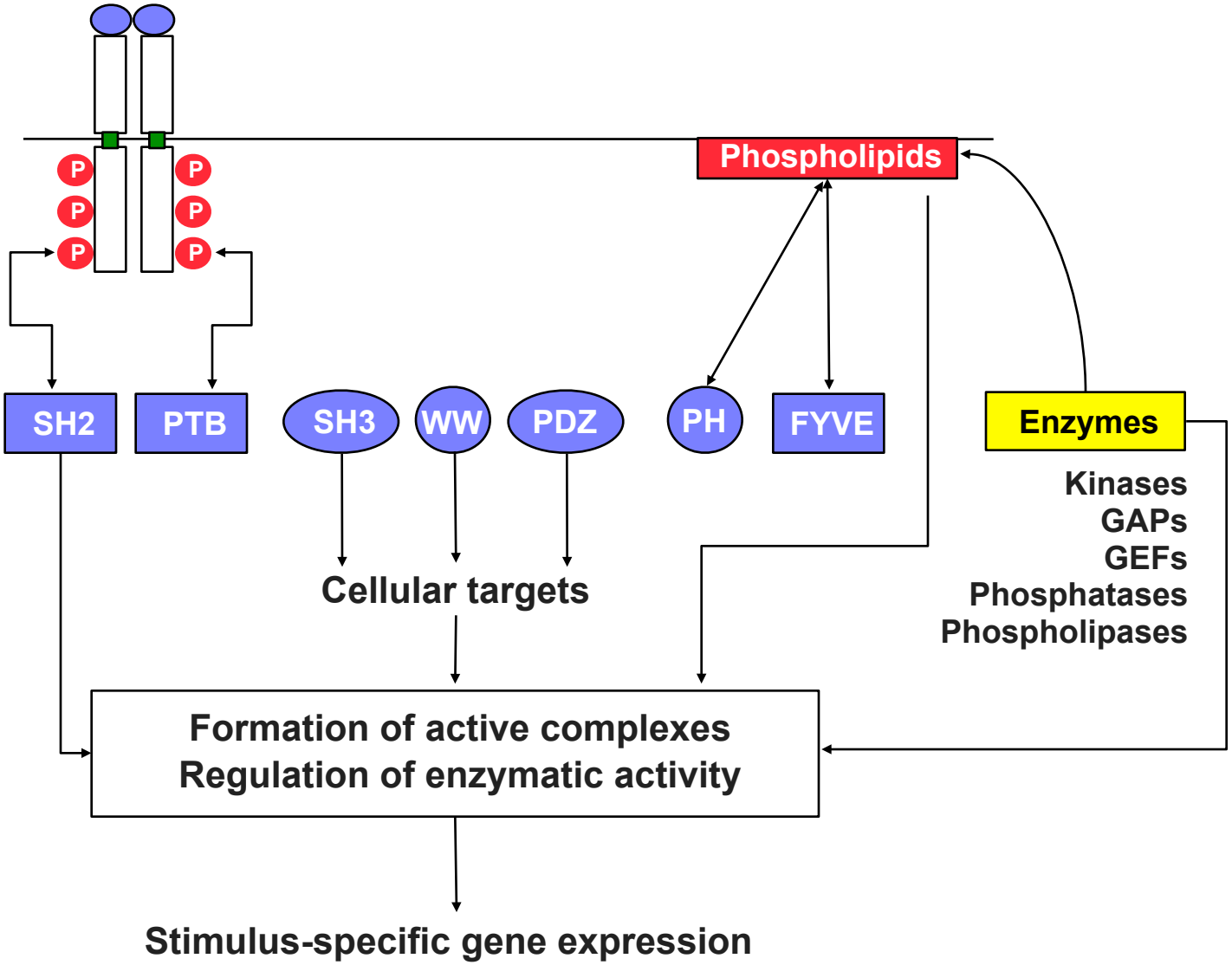
Erlotinib

Responses to drug treatment are only seen in patients with EGFR mutations that result in increased kinase activity

