

# BioMed 507

## **Week 2**

### **Outline:**

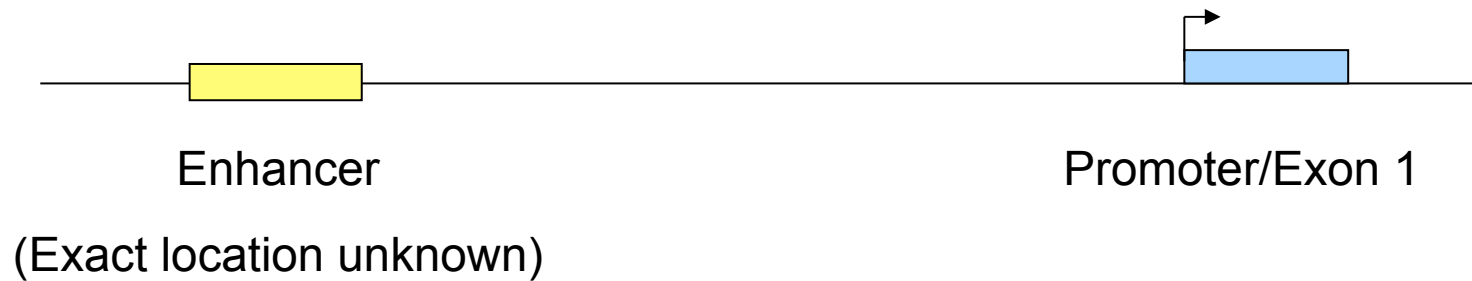
- *Regulatory Elements in Chromatin*
- *Transcriptional Activators*
- *Two-Hybrid Assays*
- *Regulated Transcription Factors*

# Identify Regulatory Elements (e.g. Enhancers) in Chromatin

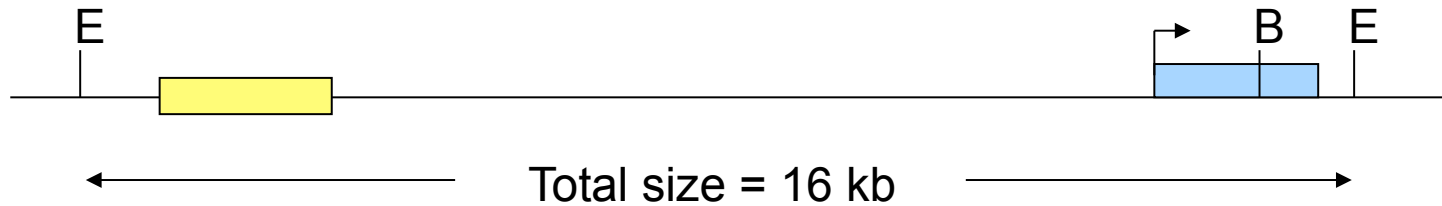
- *Regulatory elements have proteins bound*
- *Regulatory elements have altered chromatin structure*
- *Often identified as DNaseI hypersensitive sites*

# DNase I sensitivity assay - I

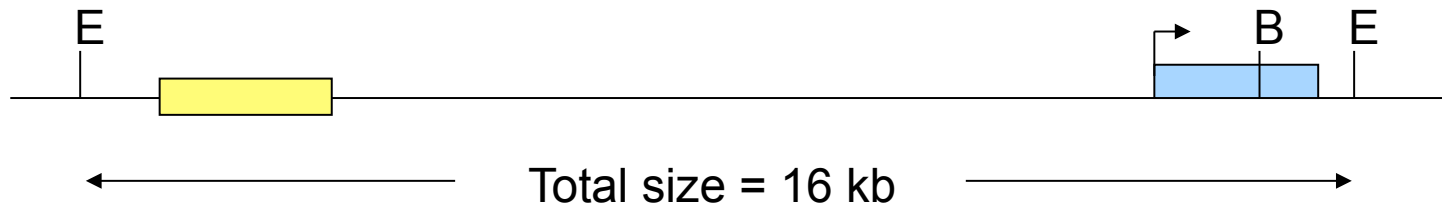
Gene Structure:



Restriction Sites:



# DNase I sensitivity assay - I



## ***Start with appropriate cells, isolate nuclei***

- *Nuclei have “holes” that allow entry of enzymes*

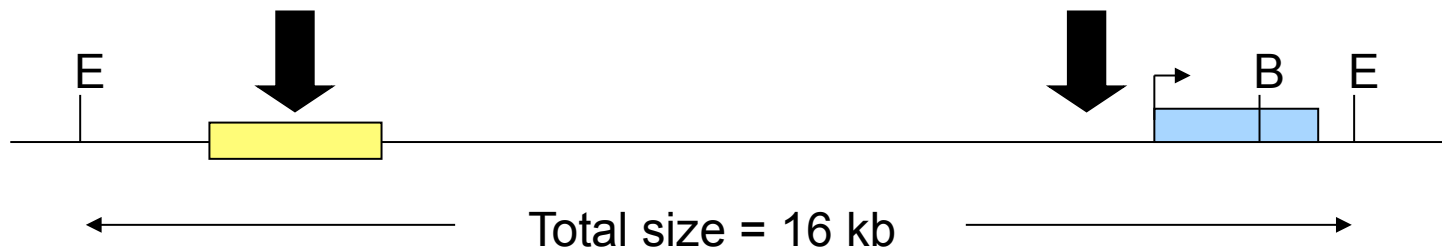
## ***Treat with limiting amounts of DNase I***

- *Partial digestion*
- *Only the most sensitive spots get cut*

## ***Purify DNA, digest with enzyme ‘E’***

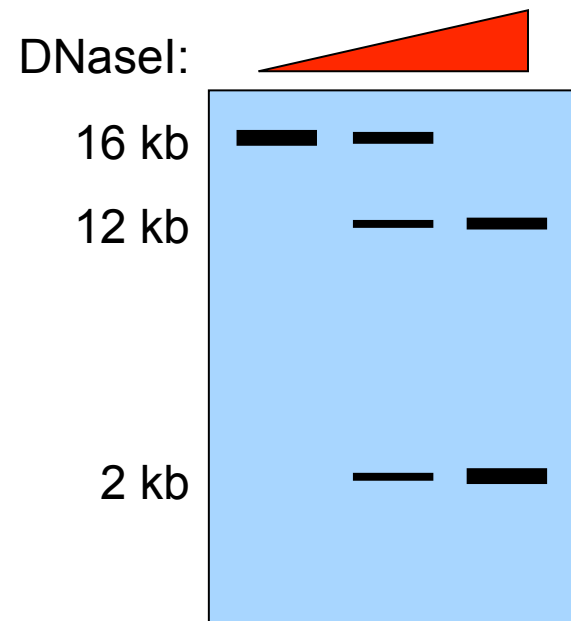
- *Analyze DNA by Southern blot*
- *Use purified B-E piece from 3'-end as probe*

# DNase I sensitivity assay - I



*Partial DNaseI digestion  
identifies hypersensitive sites*

*Size of fragments indicates  
distance from probe to  
hypersensitive sites*



# Imprinting

*H19 gene*

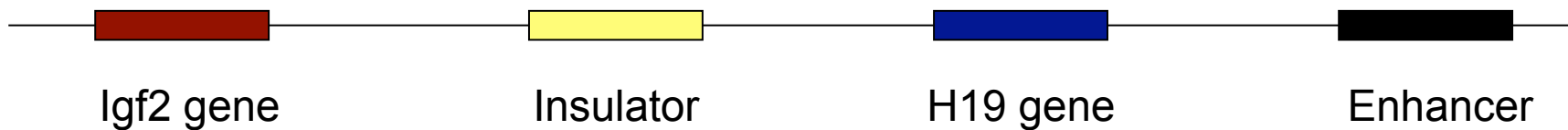
- ***Only maternal copy is expressed in offspring***

*Igf2 gene*

- ***Only paternal copy is expressed in offspring***

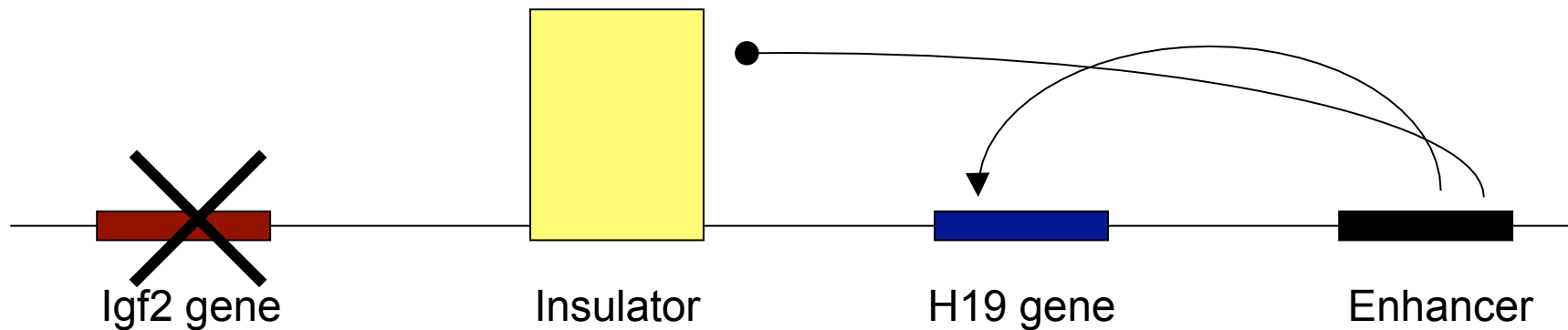
# Regulation of H19 and Igf2

- *If insulator is active, enhancer works only for H19 gene*
- *If insulator is inactive, enhancer works only for Igf2 gene*
- *Two genes compete for a single enhancer*



