Molecular Medicine

“Transcriptomics: A tool for finding efficient biomarkers for early HBV associated Liver Cancer (HCC)”

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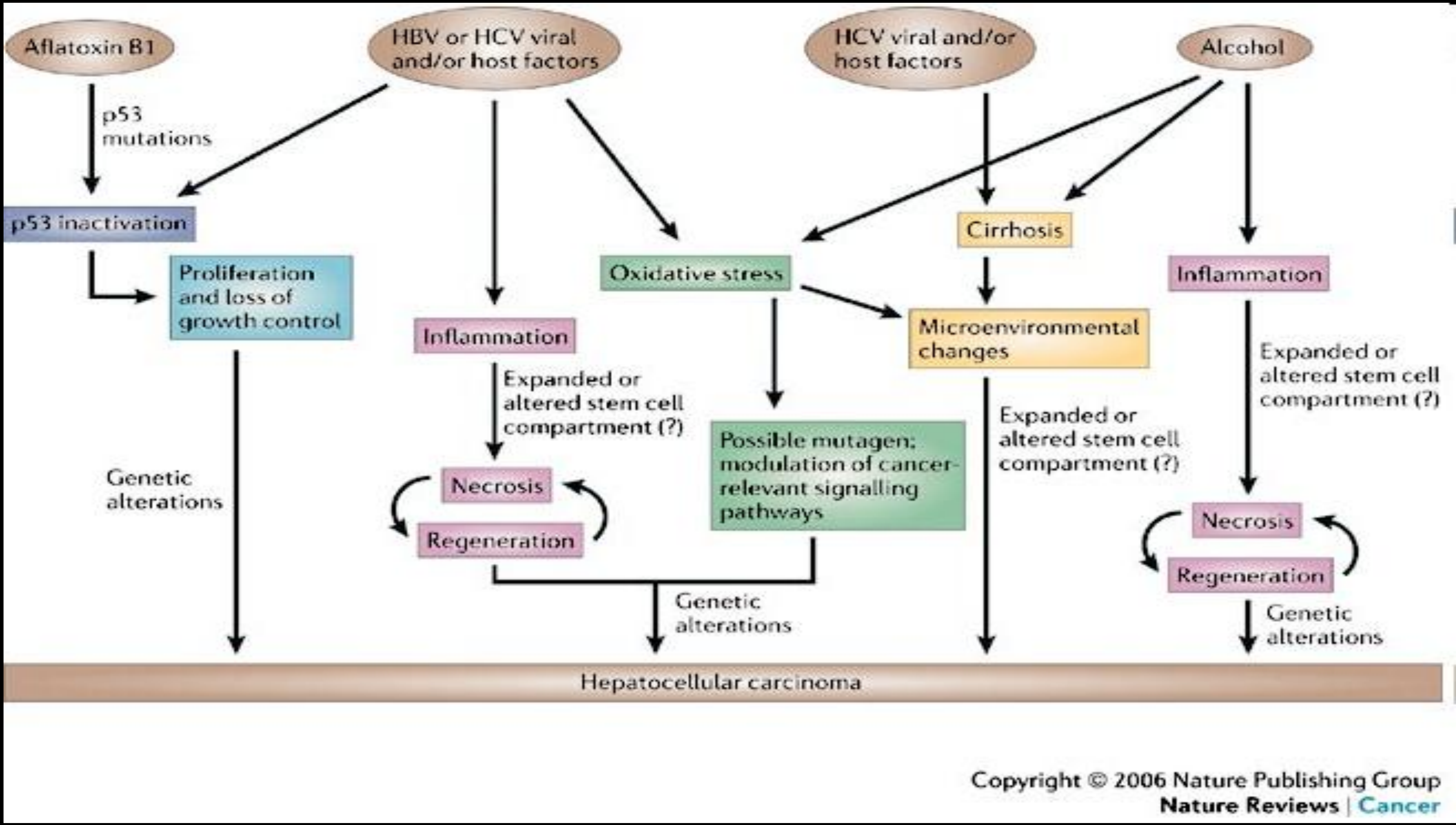
Introduction