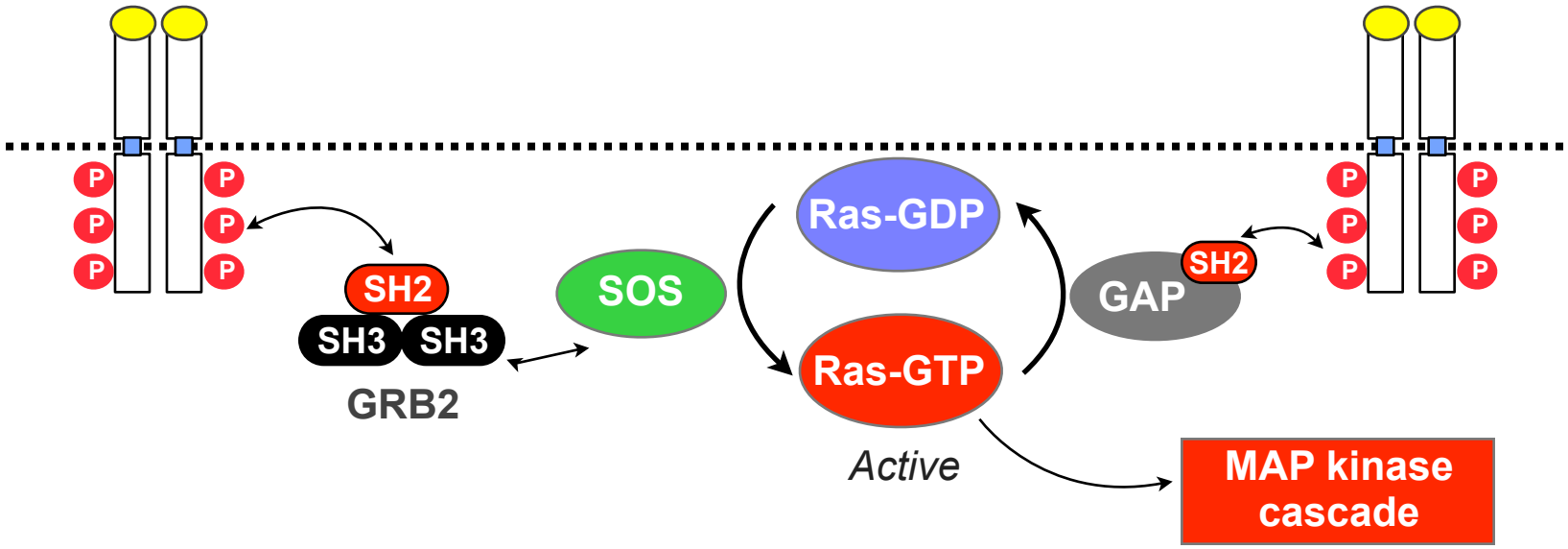


***EGFR* Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy**
J. G. Paez, et al., (2004) *Science* **304** (5676), 1497.