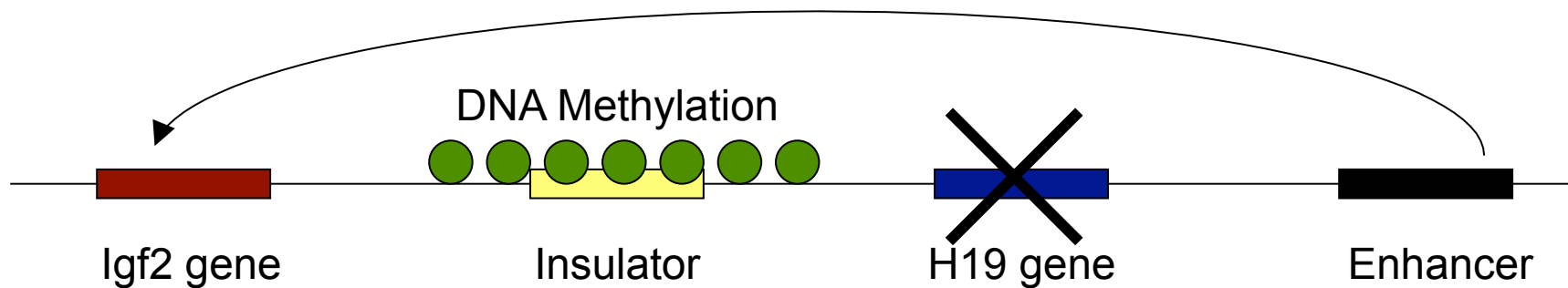
# Regulation of H19 and Igf2 - II

- *When derived from maternal cells, the insulator is not methylated, and is active*
- *So the chromosome derived from maternal cells expresses only the H19 gene and not Igf2*



# Regulation of H19 and Igf2 - III

- *When derived from paternal cells, the insulator is methylated, and is not active*
- *So the chromosome derived from paternal cells expresses only the Igf2 gene and not H19*

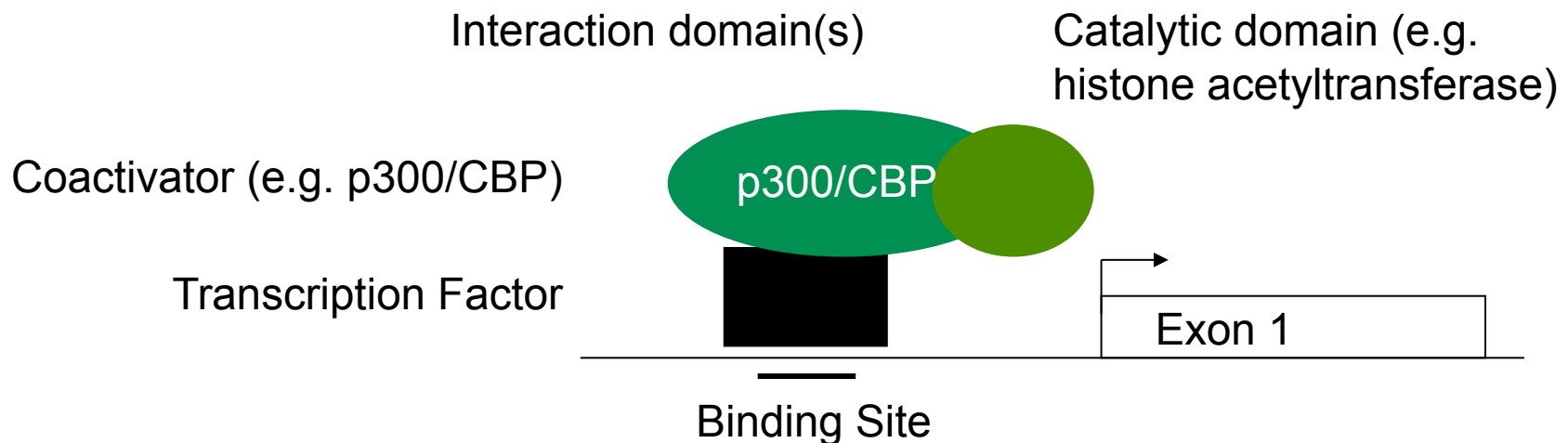


# Maintenance of DNA Methylation

- *DNA methylation is maintained after DNA replication*
- *Daughter cells have the same methylation patterns*
- *Genes inactivated by methylation remain inactivated*

# Transcriptional Coactivators

- *A major breakthrough was the finding that transcriptional co-activators and co-repressors are enzymes that modify chromatin structure*



# Coactivators as Chromatin remodeling machines

*Many different complexes*

- *SWI/SNF*
- *RSC*
- *NURF*
- *CHRAC*
- *ACF*
- *BRM*
- *E-CRF*
- *BRG1-associated*
- *hbrm-associated, etc.*

# Complexes

- *All contain SWI2/SNF2 or closely-related protein as subunit*
- *All contain ISWI, a nucleosome-stimulated ATPase (repositioning motor?)*
- *Different complexes have the same “core” components, different specificity components*

# Upstream Activator Domains

## DNA-Binding Domains

Homeo domain  
bZIP  
Helix-loop-helix  
Helix-turn-helix  
Zn-finger  
Myb  
Ets  
Pou  
Many others

## Activation Domains

**Acidic**  
**Basic**  
**Gln-rich**  
**Pro-rich**  
**others**

## Other Domains

Negative regulation  
Dimerization  
Ankyrin repeats  
Actin-binding  
Kinase  
Trans-membrane  
Others

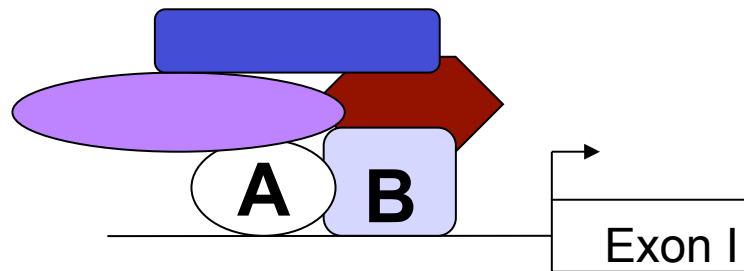
**How do activation domains work?**

# Activator Interactions

*Activators must interact with cellular components*

*Co-factors help determine activity and specificity*

*Tissue-specific co-factors could allow one TF to regulate different genes in different cell types*



# Cellular factors are limiting

- *Overexpressed activators cause “squenching”*

Set up reporter gene assay

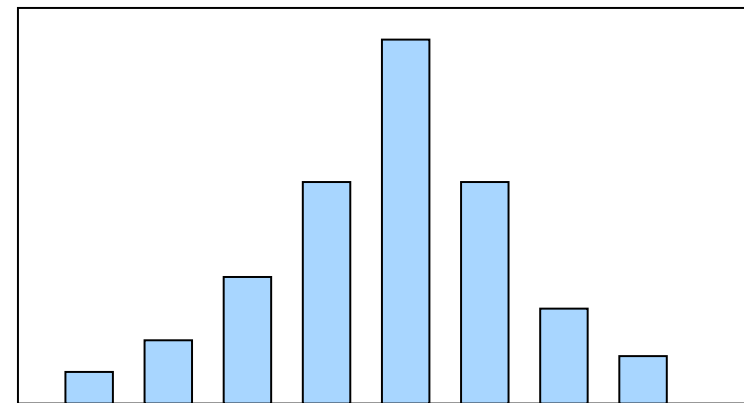
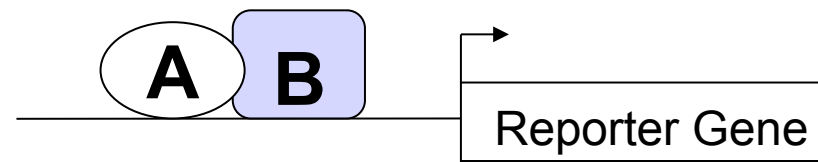
Co-transfect reporter with expression vector for TF-A

Activity of reporter increases with level of TF-A

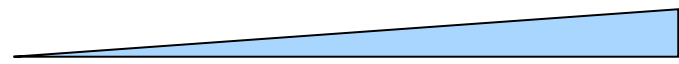
Too much TF-A inhibits

Inhibition is caused by squelching

Too much TF-A exhausts the available co-factors

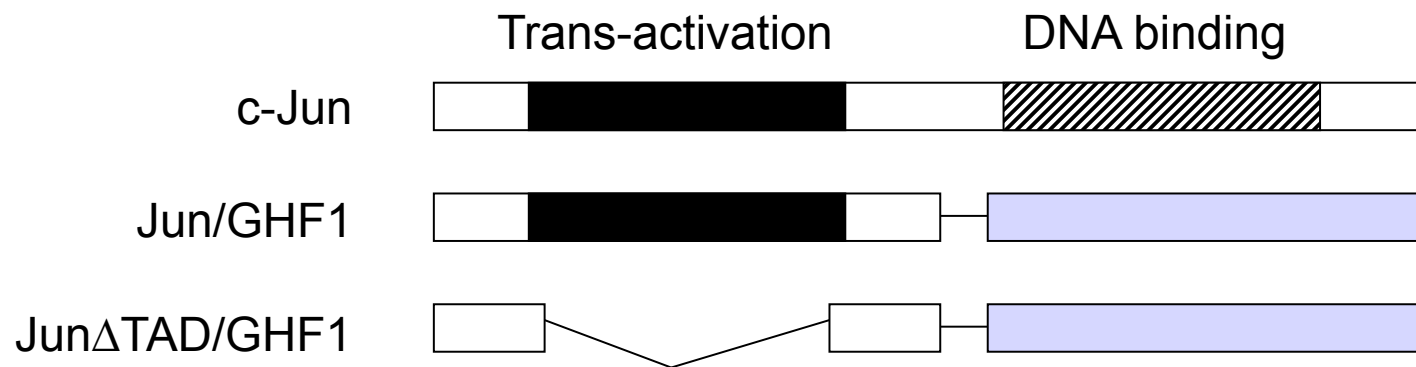


TF-A amount:



# Squelching Example - I

## *c-jun TAD binds limiting co-factors*



Construct hybrid with Jun TAD and GHF1 DNA-binding domain

Construct hybrid lacking Jun TAD

Test all three in co-transfection assay, using Jun-dependent reporter gene

# Squelching Example - II

## *Reporter gene assay (Oehler & Angel, 1992)*

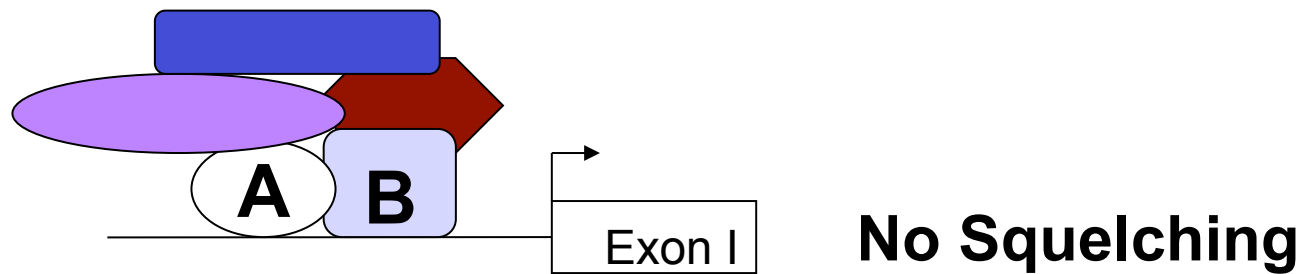
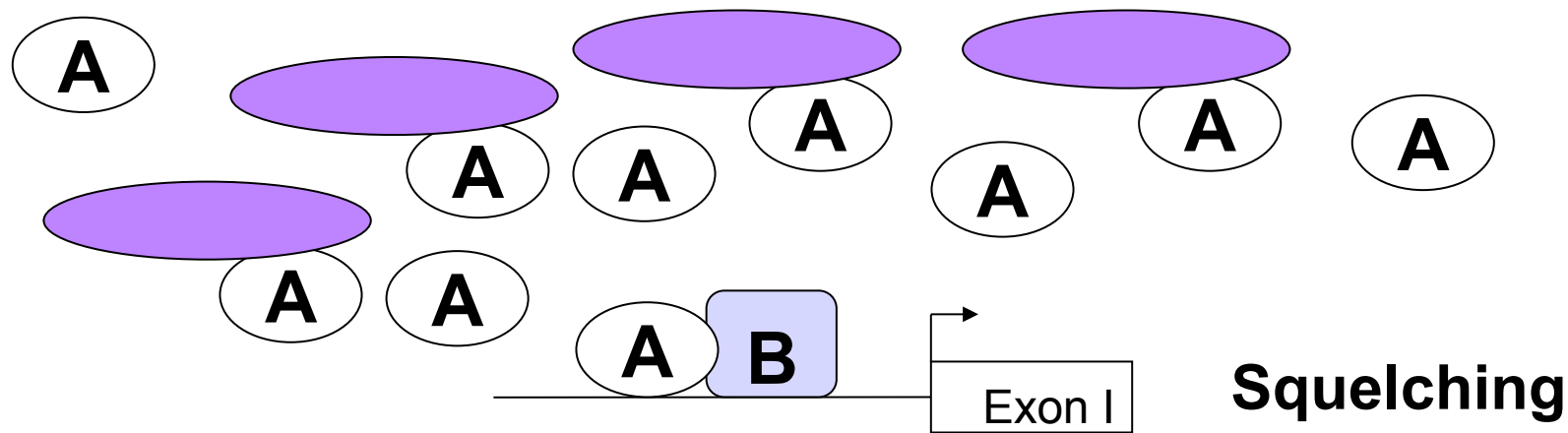
- *Use cells with very little endogenous c-Jun*
- *Transfect with reporter plus 0.5  $\mu$ g c-Jun expression plasmid*

<b>Competitor</b>	<b>Amount</b>	<b>Fold Activation</b>	
—	—	6.8	
Jun/GHF1	0.5	3.5	<b>Squelching</b>
	1.0	2.5	
	2.5	1.3	
Jun $\Delta$ TAD/GHF1	0.5	7.0	<b>No Squelching</b>
	1.0	7.7	
	2.5	7.6	

# Squelching Example - III

- *Over-expression of c-Jun TAD causes squelching*
- *Jun TAD is 'acidic' type (lots of D, E residues)*
- *Over-expression of other acidic TADs also squelches*
  - e.g. Gal4, VP16
- *No squelching by other TADs: S/T-rich or Gln-rich*
- *Data suggest that each type of TAD competes for a different limiting cellular factor*

# Squelching: Competition



# Implications of Competition

## ***Limiting co-factors***

*Not all genes will be expressed at once*

*Promoters must compete for co-factors, RNA Pol*

*Changing affinities will change activity*

e.g. post-translational modifications

*Many more ways of controlling gene expression*

Levels of TFs

Levels of co-factors

Modifications of TFs or co-factors

# Characteristics of Transcriptional Activators

- *Can be classified by amino acid composition*  
e.g. Gln-Rich, Pro-Rich, Acidic, Basic
- *Must work through protein-protein interactions*  
But what proteins do they interact with?
- *No detailed structures are known (believed?)*  
Computers predict alpha-helical structures  
Only available structures show beta-sheet structures
- *TADs are probably largely unstructured and flexible in solution, yet still specific*  
TAD structures may be modified (solidified) by protein-interactions  
“Induced Fit” mechanism

# Co-Activators, Co-Factors and Repressors

- *Co-activators mediate interactions between TADs and basal machinery*
- *dTAF110 binds Gln-rich TAD of SP1*
- *dTAF40 binds acidic domain of VP16*

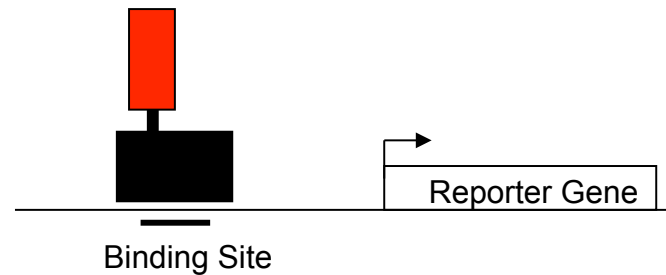
# Transcriptional Activation Domains

- *The most important structural features may not be the most obvious ones*
- *Hydrophobic residues may be more important than charged ones*
- *Hydrophobic interactions are often overlooked but are probably more important than ionic or other interactions*
  - e.g. stacked bases in DNA
  - e.g. leucine zipper structures

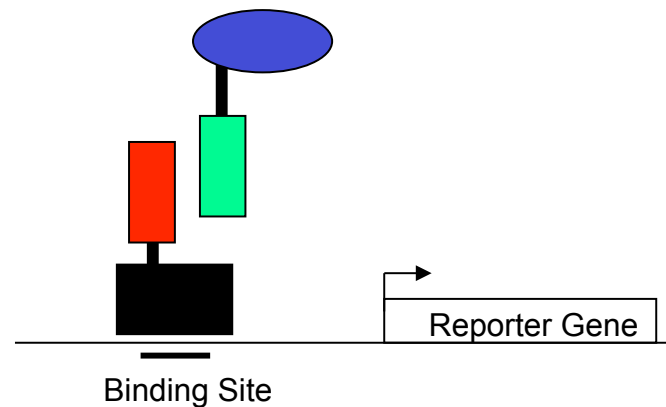
# How Do Associations Change Activity?

