莱博生物

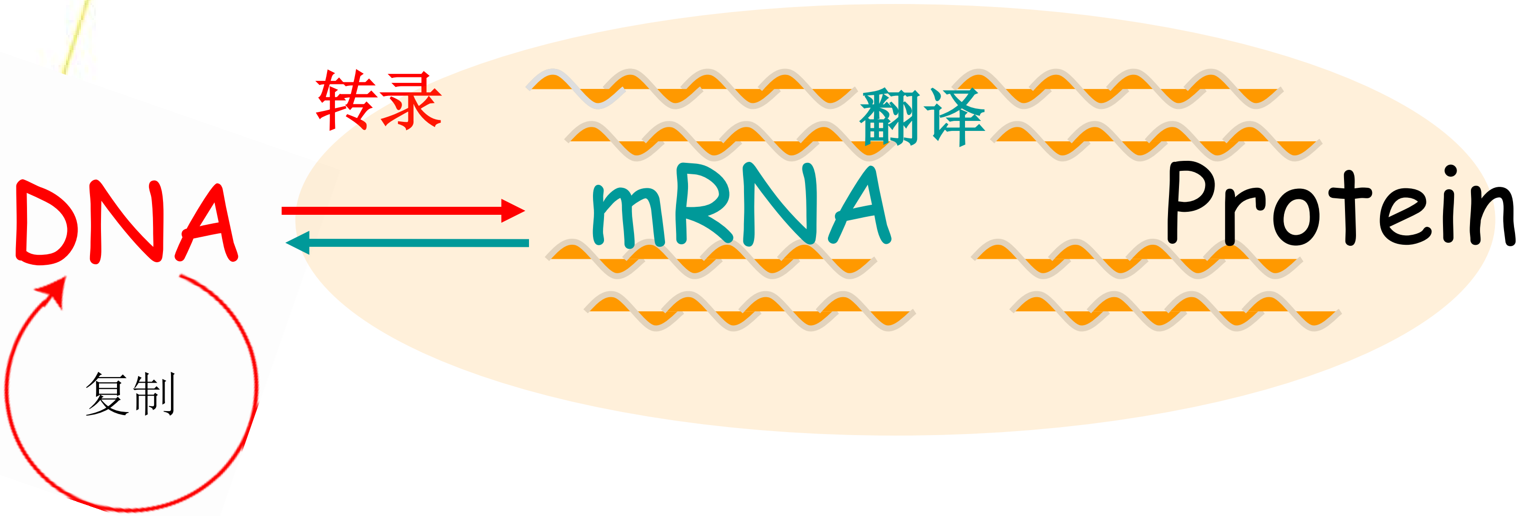
转录及转录后调控研究策略及技术

广州莱博生物科技有限公司

主要内容

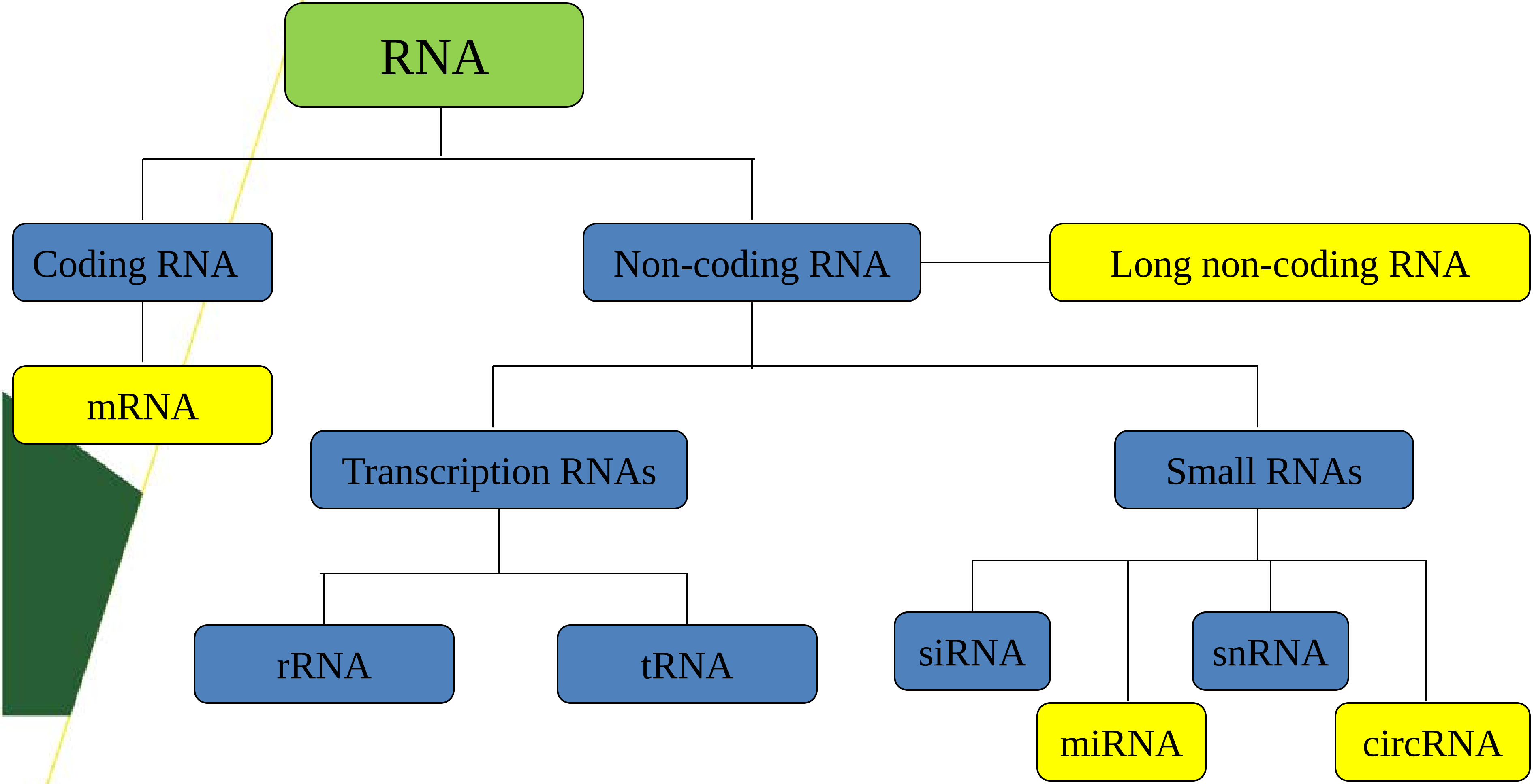
- 转录因子与启动子研究策略
- microRNA研究方法
- 信号通路研究策略
- Western blot实验技术

中心法则



高等动物基因组转录大量非编码RNA，组成细胞中高度复杂的RNA调控网络。

RNA world


RNA

Coding RNA

Non-coding RNA

Long non-coding RNA

mRNA

Transcription RNAs

Small RNAs

rRNA

tRNA

siRNA

snRNA

miRNA

circRNA

转录因子与启动子研究策略

启动子：一段能被RNA聚合酶特异性识别和结合，能正确有效的起始转录的一段DNA序列。

特点：

- 通常含一些特定的元件，如TATA box，GC box
- 具有方向性：启动下游基因的表达
- 启动子长度的不确定性
- 一个基因通常含有多个启动子，启动不同转录本的表达
- 某些启动子具有表达的时空性和组织特异性。

研究启动子的意义：

- 启动子是基因的重要组成部分，启动子就像“开关”，决定基因的活动。
- 启动子活性的异常，则可能导致基因表达的调节障碍，从而有可能导致疾病的发生
- 找到组织特异性启动子，为靶向治疗提供可能
- 找到某些疾病关键基因异常表达与启动子的关系，为基因治疗提供可能

如何获得启动子序列？

- 对于全基因组已知的物种

通过Genbank (http://www.ncbi.nlm.nih.gov/)

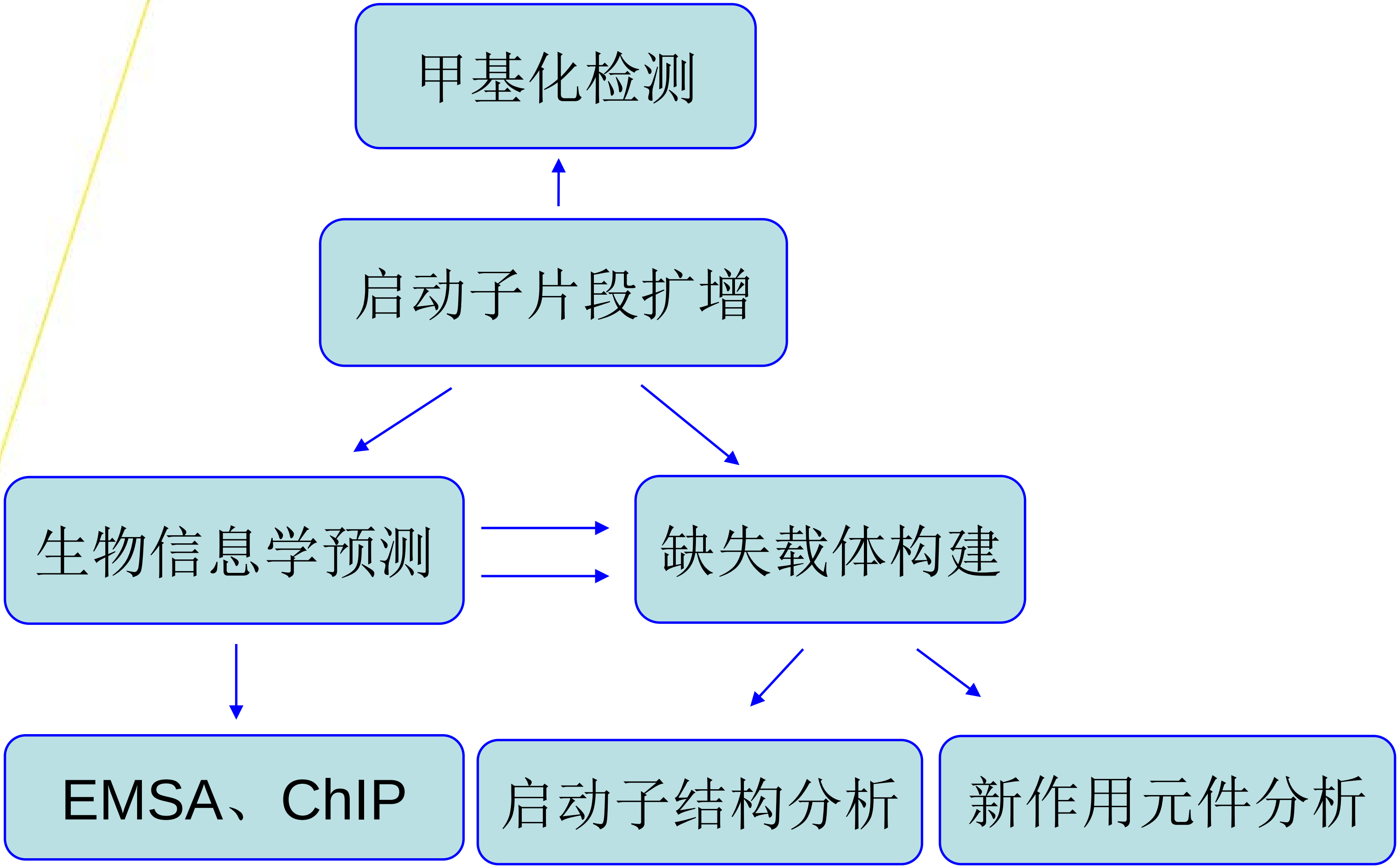
通过UCSC (http://genome.ucsc.edu/)