Signal transduction by receptor-tyrosine kinases



The link between growth factor receptors and Ras

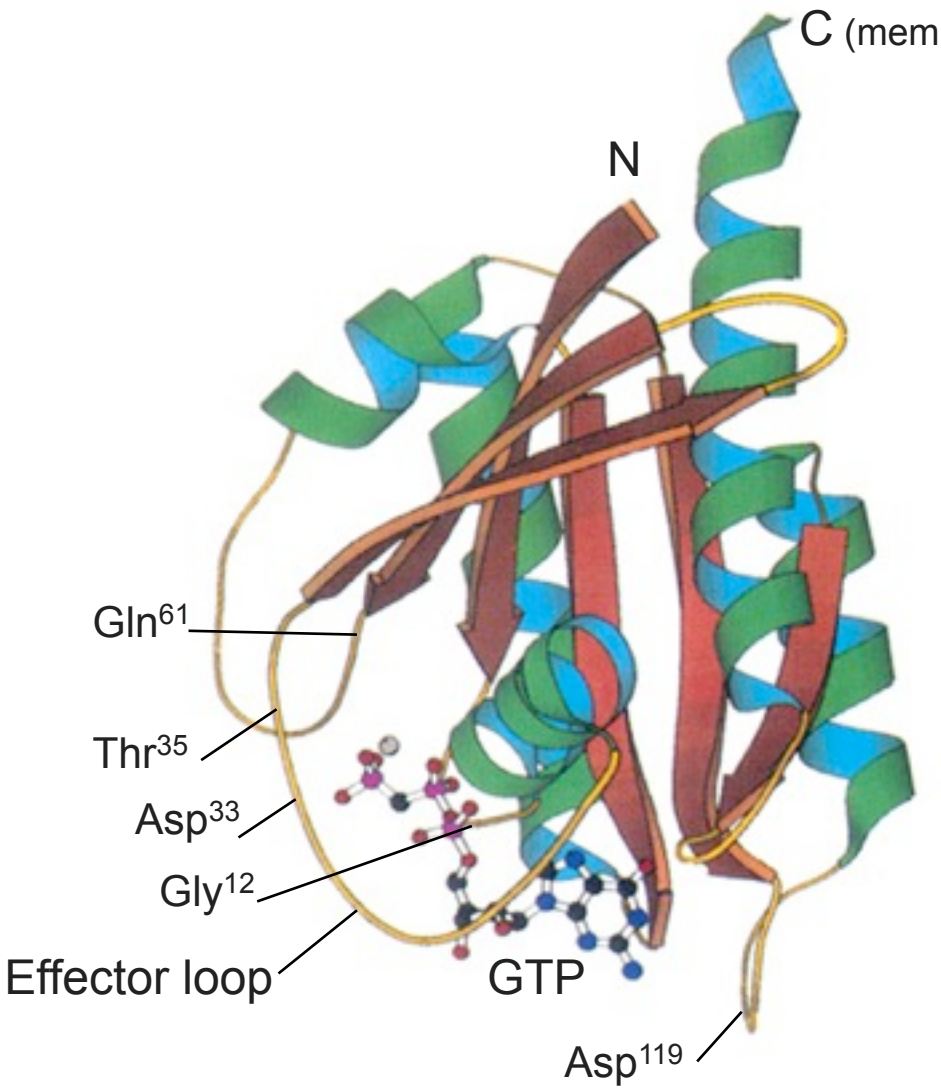


Ras cycles between an inactive, GDP-bound form and an active, GTP-bound form

This process is controlled by:

- *SOS, a guanine-nucleotide exchange factor*
- *Ras-GAP, a GTPase activating protein*

Ras mutations in cancer



Point mutations of Ras have been identified in ~30% of human tumors

The most common are at positions 12, 13, 59 and 61

- *Position 12: Reduced affinity for nucleotides*
- *Positions 13, 59, 61: Reduced GTP hydrolysis*
- *Positions 33, 35: Uncoupling from GAP*

Colon carcinoma progression - the role of the Ras oncogene

Normal epithelium → Hyper-proliferative epithelium → Early adenoma → Intermediate adenoma → Late adenoma → Carcinoma → Metastasis