- *Binding a co-activator can change a repressor to an activator*

No trans-activation domain, so DNA binding protein is a repressor (e.g. lac repressor)



Interaction with co-factor containing trans-activation domain converts repressor into an activator



# Example: Oct1, Oct2 and VP16

- *Oct1 – ubiquitous expression, weak/poor activator*
- *Oct2 – B-cell specific, strong activator*
- *Both Oct1 and Oct2 bind same OCTA site “ATGCAAAT”)*
- *VP16 – Herpes Virus protein, binds to Oct1, makes “sandwich” that is strong activator*

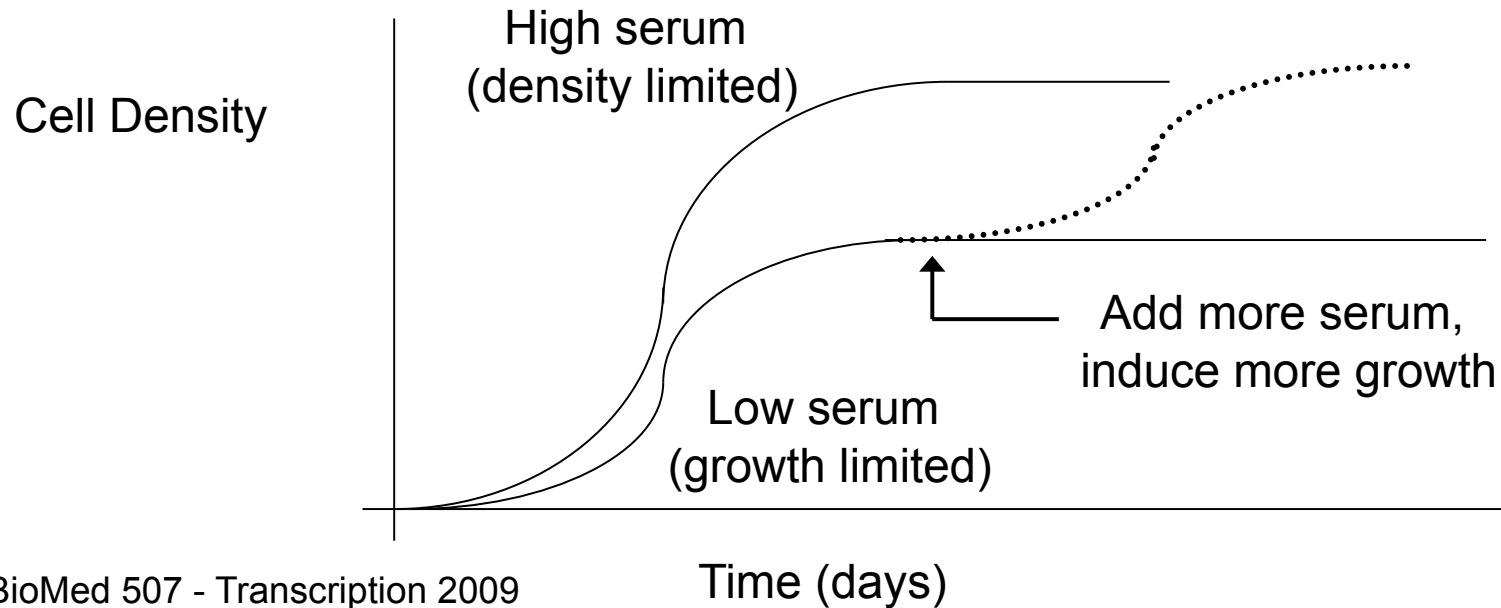
# Example: CBF/RBP-J, EBNA2 and Intracellular Notch

- *CBF/RBP-J are DNA-binding transcriptional repressors*
- *Epstein-Barr virus protein EBNA2: binds CBF/RBP-J*
- *EBNA2 has strong trans-activation domain, converts CBF/RBP-J to activator*
- *Notch is a trans-membrane receptor in plasma membrane*
- *Ligand leads to cleavage of intracellular domain: IC Notch activates transcription*

# Extracellular Signals Regulate Transcription

*Example: Serum-stimulated fibroblasts*

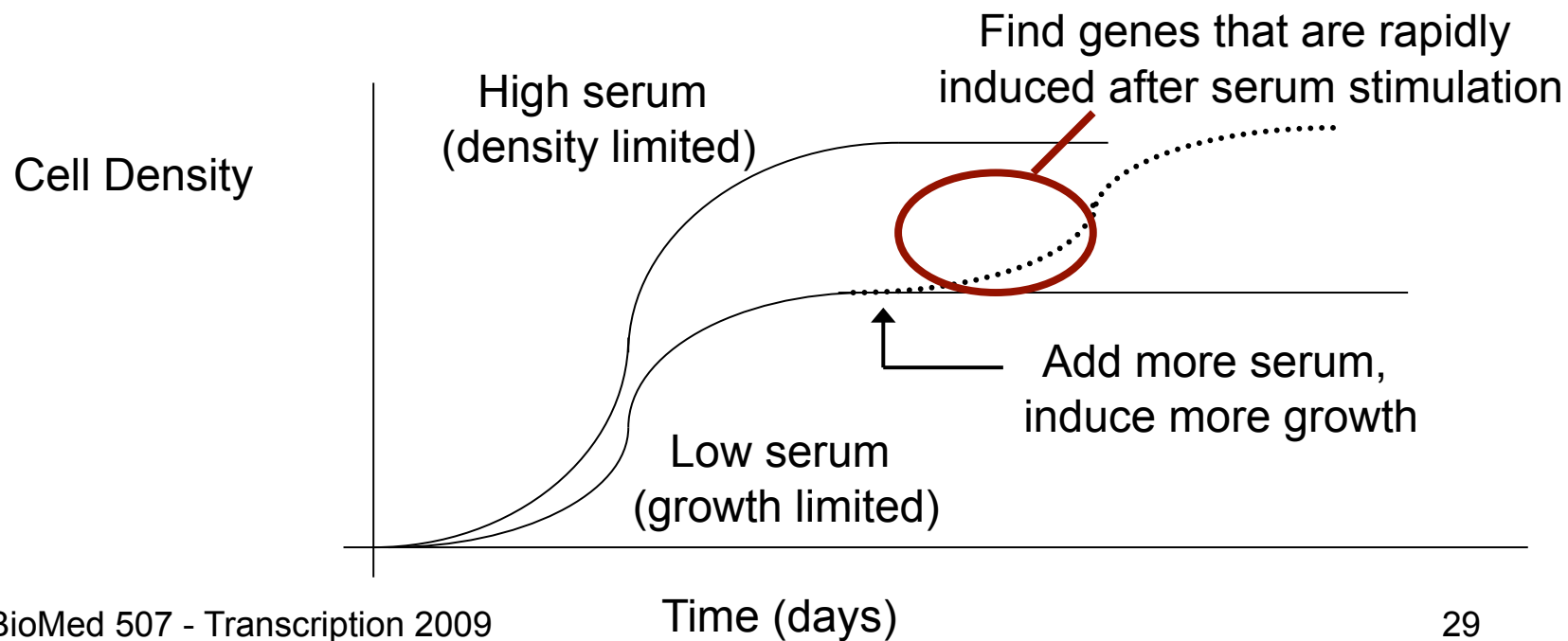
- ***Fibroblasts form monolayers in culture, 1 cell thick***
- ***Density of monolayer is medium-dependent***
- ***Growth is serum (growth factor) -dependent***



# What Genes Are Expressed First?

*Experiment: Serum-stimulate fibroblasts*

- **Start with serum-starved fibroblasts (arrested in G0)**
- **Stimulate with fresh medium plus serum**
- **Follow Gene Expression, Identify “Immediate Early Genes”**



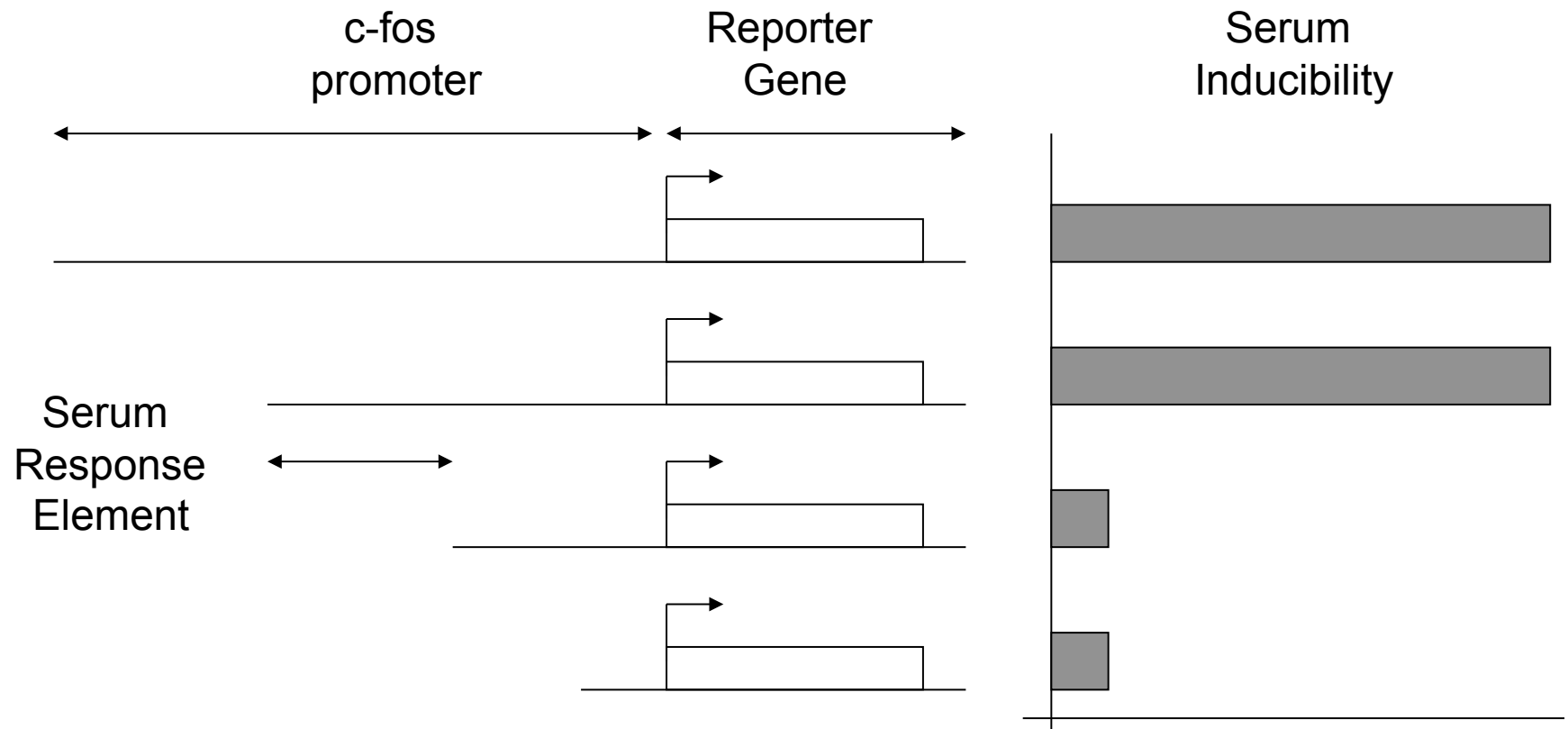
# How to Identify “Immediate Early Genes”