- 对于基因组未知的物种

反向PCR （Inverse PCR）

接头PCR （Linker adaptor PCR）

启动子研究方法



甲基化检测

启动子片段扩增

生物信息学预测

缺失载体构建

EMSA、ChIP

启动子结构分析

新作用元件分析

研究所涉及到的实验技术

1、DNA提取；

2、基因克隆；

3、载体构建；

4、生物信息学预测；

5、细胞培养及转染；

6、双荧光检测；

7、甲基化检测；

8、qRT-PCR；

9、EMSA/Super-sheft；

10、ChIP

研究方向

- 研究已知的转录因子和未知启动子的调控关系
- 在已知的启动子上找到新的顺式作用元件（发现蛋白在启动子上的结合位点）
- 寻找响应A因素（基因表达变化，药物诱导等）在启动子上的响应序列及其结合的转录因子
- 研究启动子甲基化对转录因子结合能力的影响及其对启动子活性的影响
- 发现组织或者时空特异性表达启动子的最短且活性最高的序列

案例分析1

www.impactjournals.com/oncotarget/ Oncotarget, Advance Publications 2016

COMMD7 is correlated with a novel NF-κB positive feedback loop in hepatocellular carcinoma

Lu Zheng[1], **Chang-Lin Deng**[1], **Liang Wang**[1], **Xiao-Bing Huang**[1], **Nan You**[1], **Yi-Chen Tang**[1], **Ke Wu**[1], **Ping Liang**[1], **Na Mi**[1], **Jing Li**[1]

[1] Department of Hepatobiliary Surgery, Xinqiao Hospital of Third Military Medical University, Chongqing, 400037, China

Correspondence to: Jing Li, **e-mail:** xqyyzl1@163.com

Keywords: *COMMD7, nuclear factor-kappa B, proliferation, apoptosis, hepatocellular carcinoma*

Received: *October 20, 2015* ***Accepted***: *March 31, 2016* ***Published***: *April 27, 2016*

- 研究主题：NF-κB正调控COMMD7
- 研究思路：1、临床研究— —确定COMMD7和NF-κB的表达水平及相关性
- 2、关系验证— —Luc、ChIP、EMSA/Super-sheft
- 3、功能研究— —细胞+动物

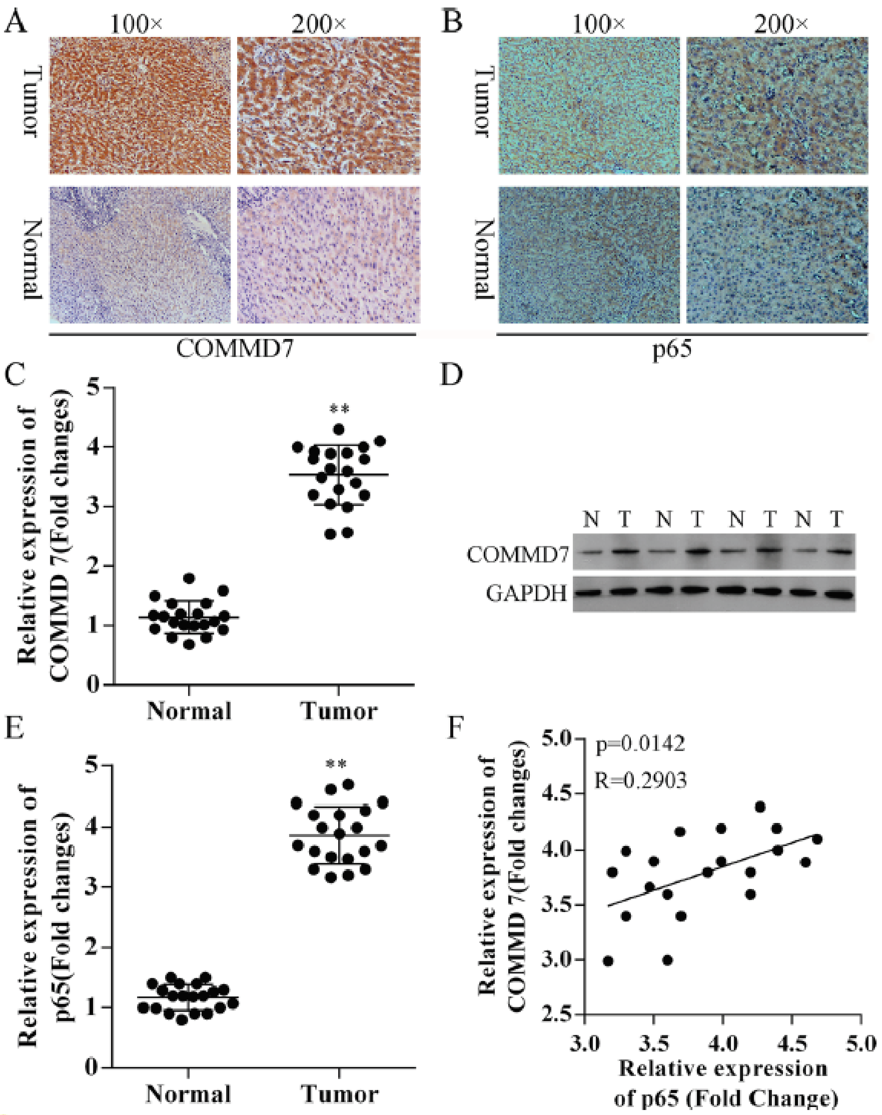
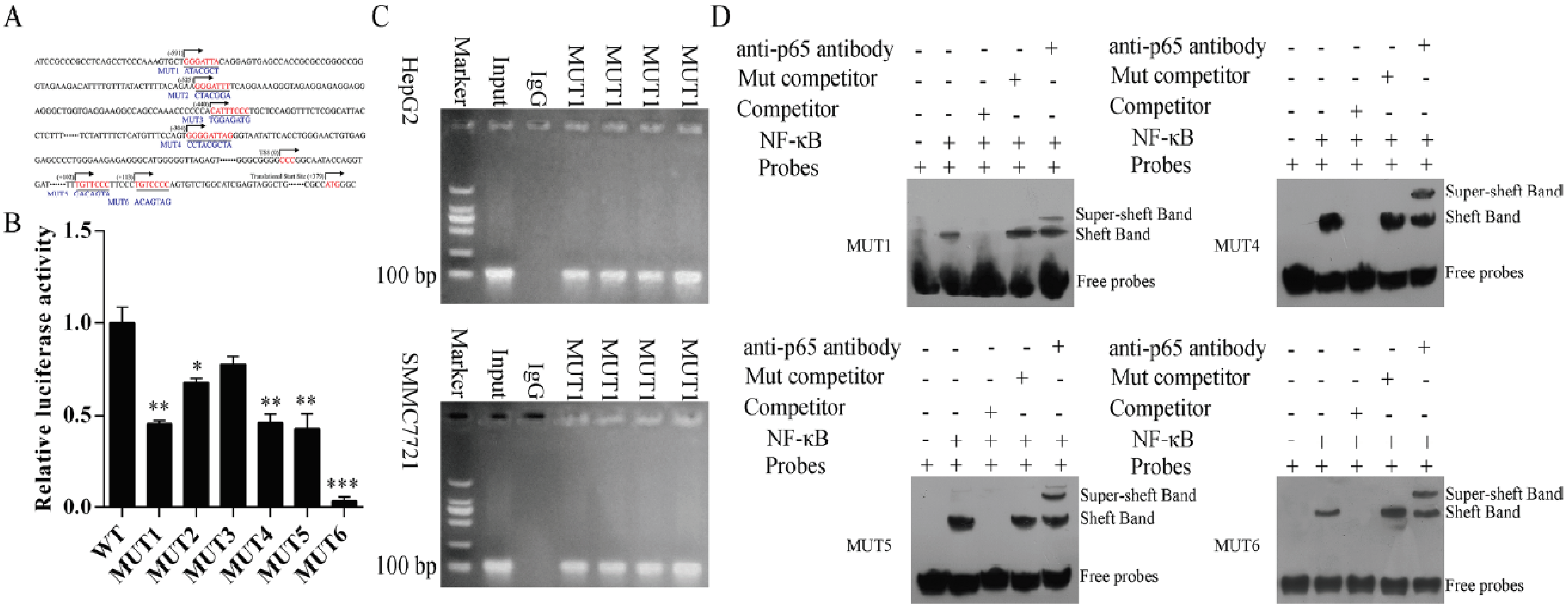
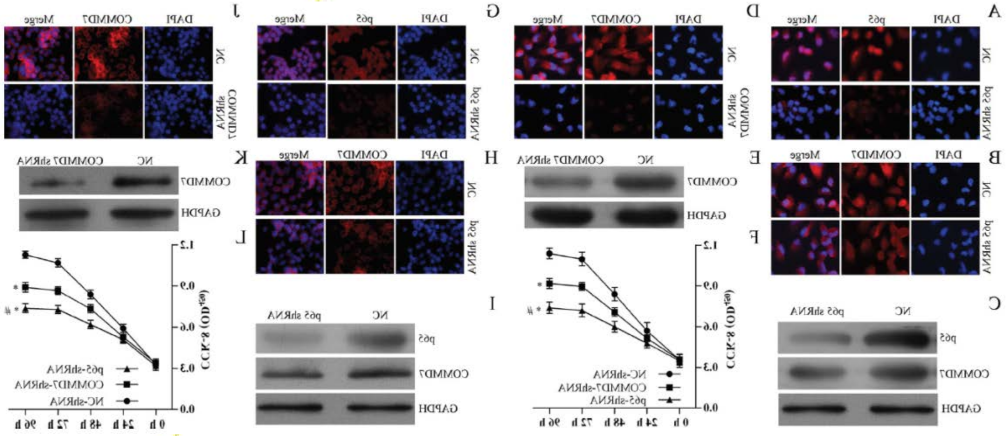


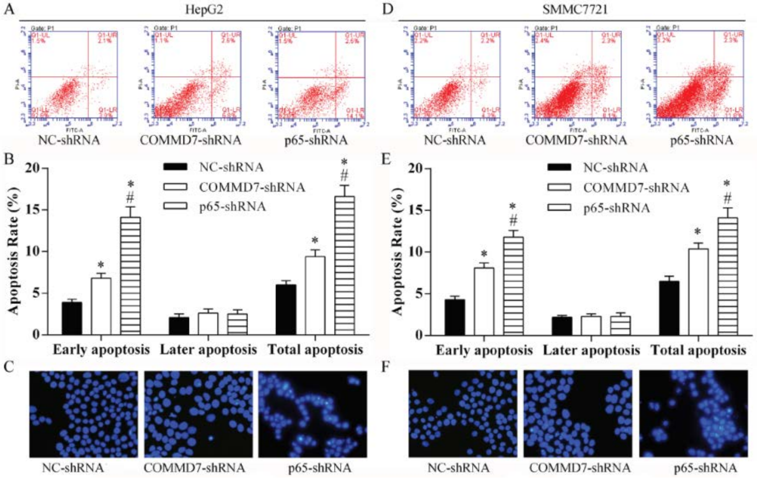
Figure 1: Correction between *COMMD7 and NF-κB in hepatocellular carcinoma*. *(A) COMMD7 and (B) p65 expression in hepatocellular carcinoma and para-carcinoma tissues using immunohistochemistry (IHC). Representative images at different magnifications from independent experiments are shown. Tumor: hepatocellular carcinoma; Normal: para-carcinoma tissue. (C) qRT-PCR and (D) Western Blot assays revealed the up-regulation of COMMD7 in hepatocellular carcinoma (tumor, T), compared with para-carcinoma tissues (normal, N). (E) qRT-PCR also revealed the up-regulation of NF-κB in hepatocellular carcinoma. (F) Correlation analysis revealed a significant correlation between COMMD7 and the expression of p65 subunit of NF-κB. **$p < 0.01$ vs. normal.*