DNA hypomethylation

Other alterations

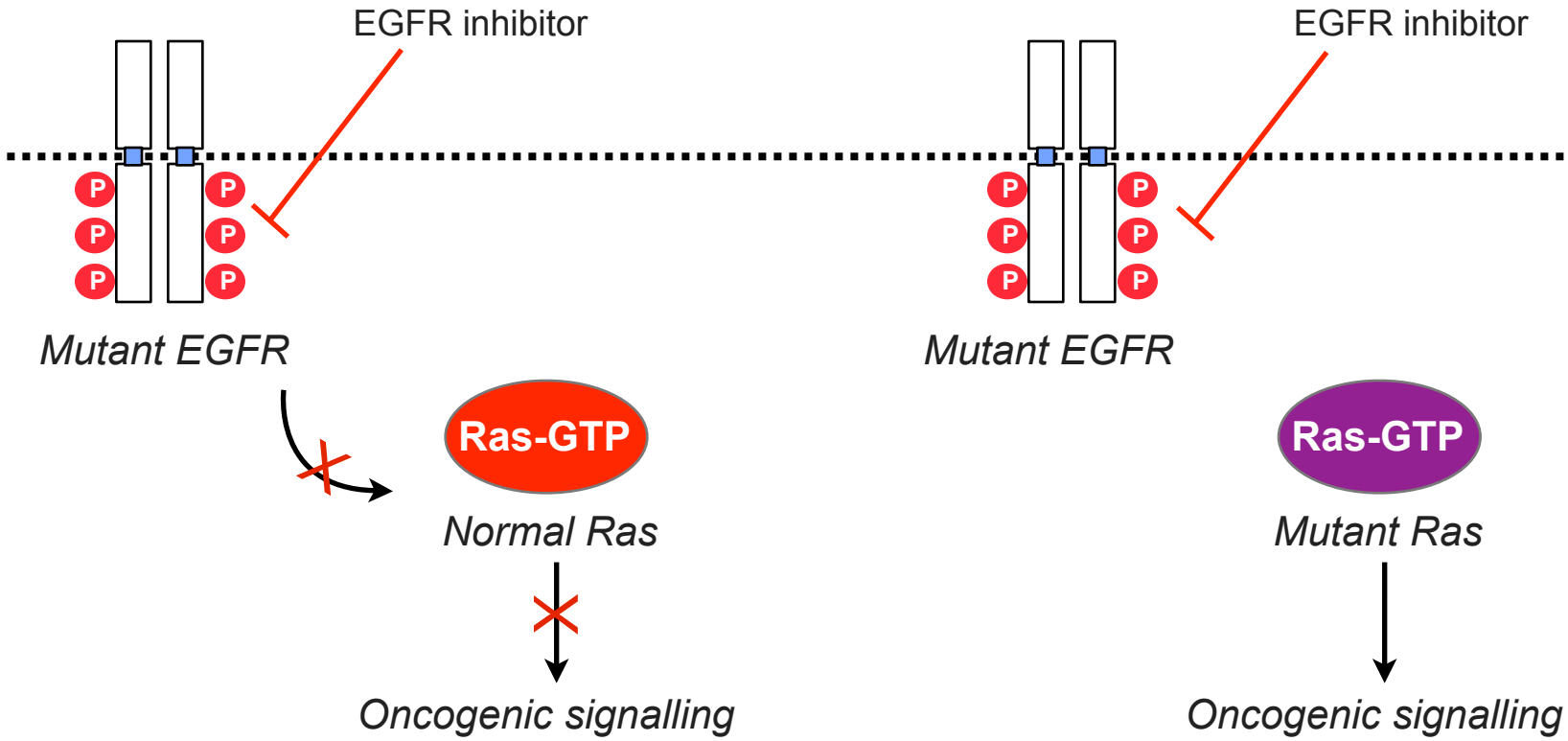
Chromosome:	5q	12p	18q	17p
Alteration:	Loss	Activation	Loss	Loss
Gene:	APC	K-ras	DCC	p53