*Experiment: Serum-stimulate fibroblasts*

- ***Start with serum-starved fibroblasts (arrested in G0)***
- ***Add Cyclohexamide to block protein synthesis***
- ***Stimulate with fresh medium plus serum***
- ***Purify mRNA after different intervals (e.g. 15 min)***
- ***Identify genes that are up-regulated***
  
- ***Could use differential colony hybridization to screen cDNA libraries***
- ***Better to use microarrays***
  
- ***These experiments identify c-fos as an immediate early gene***

# Regulation of the c-fos Promoter

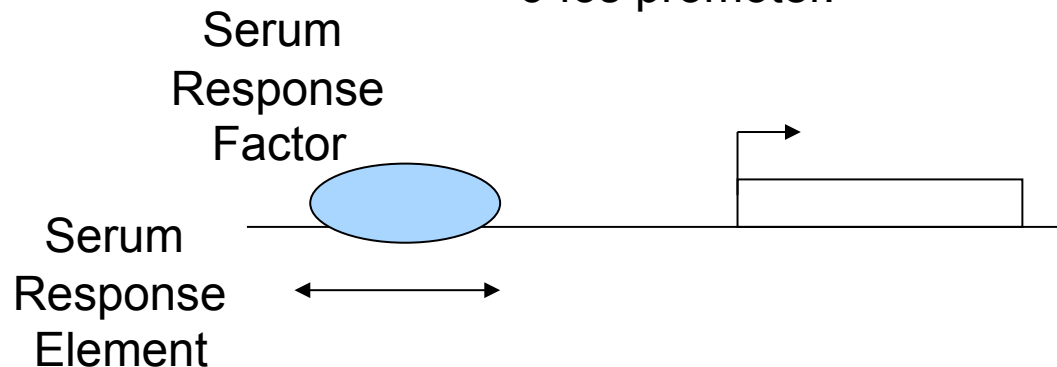
- The c-fos gene promoter is activated by serum stimulation*



# Regulation of the c-fos Promoter

- *The Serum Response Element binds a specific protein called Serum Response Factor*

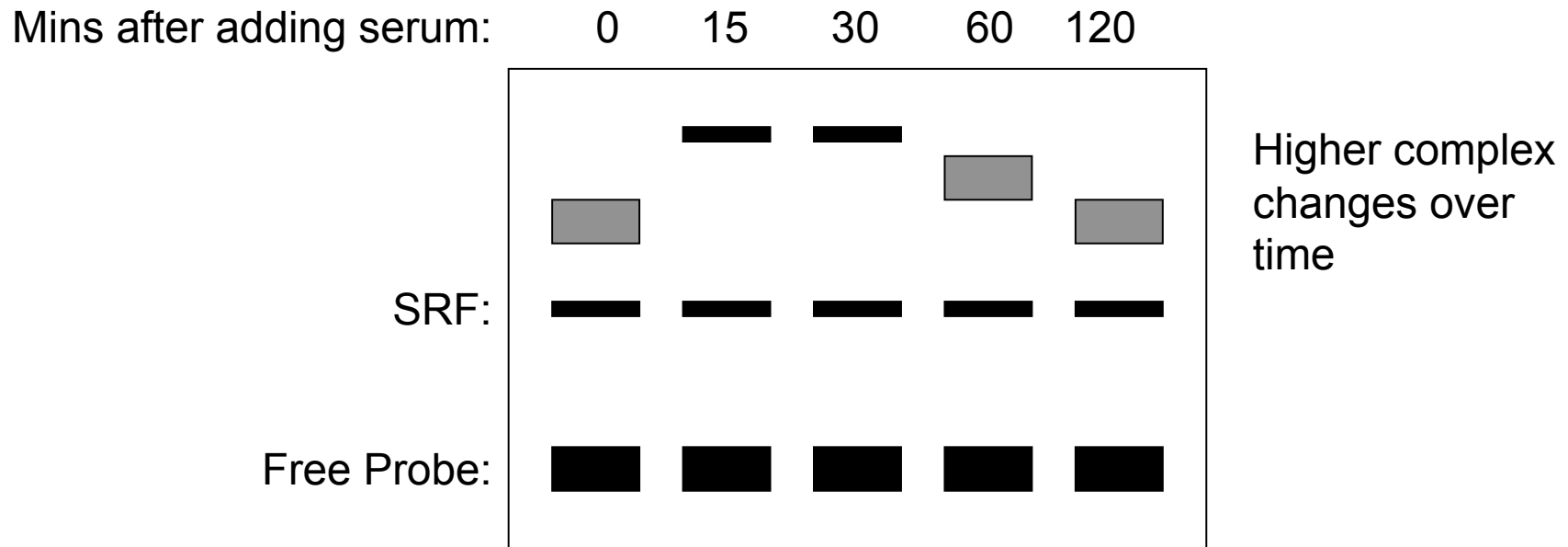
Destroying the SRE binding site or blocking the expression of SRF prevents serum stimulation of the c-fos promoter.



However, gel-shifts and in vivo binding assays (e.g. ChIP assays) showed that SRF remained expressed and bound to SRE at all times, regardless of serum stimulation. So how is c-fos promoter regulated?

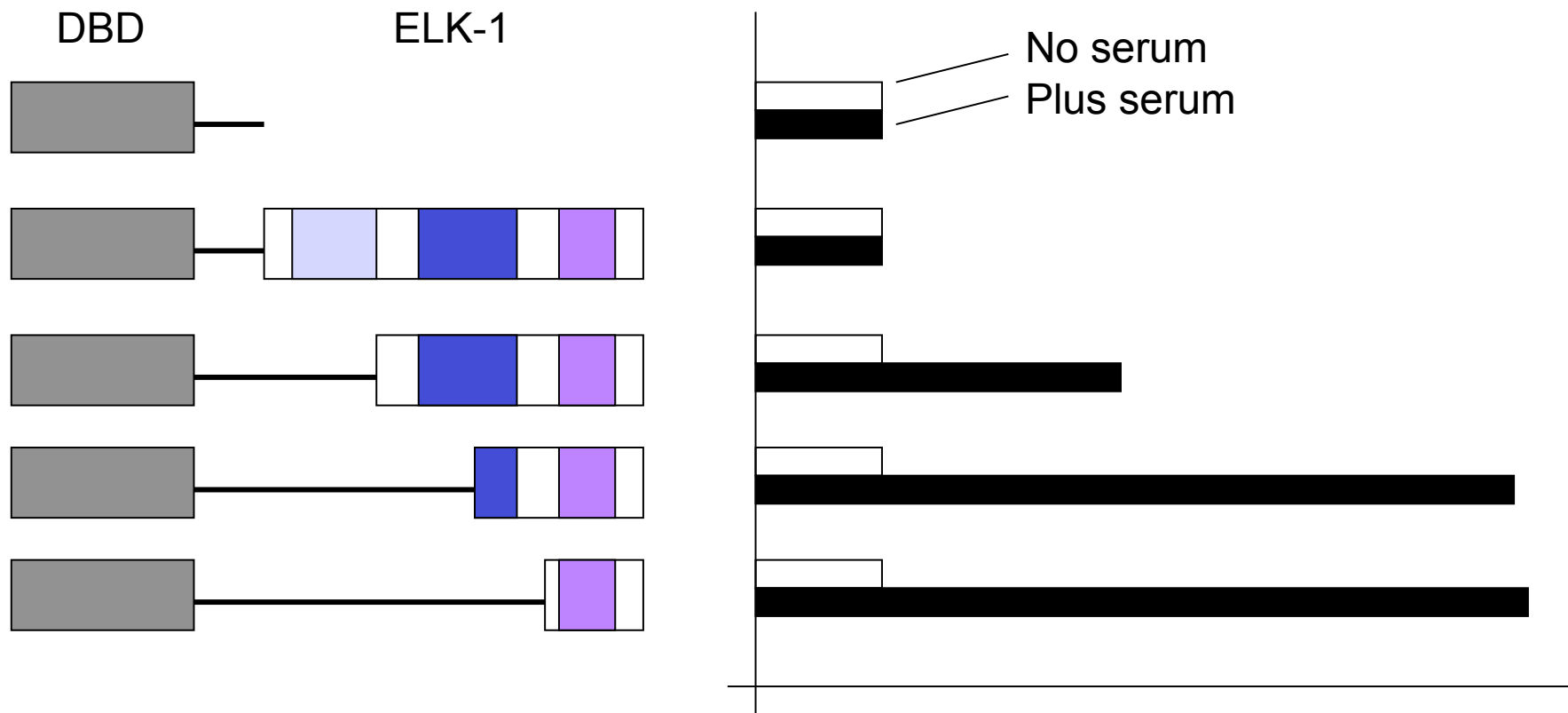
# SRF DNA Binding Activity

- *Make nuclear extracts from serum-stimulated fibroblasts*
- *Test for SRF DNA binding activity using EMSA (gel-shift)*