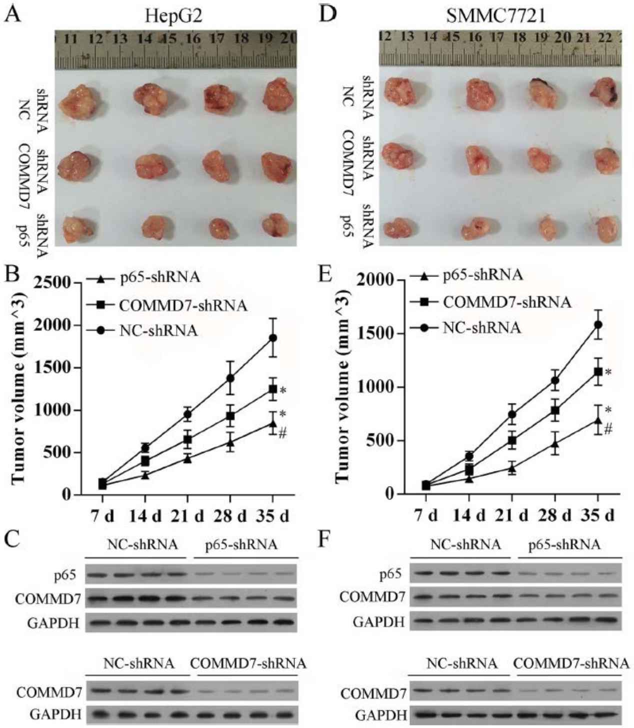
调控机制研究

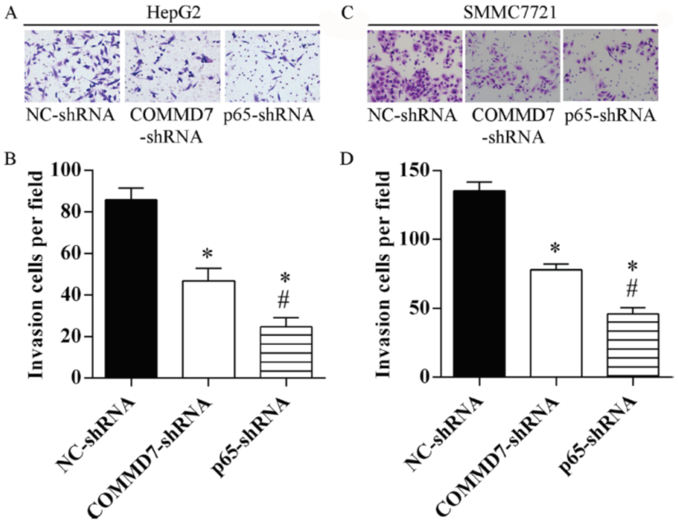

A
MUT1 ATACGCT
MUT2 CTACGGA
MUT3 TGGAGATG
MUT4 CCTACGCTA
MUT5 GACAGTA
MUT6 ACAGTAG
TSS (0)
Translational Start Site (+375)
B
Relative luciferase activity
1.5
1.0
0.5
0.0
**
*
**
**
***
WT
MUT1
MUT2
MUT3
MUT4
MUT5
MUT6
C
HepG2
Marker
Input
IgG
MUT1
MUT1
MUT1
MUT1
100 bp
SMMC7721
Marker
Input
IgG
MUT1
MUT1
MUT1
MUT1
100 bp
D
anti-p65 antibody - - - - +
Mut competitor - - - + -
Competitor - - + - -
NF-κB - + + + +
Probes + + + + +
MUT1
Super-sheft Band
Sheft Band
Free probes
MUT4
MUT5
MUT6

功能研究

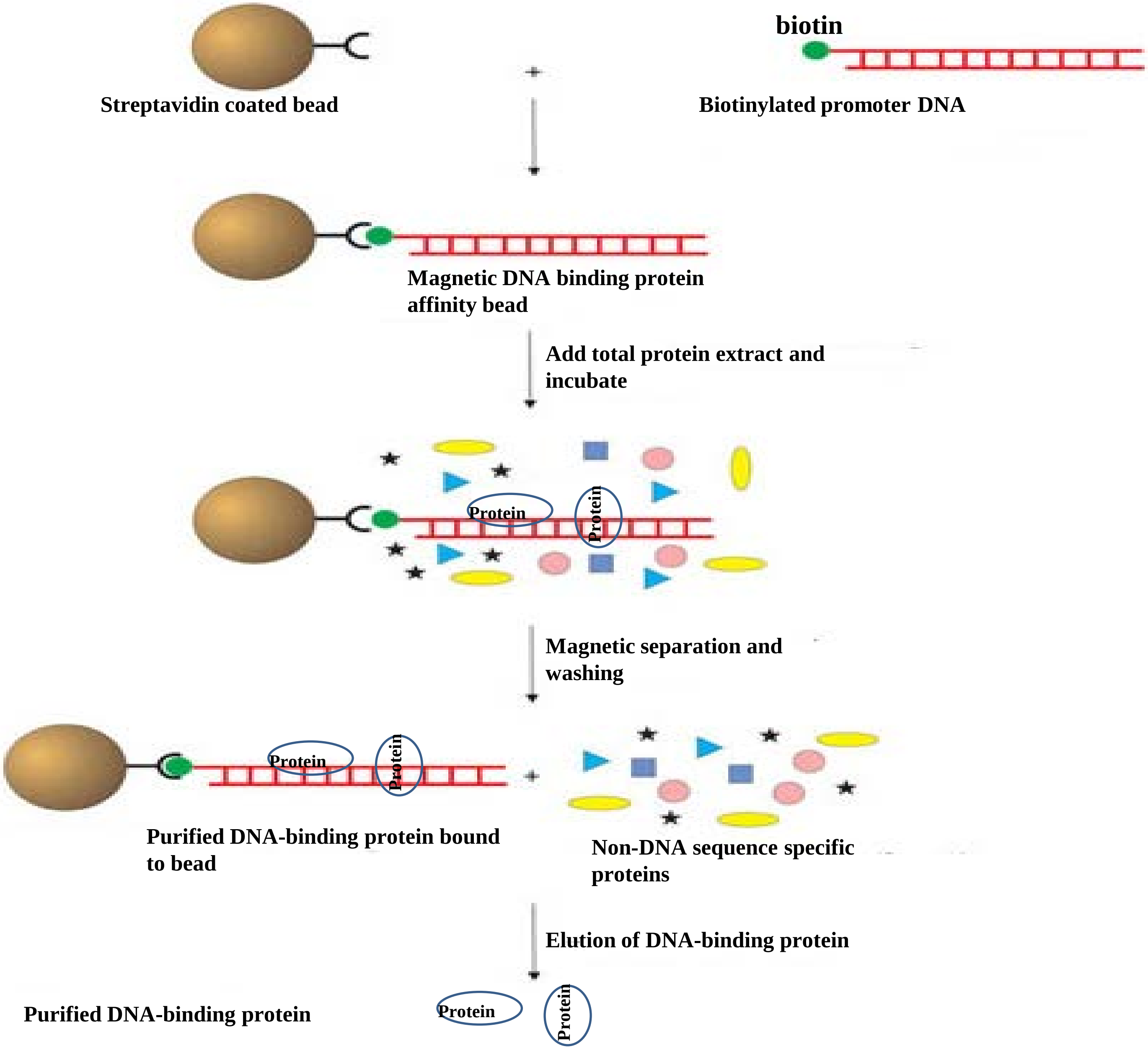

A
B
C
D
E
F
G
H
I
J
K
L
DAPI
p65
COMMD7
Merge
NC
p65 shRNA
COMMD7 shRNA
GAPDH
CCK-8 (OD450)
NC-shRNA
COMMD7-shRNA
p65-shRNA
0 h
24 h
48 h
72 h
96 h
0.0
0.3
0.6
0.9
1.2


A
HepG2
D
SMMC7721
NC-shRNA
COMMD7-shRNA
p65-shRNA
B
E
Apoptosis Rate (%)
0
5
10
15
20
Early apoptosis
Later apoptosis
Total apoptosis
*
#
C
F


A
HepG2
D
SMMC7721
NC shRNA
COMMD7 shRNA
p65 shRNA
B
E
Tumor volume (mm^3)
p65-shRNA
COMMD7-shRNA
NC-shRNA
0
500
1000
1500
2000
2500
7 d
14 d
21 d
28 d
35 d
*
#
C
F
NC-shRNA
p65-shRNA
COMMD7-shRNA
p65
COMMD7
GAPDH


A
HepG2
C
SMMC7721
NC-shRNA
COMMD7 -shRNA
p65-shRNA
B
D
Invasion cells per field
0
20
40
60
80
100
50
150
*
#
NC-shRNA
COMMD7-shRNA
p65-shRNA