- **Primary liver cancer - HCC is the most common type, accounting for 70%-85% of cases**

- **Peak in East-Asia, Mediteranean region and sub-Saharan Africa**

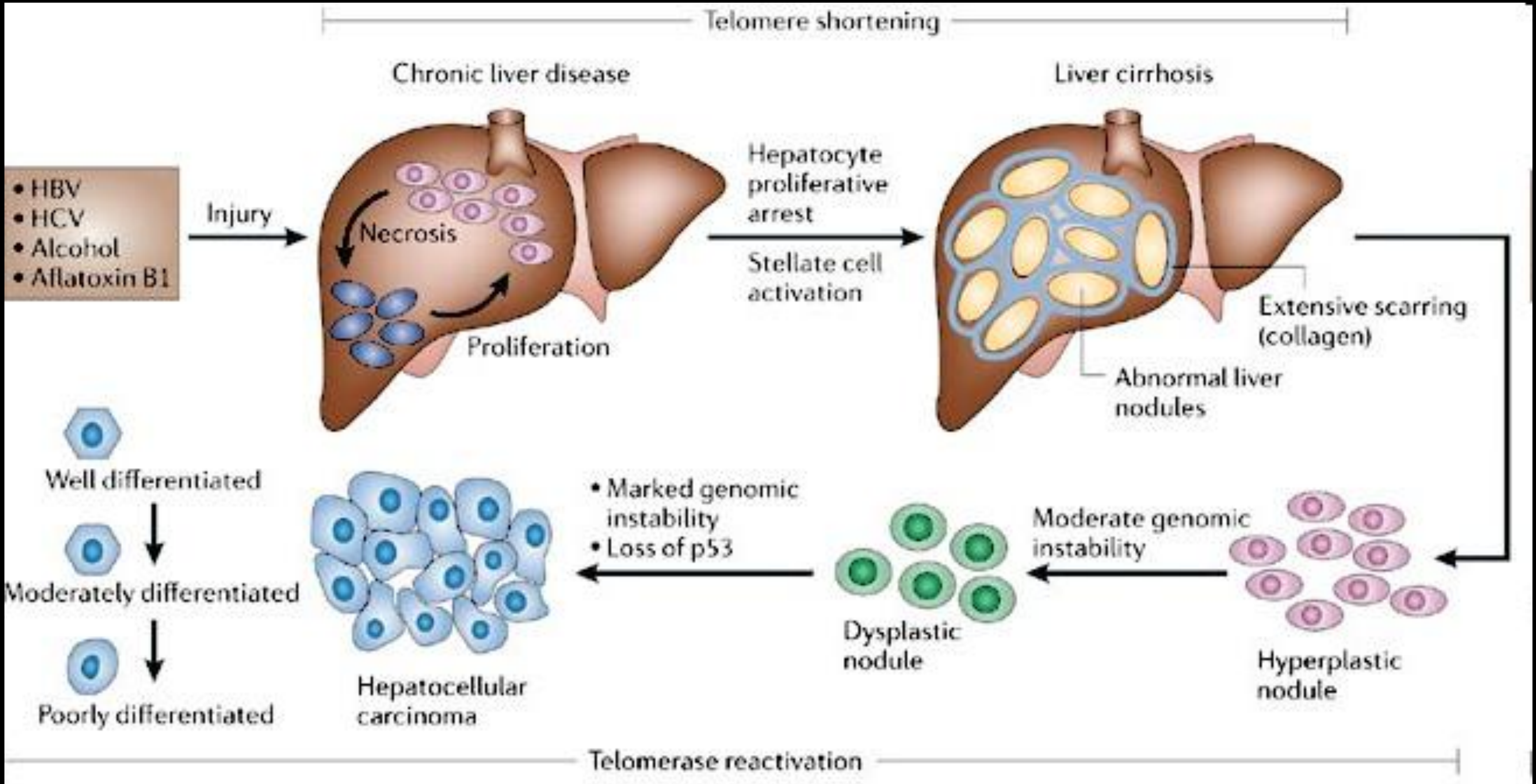
- **Third most frequent cause of cancer death in men and the sixth in women.**