# Regulating SRF

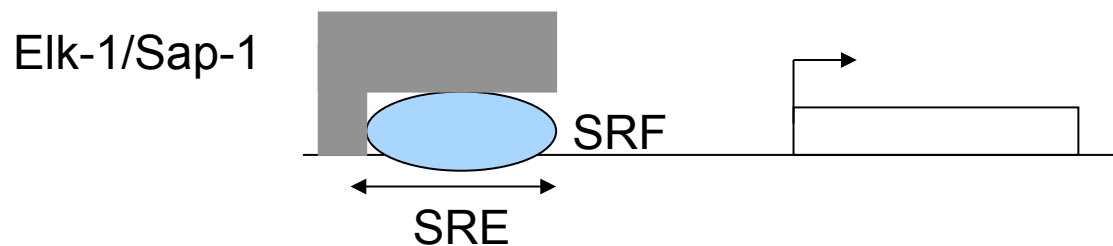
- *SRF found to interact with co-factors Elk-1 and Sap-1*
- *C-terminal domain of Elk-1 responsible for serum response*



# Regulation of SRF Activity

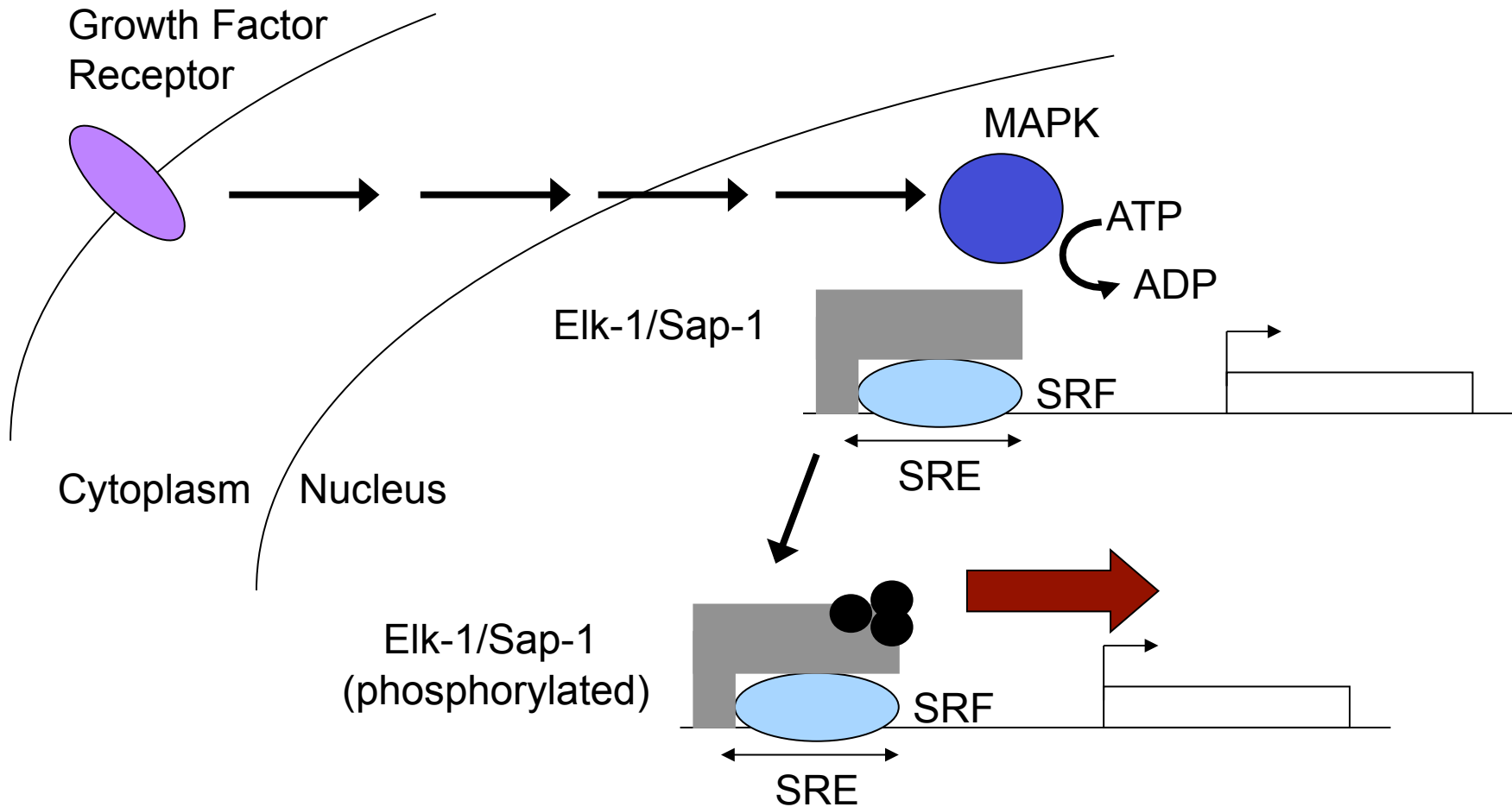
- *Higher complex caused by other proteins bound to SRF*
- *The Serum Response Factor remains bound to the SRE but its activity changes*

SRF bound to SRF binds another protein, Ternary Complex Factor, containing proteins Elk-1 or SAP-1.



TCF/SAP-1 is a target for phosphorylation by Mitogen-Activated Protein Kinases (MAPKs), which are activated following growth factor receptor stimulation.

# Regulation of SRF Activity



# *MAPK and activation of c-fos*

## ***c-Fos transcription factor family***

***Heterodimers: c-fos, fos-B, fra-1, c-Jun and Jun-D heterdimerize with***

### ***c-Jun and Jun-D***

- *10 different complexes possible*
- *All bind the same DNA sequence, all can be simultaneously co-expressed*
- *What provides the specificity?*
- *Some complexes activate (e.g. c-fos/c-Jun) others repress (e.g. c-Jun/c-Jun)*

# *Cytokines and JAK/STAT regulation*

- *Stimulating hematopoietic cells with IL-2 activates transcription of pim-1 gene*
- *Identify IL-2 responsive region in pim-1 promoter: binds STAT3 transcription factor*

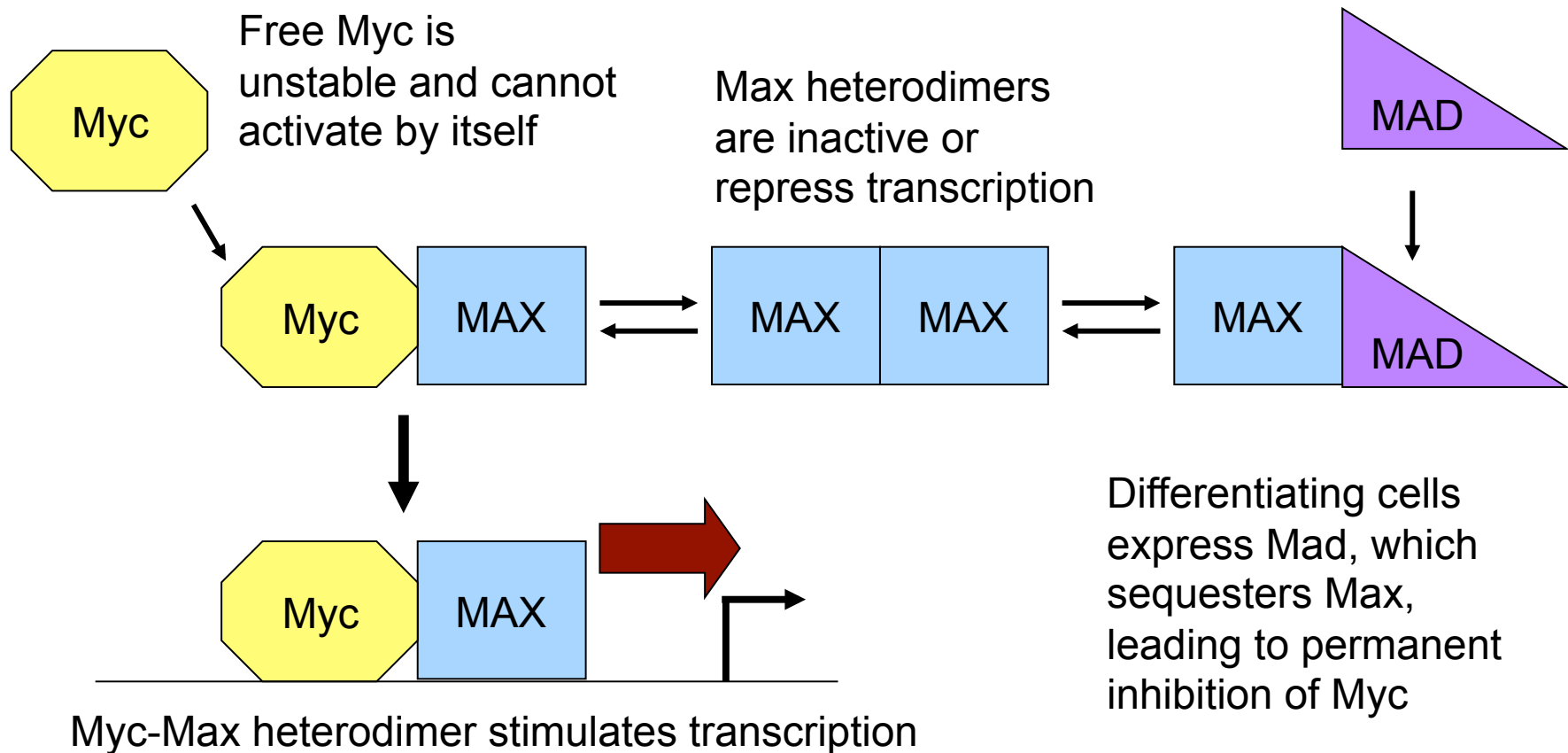
# STAT3 factor:

- *Binds DNA as dimer*
- *Present only as monomer in cytoplasm before IL-2 stimulation*
- *IL-2 treatment activates Jak-2 kinase*
- *Phosphorylates STAT3*
- *Phospho-STAT3 dimerizes, moves to nucleus, binds DNA, activates promoters.*

# Myc Regulation by Max and Mad

- *Heterodimers: Myc/Max/Mad*
- *Overexpression of Myc leads to cell growth*
- *Myc overexpressed in most tumors*
- *Myc levels are usually tightly regulated*
- *Myc only binds DNA when part of Myc/Max heterodimer*
- *Myc/Max activity regulated by Myc levels and by levels of Mad*
- *Equilibrium of Myc/Max/Mad controls proliferation and differentiation*

# Myc Regulation by Max and Mad

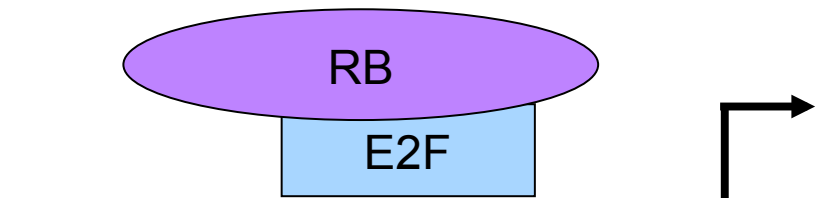


# Cell cycle regulation of transcription

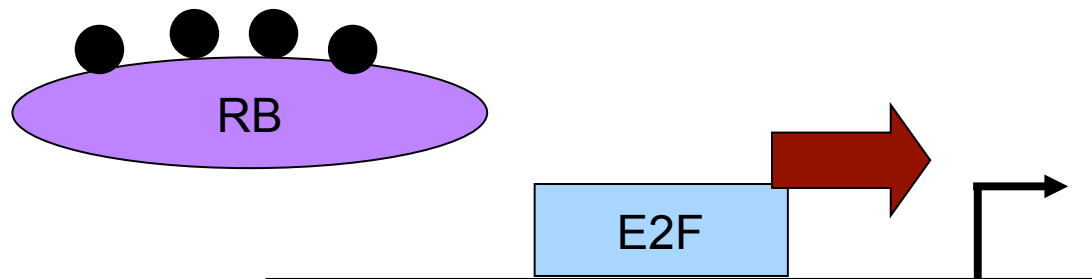
- *E2F transcription factor regulates genes involved in replication (DNA polymerase, etc.)*
- *E2F stays bound to DNA at all times.*
- *Transactivation activity of E2F is regulated by RB, which binds to and inhibits E2F on promoters.*

# Cell cycle regulation of transcription

RB binds to E2F, blocks its transactivation activity



At end of G2 phase, Cyclin D1/CDK6 phosphorylates RB, making it let go



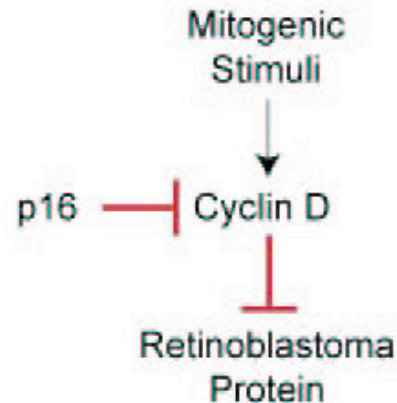
E2F regulates promoters involved in S phase

# Retinoblastoma Protein

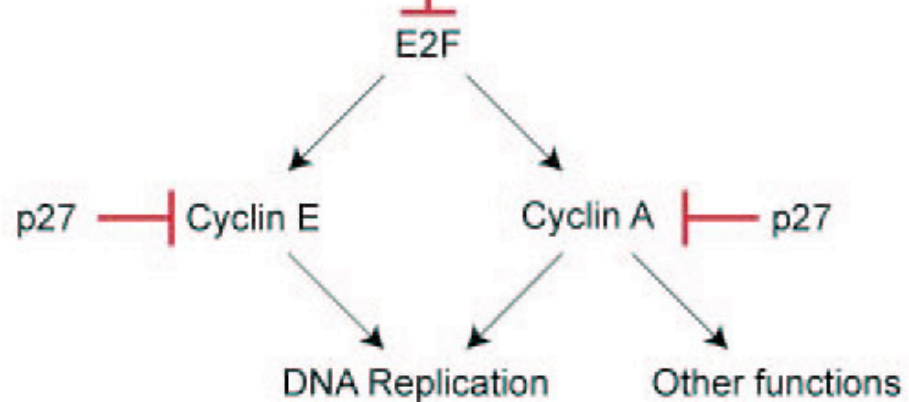
- *RB is found mutated in Retinoblastomas*
- *RB is a tumor suppressor, since expression stops cell growth, mutations lead to cancer*

# Transcription and Cell Cycle: CDK Inhibitors

**Early G1**



**Late G1**



**S, G2/M**

# Regulation of p27 degradation

***Up to 50% of human tumors lack detectable p27, mainly due to increased proteolysis***

***Low p27 levels are associated with low survival rates in cancer patients***

# Properties of p27 Tumor Suppressor

***Loss of one allele of p27 increases risk of tumors***

***Unlike other tumor suppressors, the second allele does not need to be mutated***

***This has important implications for human cancer genomics, screening***

# Regulation of p27 Localization

***p27 must be nuclear to suppress CDK activity***

***Cytoplasmic localization found in tumors***

- *40% of breast cancer cells*
- *>30% of colon cancer cells*

***In some tumors, cytoplasmic localization is an indicator of poor prognosis***

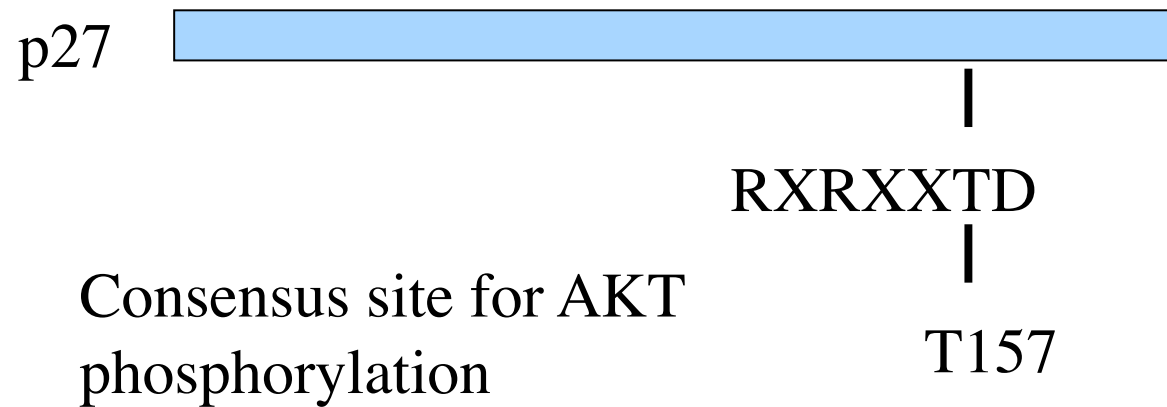
# AKT Kinase

## ***Phosphorylates key regulatory molecules***

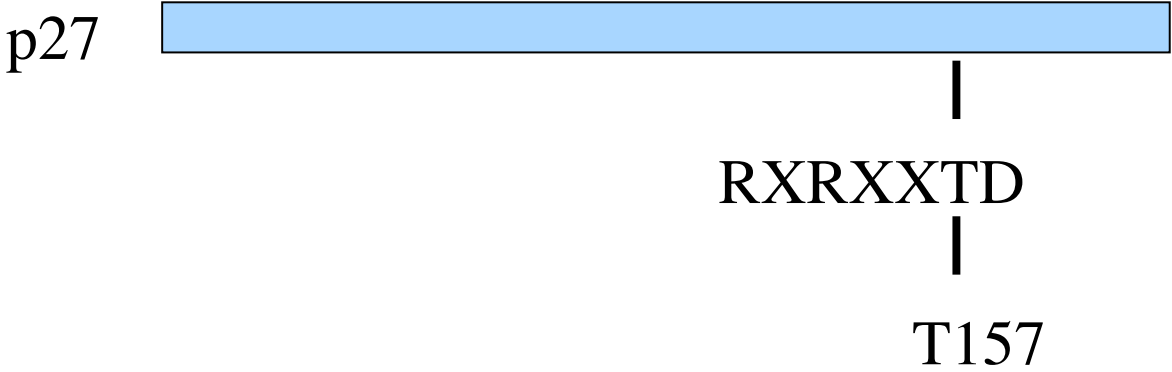
- *Bad (regulation of apoptosis)*
- *Caspase-9 (regulation of apoptosis)*
- *AFX, FKHRL1 (DAF-16-like Forkhead txn factors)*
- *p21 CIP1 (CDK-inhibitor)*