未知转录因子筛选



Streptavidin coated bead

Biotinylated promoter DNA

Magnetic DNA binding protein affinity bead

Add total protein extract and incubate

Magnetic separation and washing

Purified DNA-binding protein bound to bead

Non-DNA sequence specific proteins

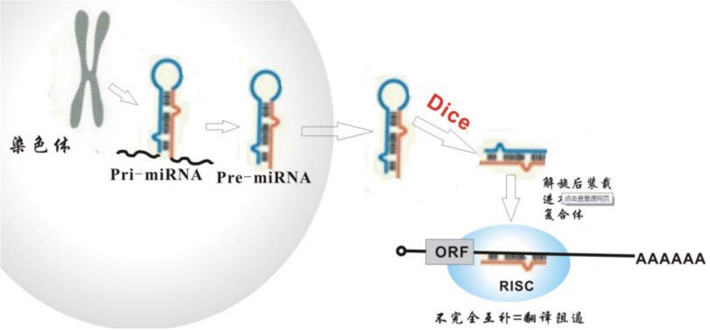
Elution of DNA-binding protein

Purified DNA-binding protein

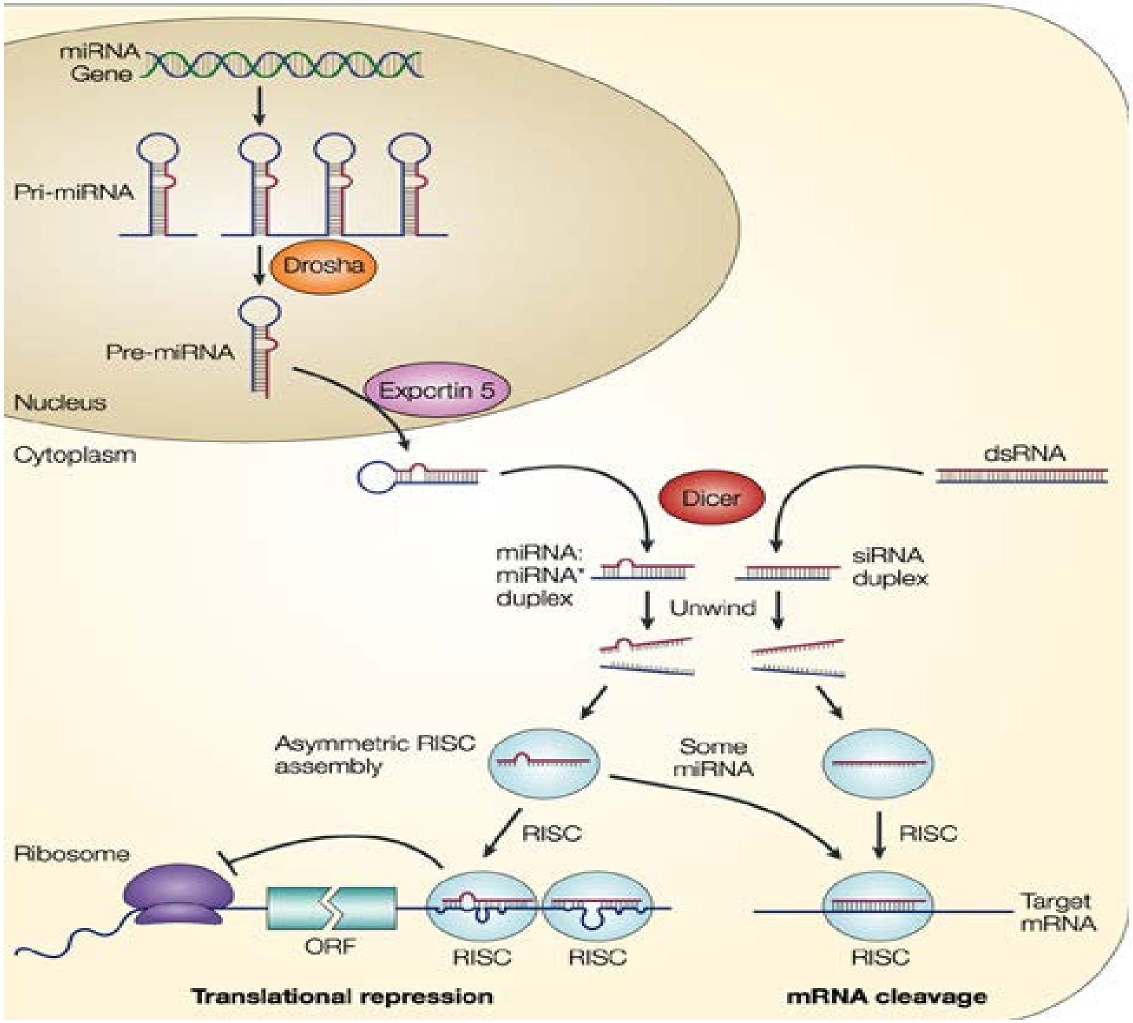
定义

microRNAs（miRNAs）是一种小的内源性非编码RNA分子，大约由18-25个核苷酸组成。这些小的miRNA通常靶向一个或者多个mRNA，通过翻译水平的抑制或断裂靶标mRNAs而调节基因的表达.

自从let-7发现以来，应用随机克隆和测序、生物信息学预测的方式，又分别在众多生物体如病毒、家蚕和灵长类动物中发现了成千上万的miRNAs。被鉴定的miRNAs均被miRBase（www.mirbase.org）网站整理并加以注释。



microRNA作用的分子机制

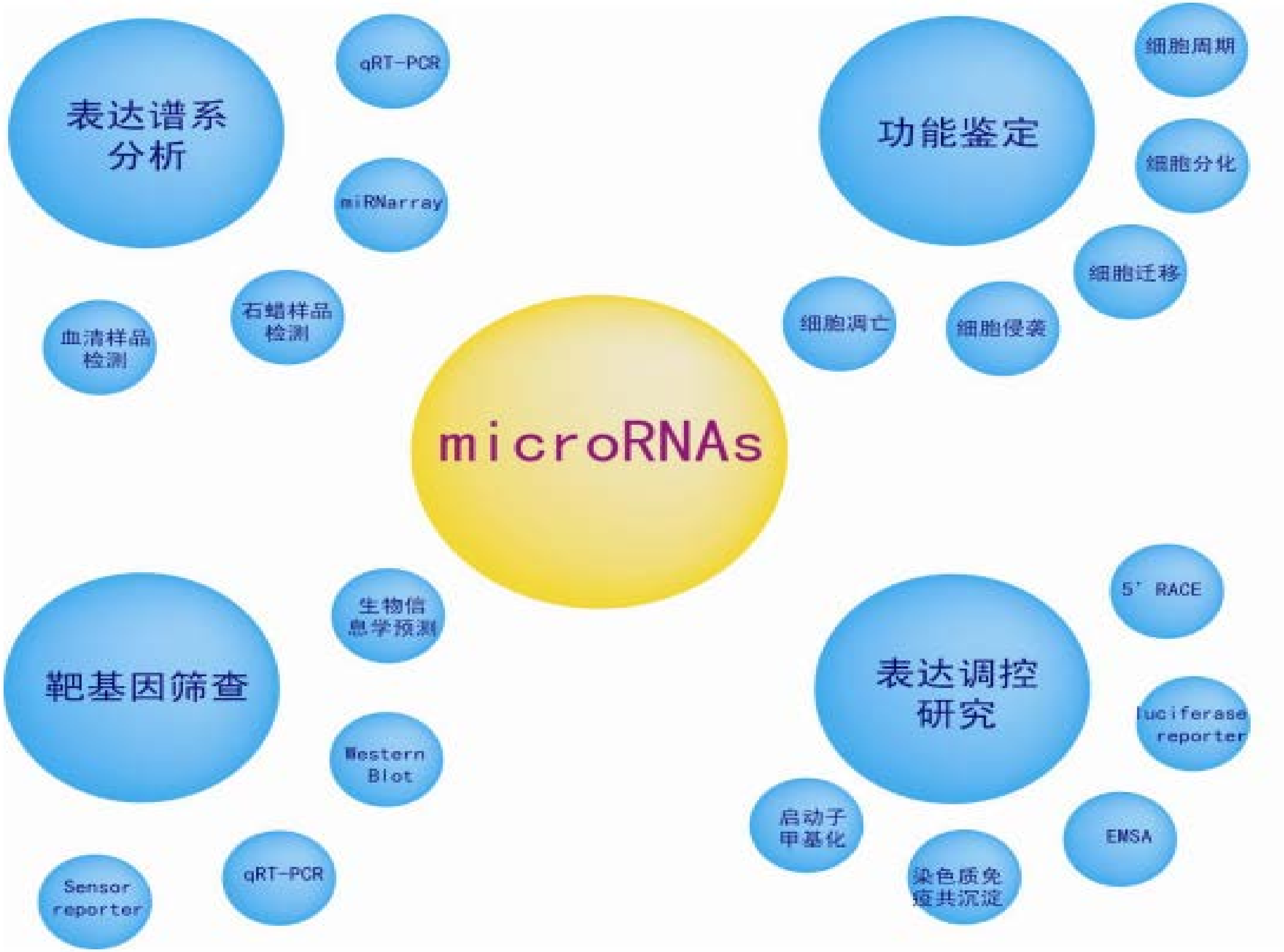


Initiation

Execution

RISC：

RNA-induced silencing complex



microRNAs

表达谱系分析
- qRT-PCR
- miRNarray
- 石蜡样品检测
- 血清样品检测

功能鉴定
- 细胞周期
- 细胞分化
- 细胞迁移
- 细胞侵袭
- 细胞凋亡

靶基因筛查
- 生物信息学预测
- Western Blot
- qRT-PCR
- Sensor reporter

表达调控研究
- 5' RACE
- luciferase reporter
- EMSA
- 染色质免疫共沉淀
- 启动子甲基化

案例分析2

www.impactjournals.com/oncotarget/ Oncotarget, Advance Publications 2016

MicroRNA-375 suppresses human colorectal cancer metastasis by targeting Frizzled 8

Lingling Xu[1,*], Tao Wen[2,*], Zhe Liu[1], Feng Xu[1], Lei Yang[2], Jian Liu[2], Guosheng Feng[1], Guangyu An[1]

[1] Department of Oncology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China

[2] Medical Research Center, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China

[*] The two authors contributed equally to this work

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Guosheng Feng, **e-mail:** *fgs010@163.com*

Keywords: *colorectal cancer, microRNA-375, metastasis, FZD8*

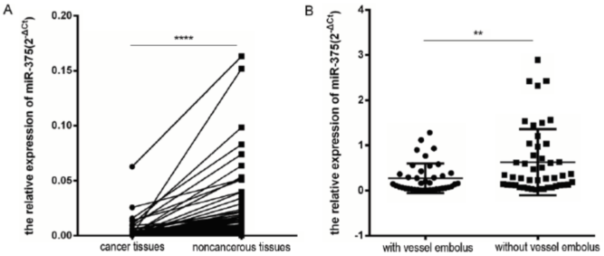
Received: *December 20, 2015* **Accepted:** *May 17, 2016* **Published:** *June 3, 2016*

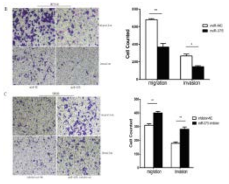
- 研究主题：miR-375靶向调控Frizzled 8抑制结直肠癌转移
- 研究思路：1、临床研究— —确定miR-375的表达水平
- 2、功能研究— —迁移、侵袭、动物模型
- 3、机制探讨— —Wnt/β-catenin pathway、FZD 8