- **Most of the patients currently identified in clinics are often at advanced stage of disease .**
- **All therapy regimens have provided limited success.**



Nature Reviews | Cancer

- **Variations in the prevalence- etiological factors mirror the geographical distribution of the incidence of HCC.**



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ANIMAL MODELS

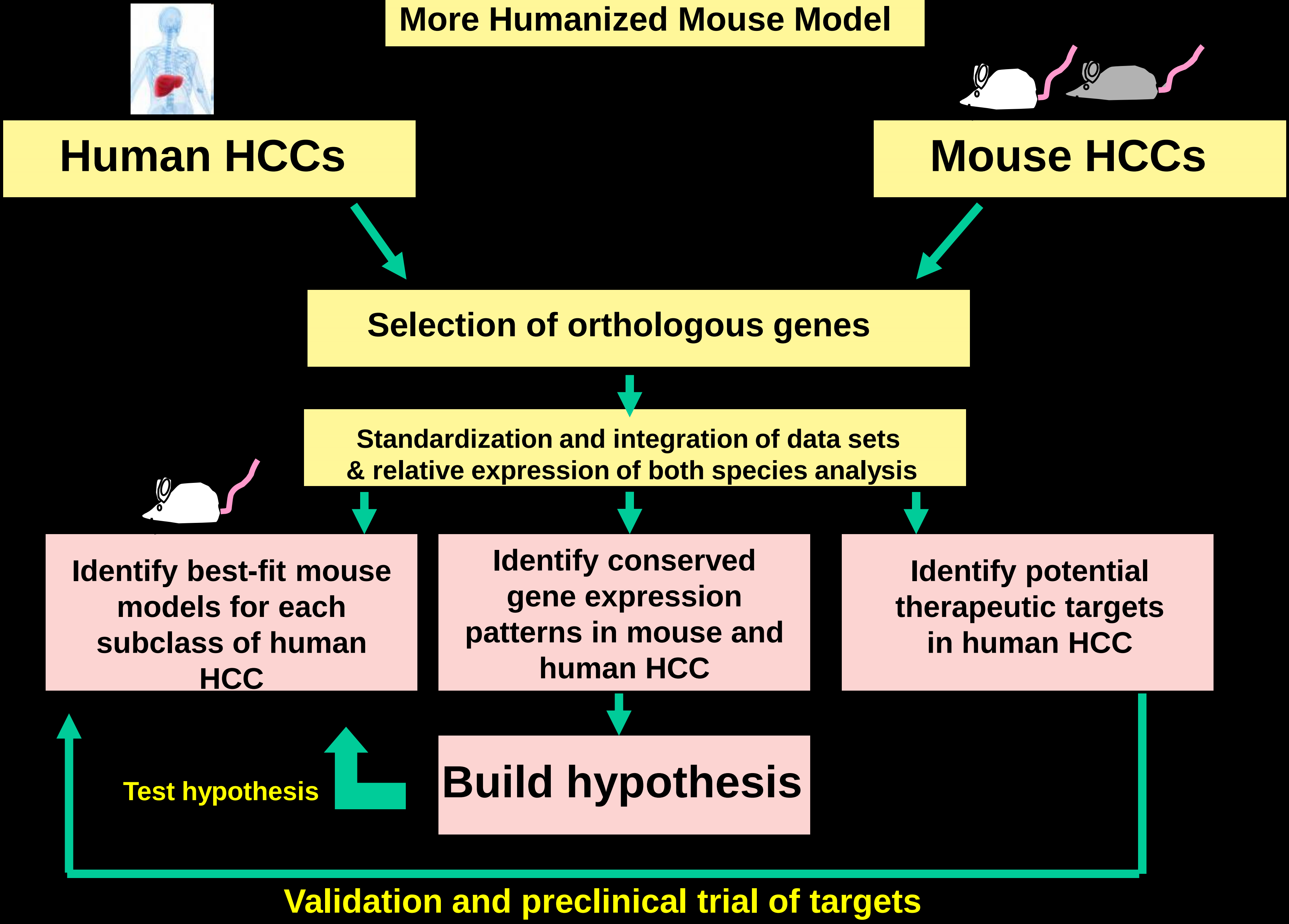
- Carcinogen Induced Mouse models of HCC
- Implantation models of HCC
- Genetically Engineered Mouse (GEM) Models for HCC
- Viral Hepatocarcinogenesis-HBV and HCV associated mouse models of HCC