***AKT is a downstream effector of HER2/Neu, IGFR, EGFR, PI-3-kinase (PI3K)***

# AKT Phosphorylates p27



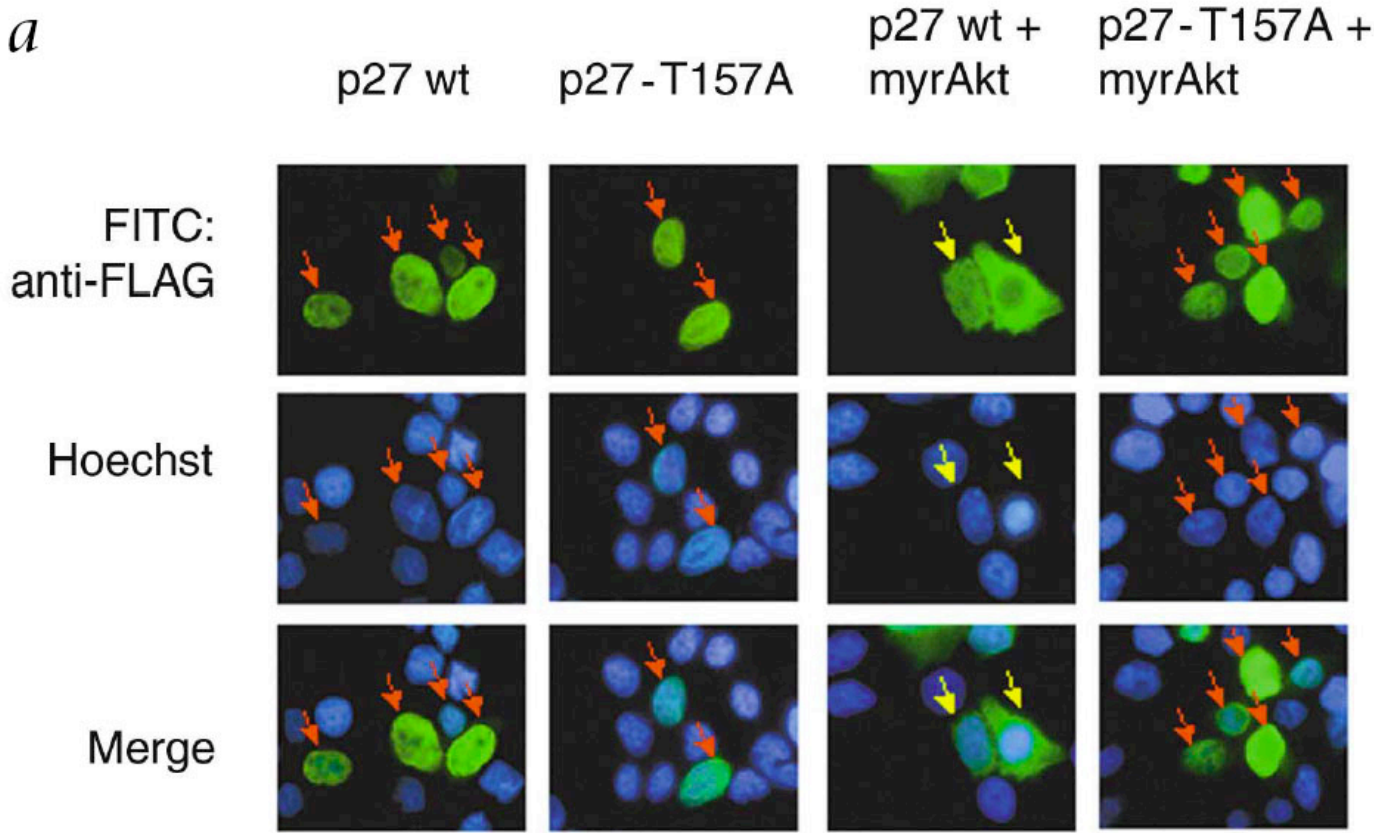
# AKT Phosphorylates p27



T157A cannot be phosphorylated by AKT

T157D mimics phosphorylation by AKT

# T157A Phosphorylation Regulates Localization of p27



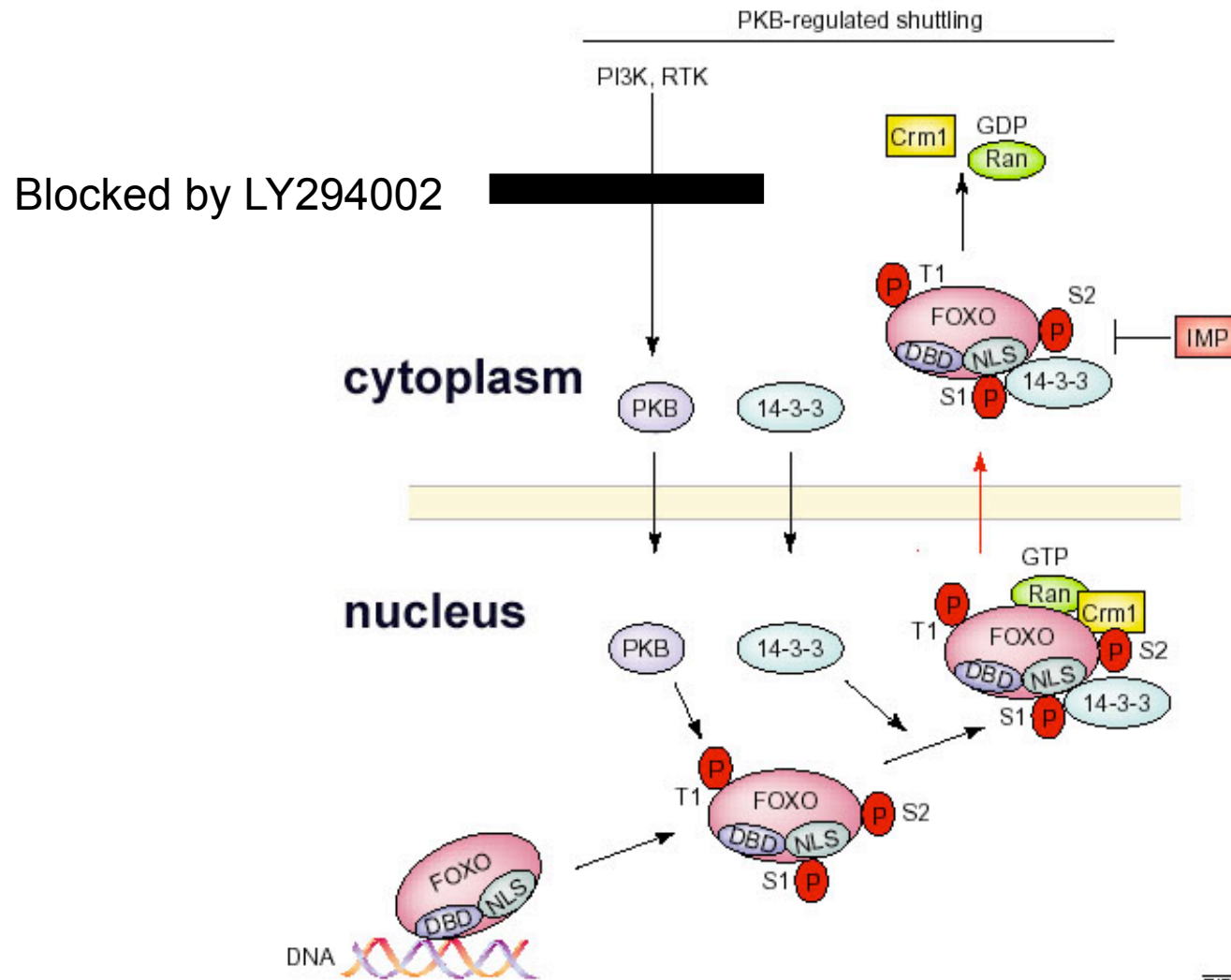
# AKT Phosphorylation Affects Forkhead (FOXO) Transcription Factors

***AKT/PKB protein kinase also phosphorylates FOXO transcription factors***

***FOXO proteins regulate the p27 gene***

***Activation of AKT leads to lower expression of p27***

# Forkhead (FOXO) Protein Shuttling



# Dual Action of AKT/PKB on p27

***AKT phosphorylation of p27 causes re-localization to the cytoplasm***

***Cytoplasmic p27 no longer blocks cell cycle***

***AKT phosphorylation of FOXO transcription factors inactivates them***

***Inactive FOXO proteins no longer induce p27 gene expression***

# Paper for Next Time

Molecular Cell 27, 107-119 (2007)

S. Tyagi, A.L. Chabes, J. Wysocka and W. Herr

E2F Activation of S Phase Promoters via Association with HCF-1 and the MLL Family of Histone H3K4 Methyltransferases

# Keywords and Learning Issues

## **Major**

Yeast two-hybrid assay

“bait”, “prey”

Retinoblastoma

Double Thymidine Block

Histone 3 Lysine Methylation

MLL, Set1

## **Minor**

Leucine Zipper

Nuclear Localization Signal

Nuclear Export Signal

Ortholog

Synchronized cells