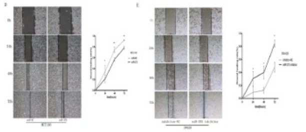
样品选择

90例癌组织，90例癌旁组织

qRT-PCR

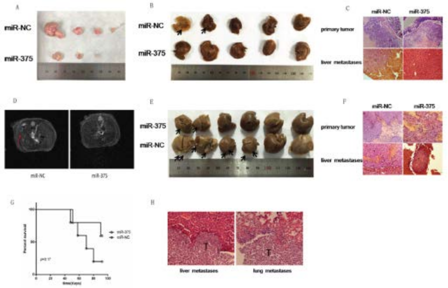






功能研究（体内）

细胞迁移、细胞侵袭、细胞划痕

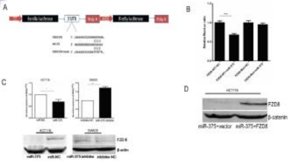


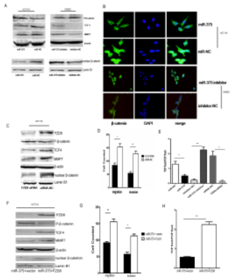
功能研究（体外）

裸鼠转移模型

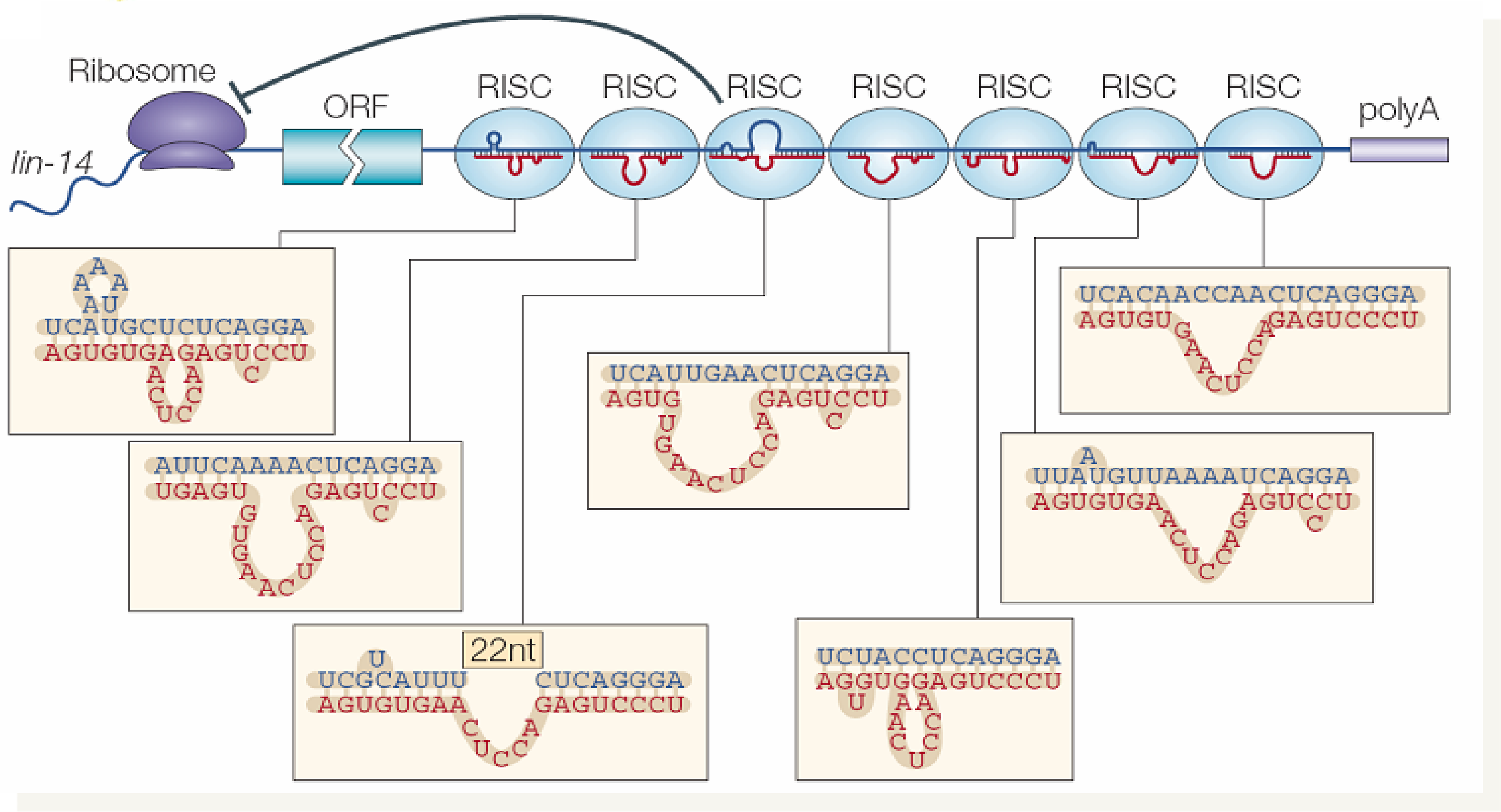
机制探讨

FZD 8，Wnt/β-catenin pathway





miRNA靶基因的筛选



miRNAs主要结合于靶基因的3’UTR

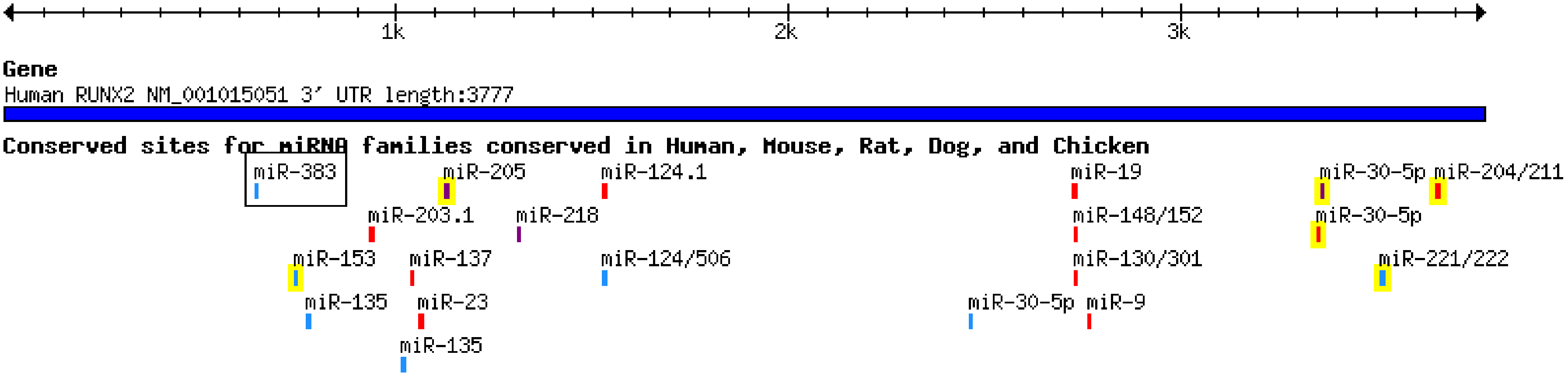
生物信息学的分析（在线搜寻工具）

表1　高等生物miRNA靶基因预测软件

软件	适用范围	网址	本地运行	参考文献
miRanda	脊椎动物	http://www.microrna.org/	是	[12]
DIANA-microT	所有哺乳动物	http://www.diana.pcbi.upenn.edu/	否	[14]
RNAhybrid	所有哺乳动物	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	是	[15]
TargetScan	脊椎动物	http://www.targetscan.org/	是	[16-17]
MicroInspector	所有哺乳动物	http://mirna.imbb.forth.gr/microinspector/	否	[18]
PicTar	所有哺乳动物	http://pictar.bio.nyu.edu/	否	[19]
TargetBoost	线虫和果蝇	https://demo1.interagon.com/targetboost/	否	[20]
miTarget	所有哺乳动物	http://cbit.snu.ac.kr/~miTarget/	否	[22]
RNA22	所有哺乳动物	http://cbcsrv.watson.ibm.com/rna22.html	是	[23]
microTar	线虫、果蝇和小鼠	http://tiger.dbs.nus.edu.sg/microtar/	是	[27]

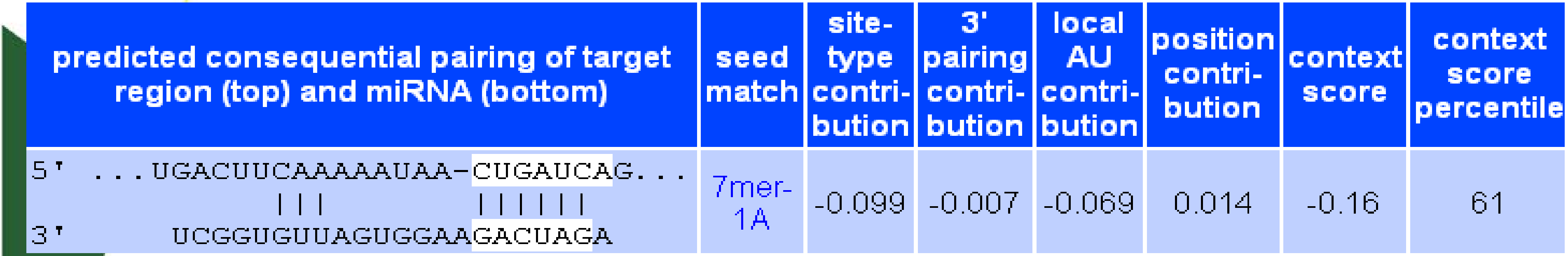
miRBase

Human RUNX2 3' UTR



Human RUNX2 NM_001015051 3' UTR length:3777

Conserved sites for miRNA families conserved in Human, Mouse, Rat, Dog, and Chicken



<table>
<tr><th>predicted consequential pairing of target region (top) and miRNA (bottom)</th><th>seed match</th><th>site-type contri-bution</th><th>3' pairing contri-bution</th><th>local AU contri-bution</th><th>position contri-bution</th><th>context score</th><th>context score percentile</th></tr>
<tr><td>5' ...UGACUUCAAAAAUAA-CUGAUCAG...
||| |||||||
3' UCGGUGUUAGUGGAAGACUAGA</td><td>7mer-1A</td><td>-0.099</td><td>-0.007</td><td>-0.069</td><td>0.014</td><td>-0.16</td><td>61</td></tr>
</table>