Transgenic mouse models for HCC

Transgene	Promoter	Mouse strain	Percentage HCCs	Reference
TGF-α	MT	CD1	50% in males > 12 months	1.Jhappan C, *et.al.*, 1990. 2. Lee GH, *et.al.*, 1992
c-myc	Alb	C57BL/6 × CBA/J 65	65% in males at 20 months	Santoni-Rugiu E, *et.al.*, 1996
c-myc/TGF-α	Alb, MT	C57BL/6 × CBA/J × CD1	100% in males at 8 months	Murakami *et.al.*, 1993 Santoni-Rugiu E, *et.al.*, 1996
SV40 T-Ag	AT III	C57BL/6 × DBA2	100% at 8 months	Dubois N, *et.al.*, 1991
E2F-1	Alb	C57BL/6 × CBA/J	33%-60% at 12 months	1. Conner EA, *et.al.*, 2000 2. Calvisi DF, *et.al.*, 2005
c-myc/E2F-1	Alb	C57BL/6 × CBA/J	100% at 9 months	Calvisi DF, *et.al.*, 2005

❖Alb, albumin; AT III, Antithrombin III; MT, Metallothionein

Comparative Genomic Analysis
More Humanized Mouse Model



Human HCCs

Mouse HCCs

Selection of orthologous genes

Standardization and integration of data sets
& relative expression of both species analysis