Identification of sub-groups of patients that can benefit from inhibitors

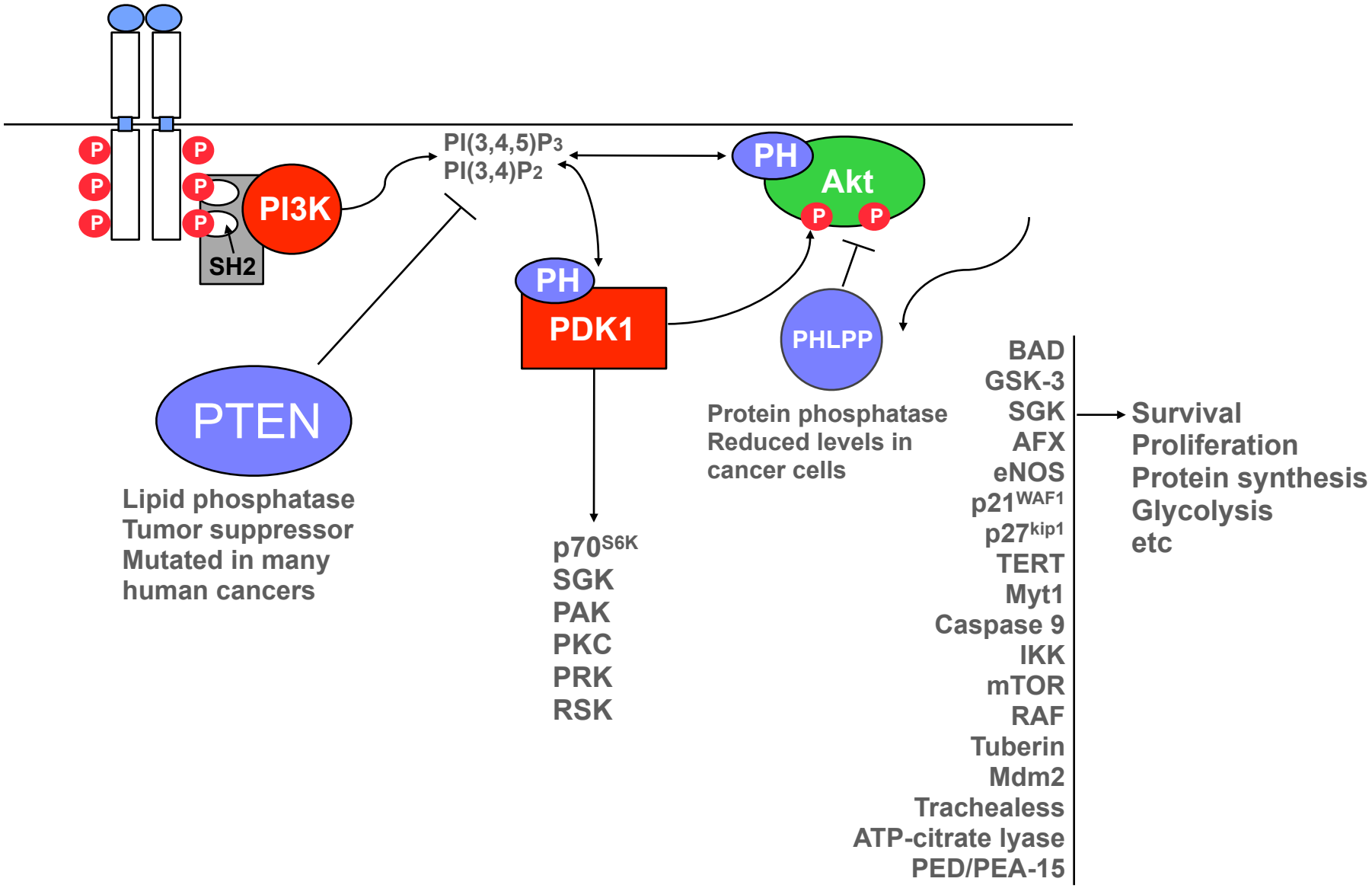
Metastatic colorectal carcinomas often have mutations in the EGF receptor