搜索结果

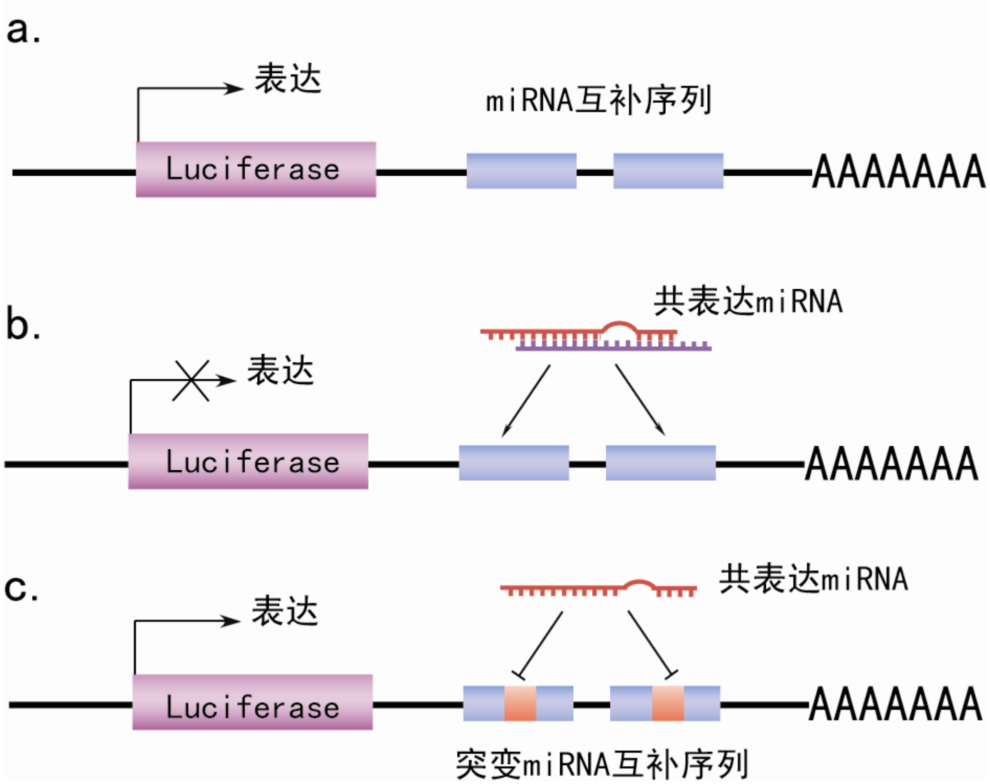
1. 基因 预测miRNA　　2. miRNA 预测targets

miRNA靶基因的验证

1. qRT-PCR：部分有效或间接作用
2. Western Blot：可能是间接作用
3. Sensor reporter

直接证据

综合应用上述方法。



Sensor reporter assay

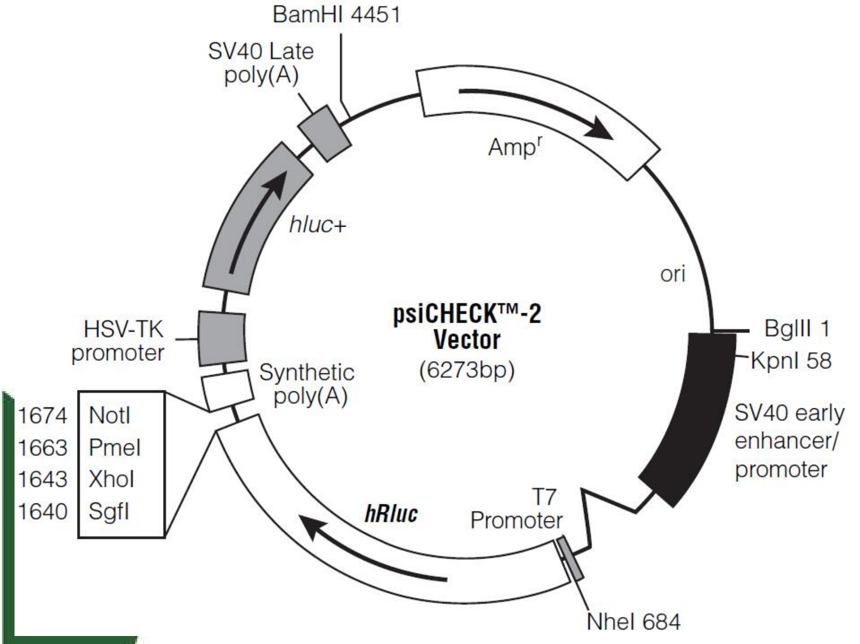
miRNA靶基因功能验证

对miRNA靶基因的功能进行研究，是深入了解miRNA功能的一种重要手段。研究方法：

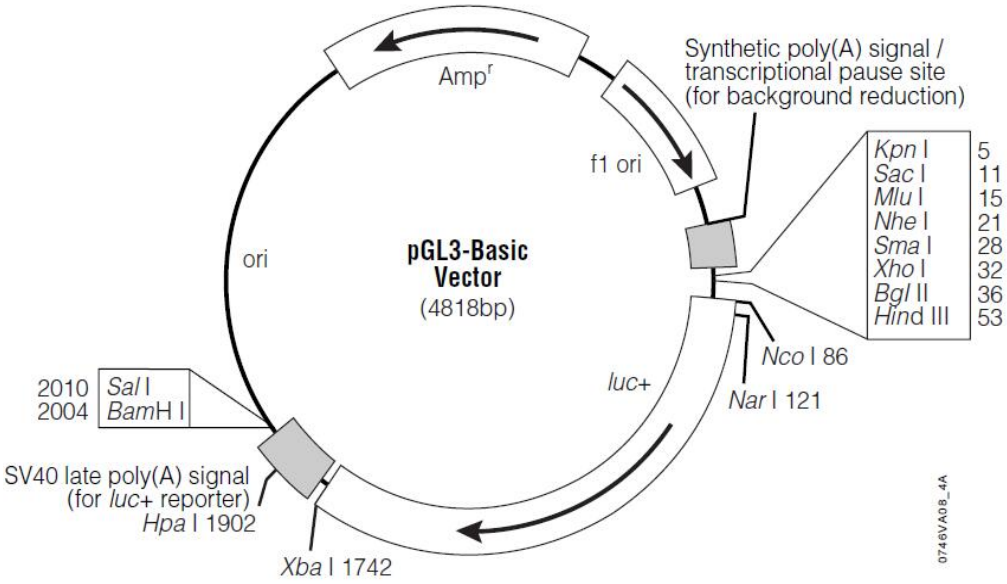
Loss-of-function

Gain-of-function

双荧光素酶报告质粒比较



miRNA-Targets



TF-Promoter

信号通路研究策略

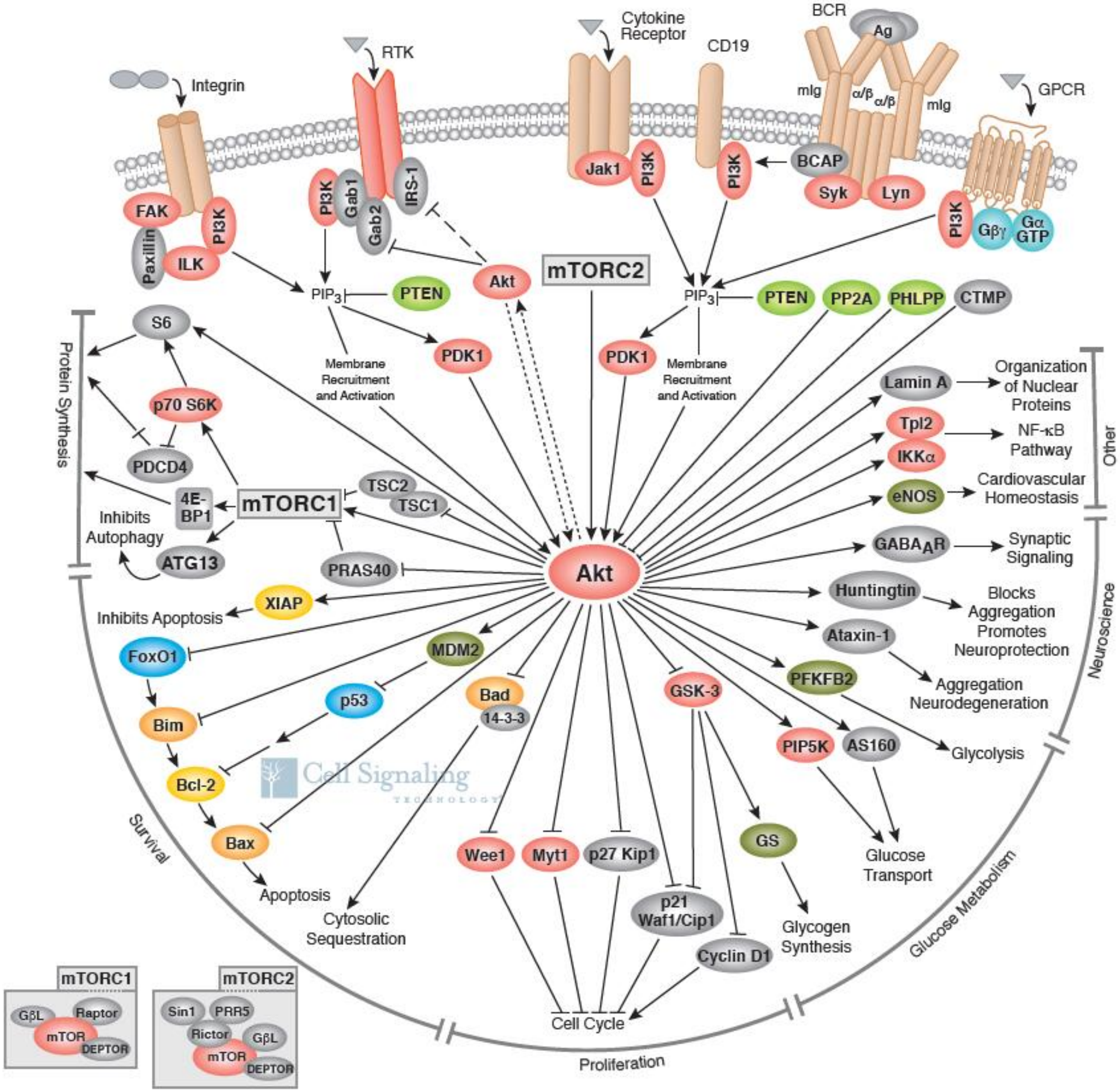
定义

信号通路是指当细胞里要发生某种反应时，信号从细胞外到细胞内传递了一种信息，细胞要根据这种信息来做出反应的现象。

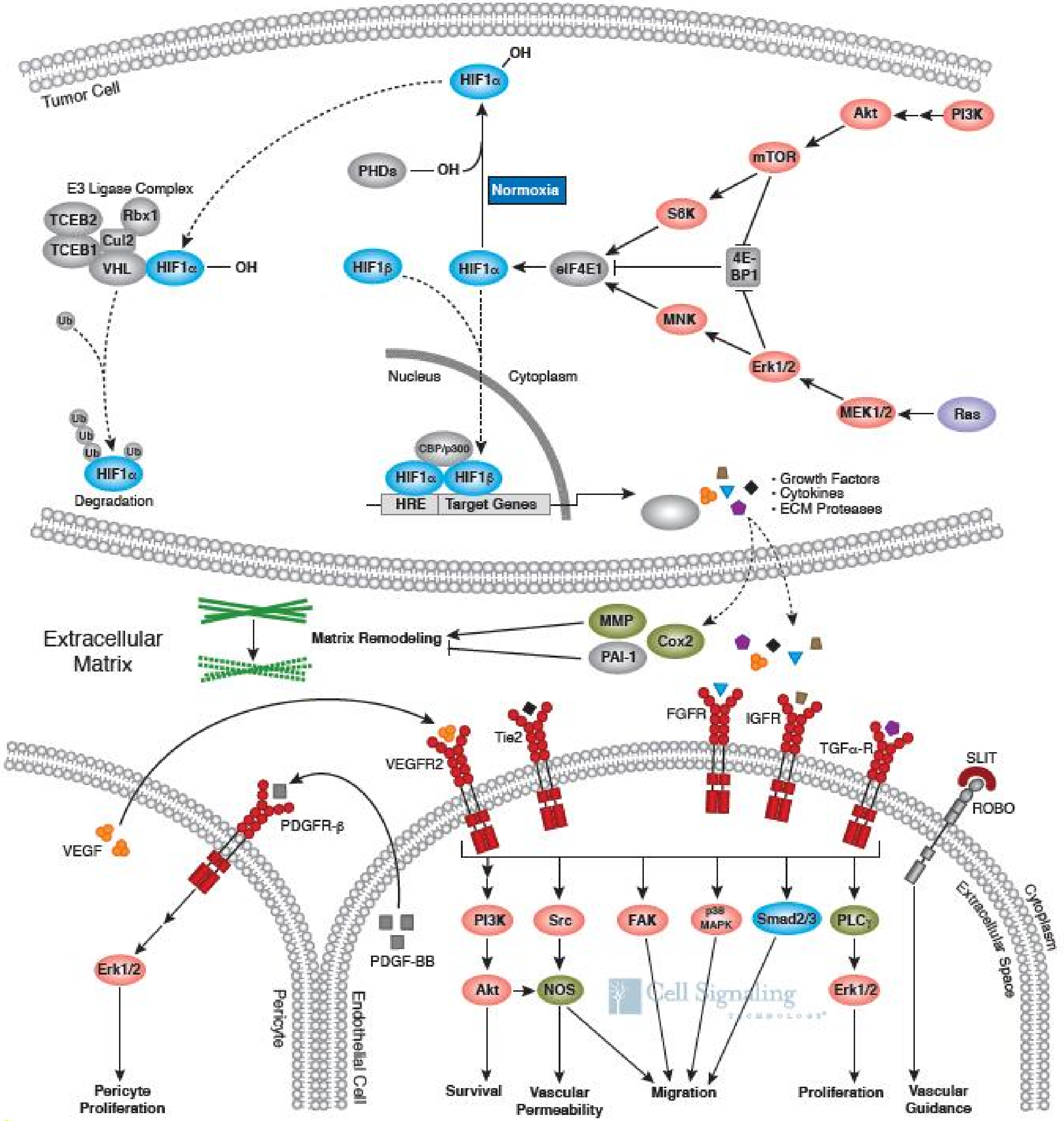
即： 能将细胞外的分子信号经细胞膜传入细胞内发挥效应的一系列酶促反应通路。

细胞外的分子信号（称为配体，ligand）包括激素、生长因子、细胞因子、神经递质以及其它小分子化合物等

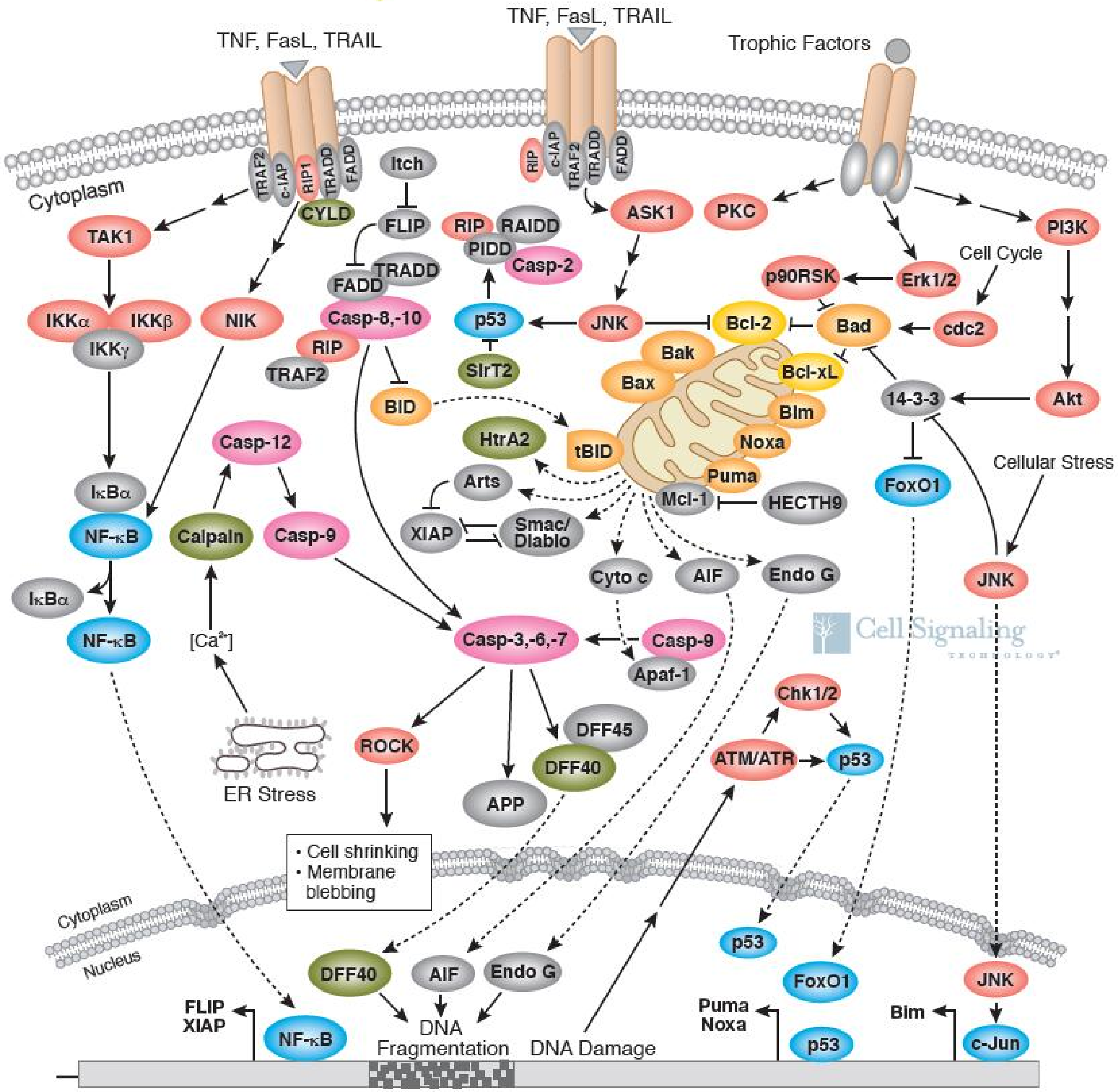
PI3K/AKT


Integrin
RTK
Cytokine Receptor
CD19
BCR
Ag
mIg
α/β
α/β
mIg
GPCR
FAK
Paxillin
PI3K
ILK
PI3K
Gab1
Gab2
IRS-1
Jak1
PI3K
PI3K
BCAP