Identify best-fit mouse models for each subclass of human HCC

Identify conserved gene expression patterns in mouse and human HCC

Identify potential therapeutic targets in human HCC

Build hypothesis

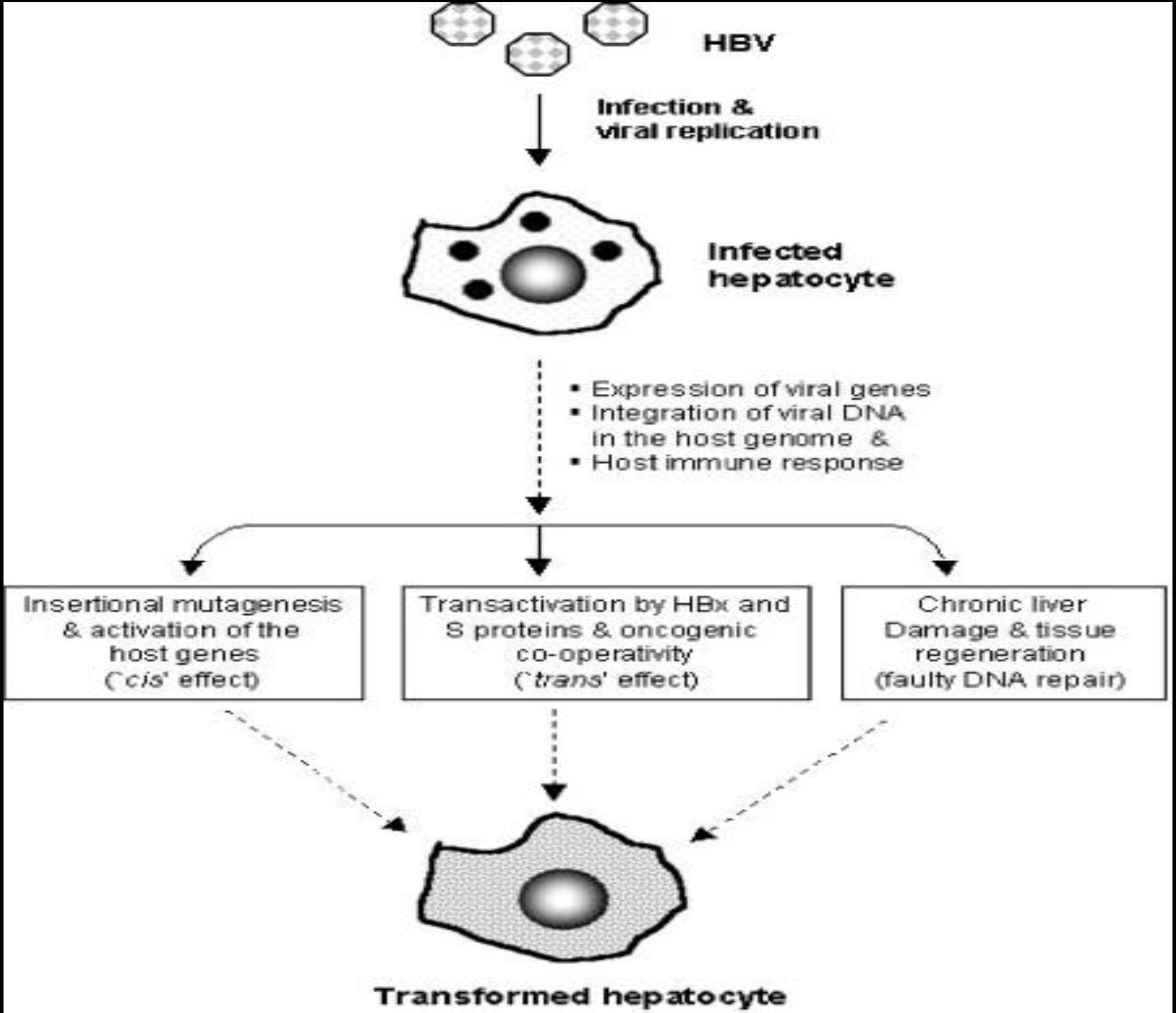
Test hypothesis

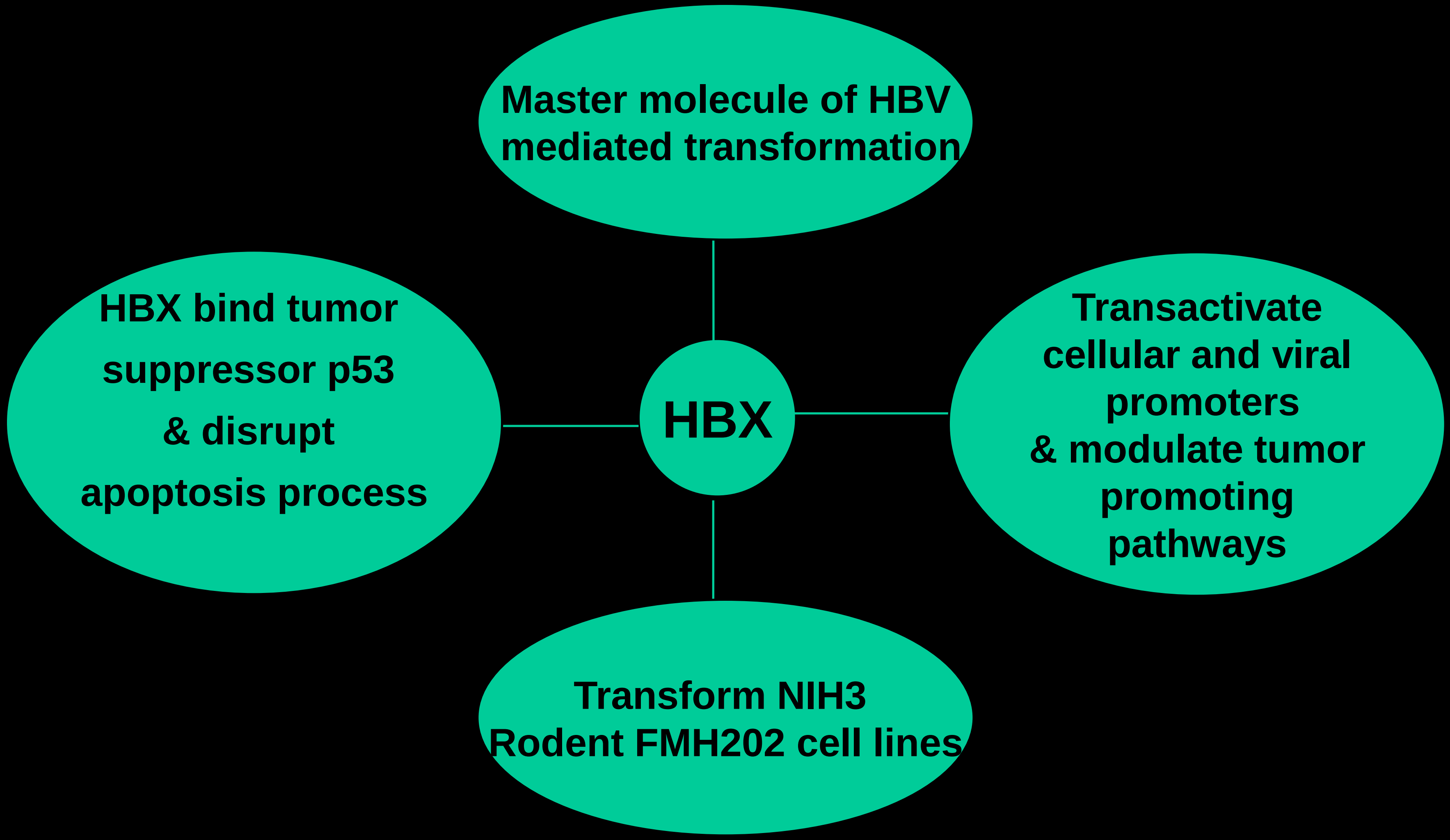
Validation and preclinical trial of targets