Q: Do EGFR inhibitors work as anti-tumor agents?

A: Only if Ras is not mutated

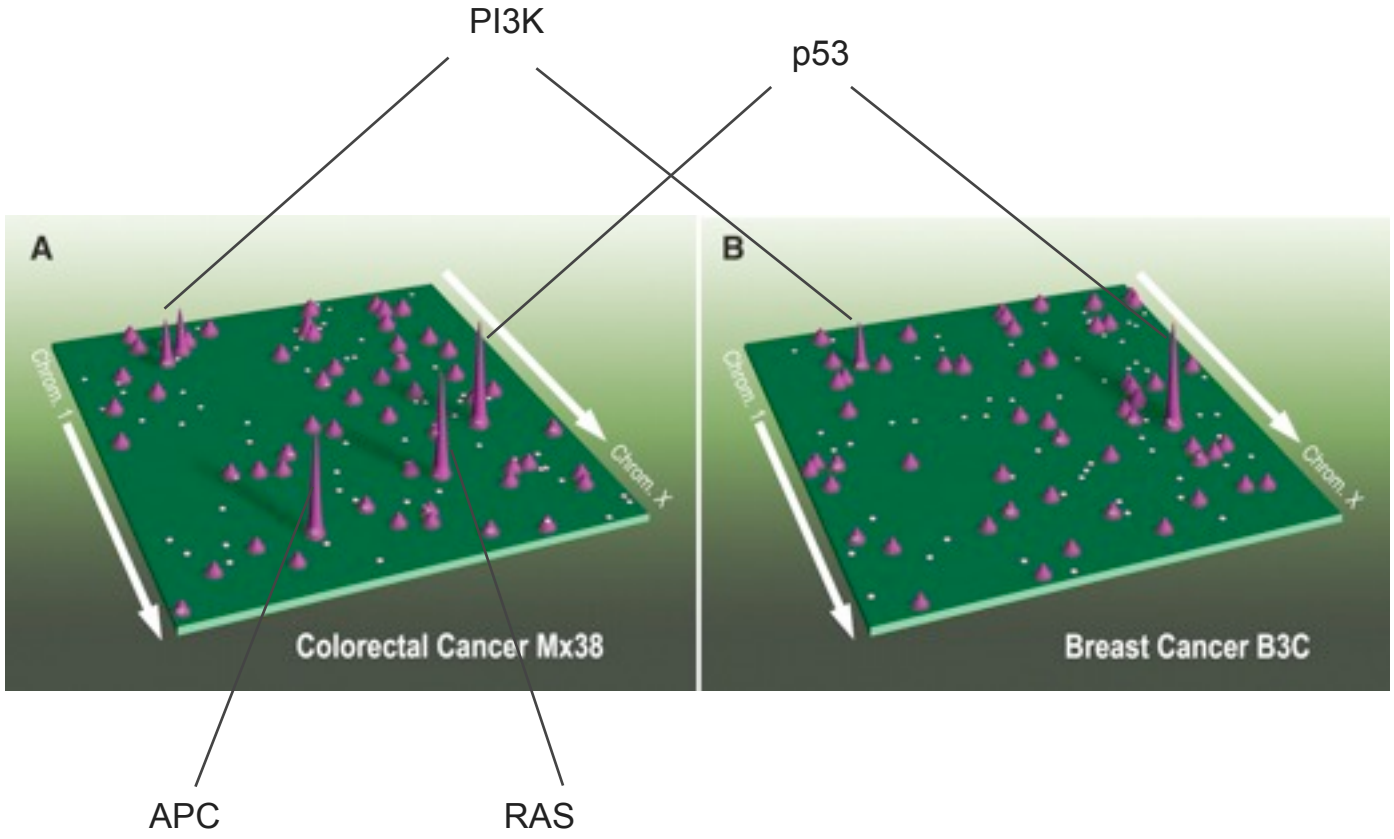


Protein targets of PI 3-kinase products



Genomic landscapes of human cancer

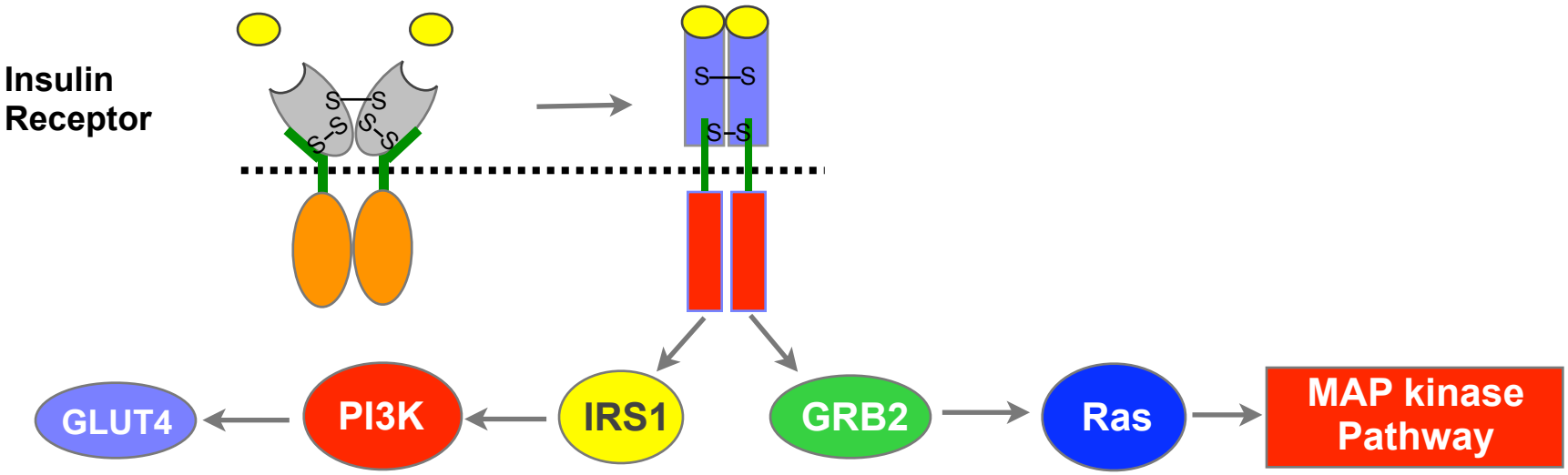
Analysis of mutations in 18191 genes from 11 colorectal and 11 breast cancer samples



Small number of commonly mutated “mountains” and large number of infrequently mutated gene “hills”

L. D. Wood et al., Science 318, 1108 -1113 (2007)

Insulin Receptor and NIDDM



Mutations on the Insulin Receptor gene, associated with diabetes:

Class 1: impaired receptor biosynthesis

Class 2: impaired transport of receptors to the cell surface

Class 3: decreased affinity of insulin binding

Class 4: impaired tyrosine kinase activity

Class 5: accelerated receptor degradation