Syk
Lyn
PI3K
Gβγ
Gα GTP
PIP3
PTEN
Akt
mTORC2
PIP3
PTEN
PP2A
PHLPP
CTMP
S6
PDK1
Membrane Recruitment and Activation
PDK1
Membrane Recruitment and Activation
Protein Synthesis
p70 S6K
PDCD4
4E-BP1
mTORC1
TSC2
TSC1
Inhibits Autophagy
ATG13
PRAS40
Akt
Lamin A
Organization of Nuclear Proteins
Tpl2
IKKα
NF-κB Pathway
eNOS
Cardiovascular Homeostasis
Other
GABAAR
Synaptic Signaling
Huntingtin
Blocks Aggregation Promotes Neuroprotection
Ataxin-1
Aggregation Neurodegeneration
Neuroscience
PFKFB2
Glycolysis
XIAP
Inhibits Apoptosis
FoxO1
MDM2
p53
Bim
Bad
14-3-3
Bcl-2
Bax
Apoptosis
Cytosolic Sequestration
Survival
GSK-3
PIP5K
AS160
GS
Glucose Transport
Glycogen Synthesis
Glucose Metabolism
Wee1
Myt1
p27 Kip1
p21 Waf1/Cip1
Cyclin D1
Cell Cycle
Proliferation
Cell Signaling TECHNOLOGY
mTORC1
GβL
Raptor
mTOR
DEPTOR
mTORC2
Sin1
PRR5
Rictor
GβL
mTOR
DEPTOR

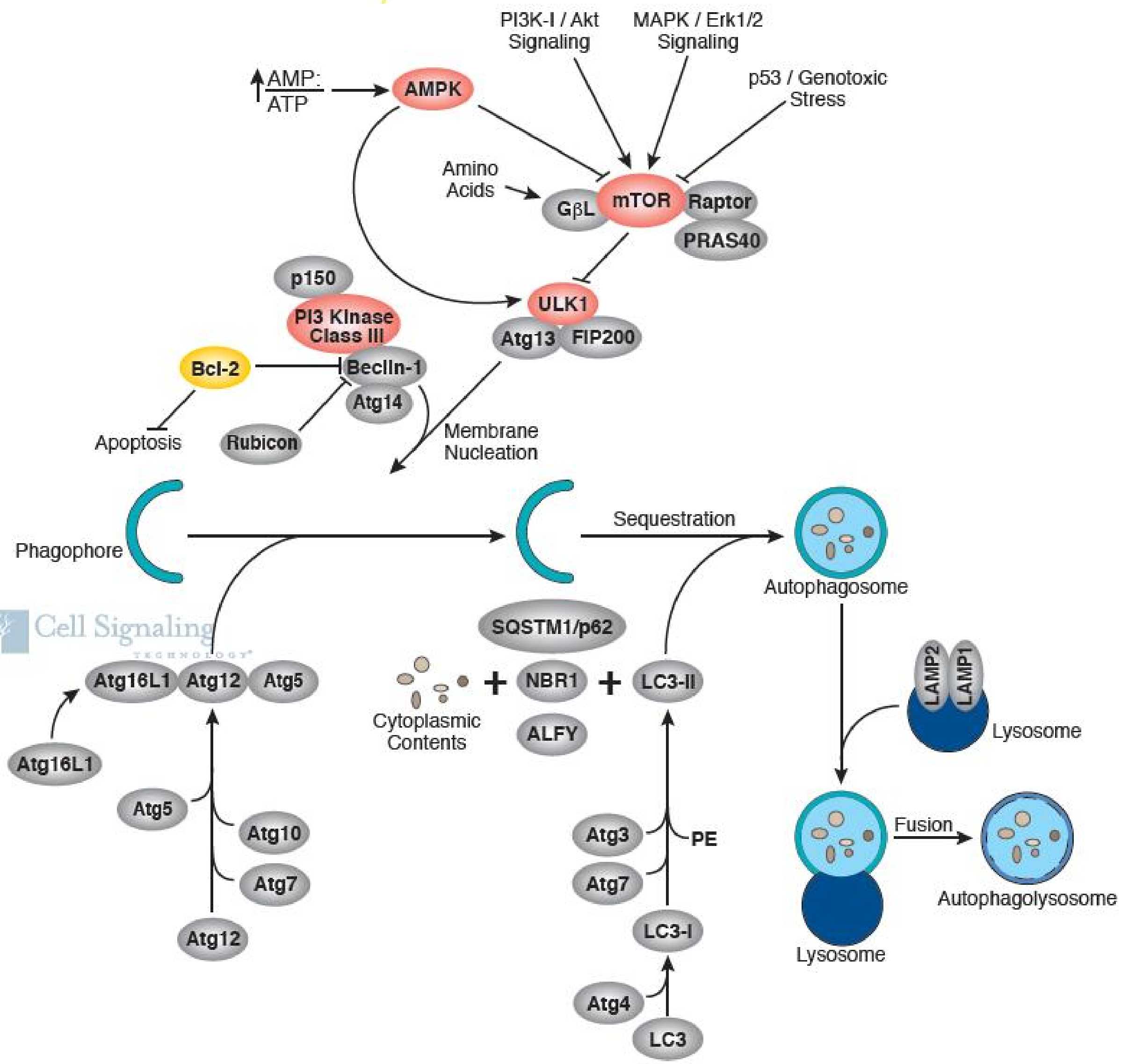
Angiogenesis



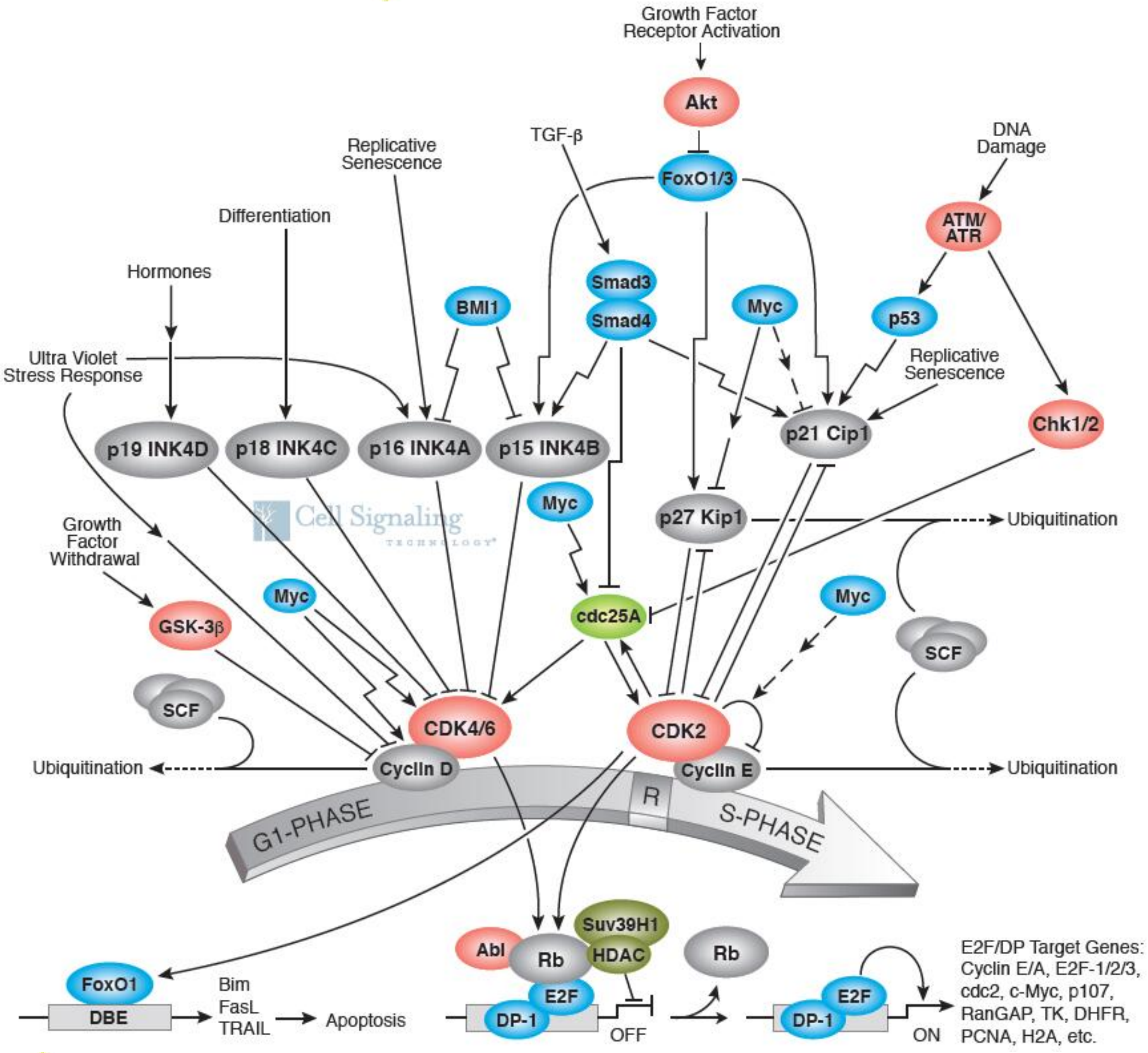
Apoptosis


TNF, FasL, TRAIL
TNF, FasL, TRAIL
Trophic Factors
Cytoplasm
TRAF2
c-IAP
RIP1
TRADD
FADD
CYLD
Itch
FLIP
RIP
c-IAP
TRAF2
TRADD
FADD
ASK1
PKC
PI3K
TAK1
RIP
RAIDD
PIDD
Casp-2
TRADD
FADD
Casp-8,-10
RIP
TRAF2
IKKα
IKKβ
IKKγ
NIK
p53
SIrT2
JNK
Bcl-2
Bad
cdc2
p90RSK
Erk1/2
Cell Cycle
Bak
Bax
Bcl-xL
Bim
Noxa
Puma
Mcl-1
HECTH9
14-3-3
Akt
BID
tBID
HtrA2
Arts
XIAP
Smac/ Diablo
Cyto c
AIF
Endo G
FoxO1
Cellular Stress
JNK
Casp-12
Calpain
Casp-9
IκBα
NF-κB
IκBα
NF-κB
[Ca²⁺]
Casp-3,-6,-7
Casp-9
Apaf-1
Cell Signaling TECHNOLOGY
Chk1/2
ATM/ATR
p53
ROCK
DFF45
DFF40
APP
ER Stress
• Cell shrinking
• Membrane blebbing
Cytoplasm
Nucleus
DFF40
AIF
Endo G
p53
FoxO1
JNK
FLIP
XIAP
NF-κB
DNA Fragmentation
DNA Damage
Puma
Noxa
p53
Bim
c-Jun

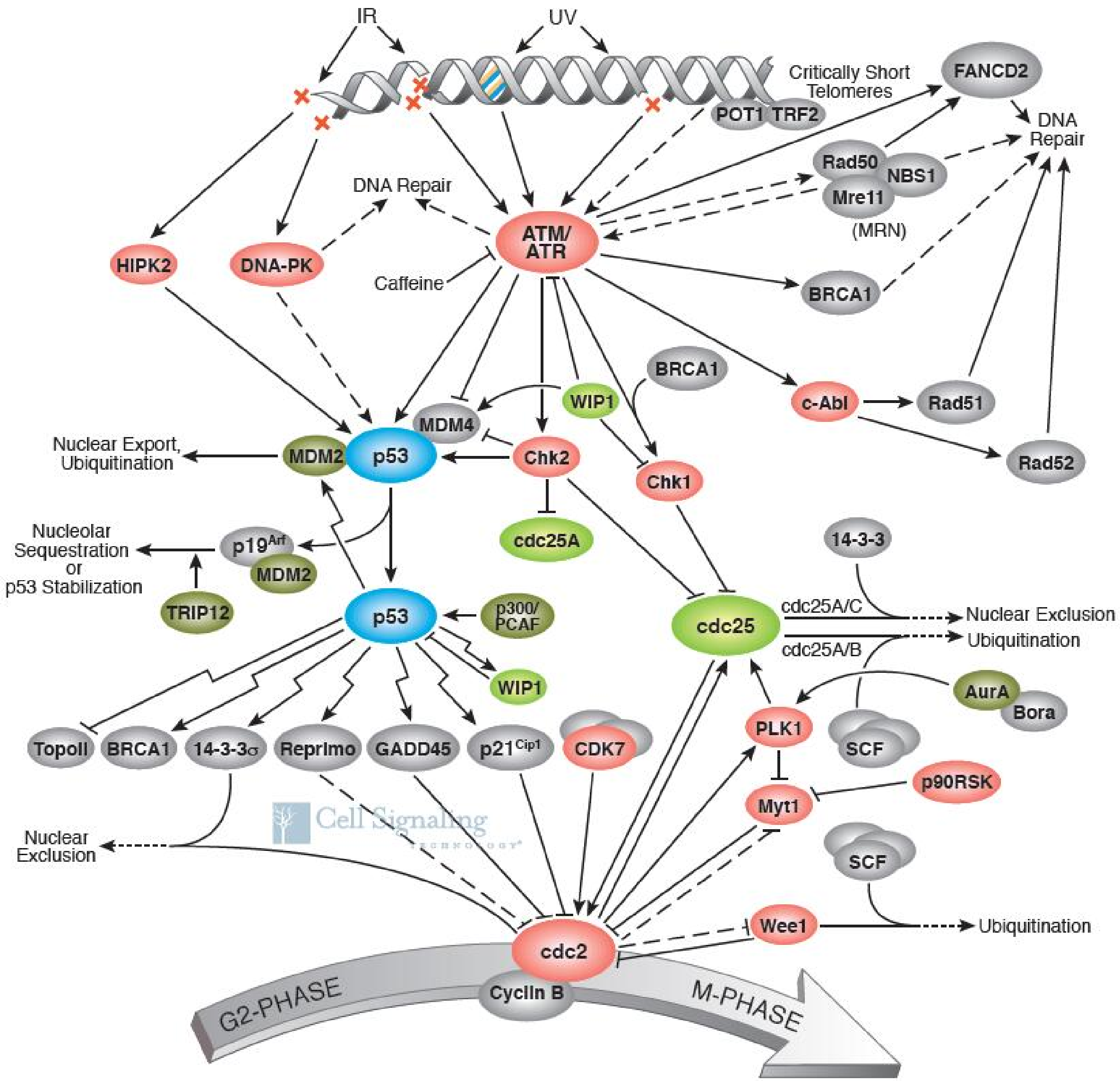
Autophagy Signaling



Cell Cycle Control: G1/S Checkpoint



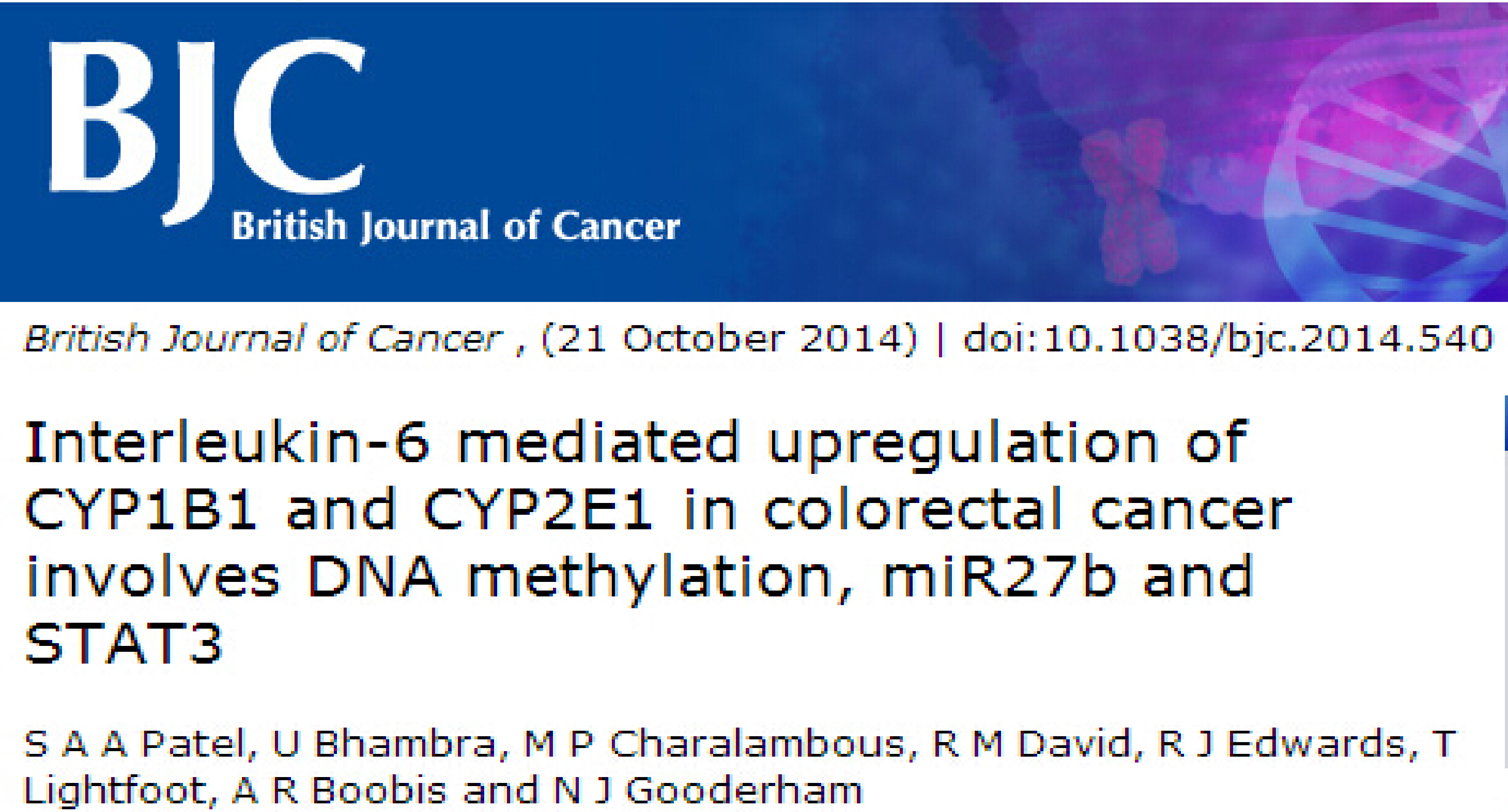
Cell Cycle Control: G2/M DNA Damage Checkpoint



Other Cell signaling

- AMPK
- B Cell Antigen
- Death Receptor
- Histone Methylation
- Insulin Receptor
- Jak/Stat/IL-6
- Notch、MAPK、mTOR、Toll-Like、 NF-kappaB、 Wnt/beta-Catenin, *et al.*

案例分析3



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Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3

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信号通路调控甲基化影响miRNA