Viral Hepatocarcinogenesis

- More than 80% of HCC in human -HBV or HCV or both
- WHO- 400 million people-Chronic HBV-2000
- Infected person may take 20 years to develop HCC
- Undergo multiple steps of genetic alteration
- Human tropism virus
- WHV and GSHV- *in vivo* studies
- Human hepatocyte transplanted model
- Transgenic mice expressing HBV proteins represent the best model

Possible mechanisms for the HBV-associated HCC development





HBX

Master molecule of HBV
mediated transformation

HBX bind tumor
suppressor p53
& disrupt
apoptosis process

Transactivate
cellular and viral
promoters
& modulate tumor
promoting
pathways

Transform NIH3
Rodent FMH202 cell lines

HBV-related transgenic mouse models of HCC

Chisari FV, et.al., 1985 and Babinet C,et.al., 1985

Transgene	expressed Promoters	used Mouse strain	Pathology	Reference
Large and major S	Alb-HBV	C57BL/6 x SJL	HCC in the second half of animals’ life span	Chisari FV et.al., 1989
HBx	Xp	CD1	,,	Kim CM et.al.,1991
HBx and c-myc in separate lines	Xp for HBx & WHV for c-myc	C57BL/6 x SJL/J	,,	Terradillos O,et.al., 1997
HBx	Xp	C57BL/6 x DBA	,,	Yu DY et.al., 1999

- Alb, albumin; Cp, core gene promoter; S, surface antigen; WHV, woodchuck hepatitis virus; Xp, X gene promoter.

Accelerated HCC - HBX Tg mouse & WHV/c-myc Tg mice hybrid offspring's

But still not fast as the pathogenic studies demanded

Bicistronic DNA construct, X-myc

X15-myc
(58-150aa of X protein)-Exon 2and 3

Minimal transactivation domain of HBX
(Kumar et.al., 1996)

Murine c-myc protein

Reasons for choosing myc gene

- Selective amplification of c-myc gene found in HBV related HCC cases **(Peng *et.al*., 1993)**
- The activation of N-myc and c-myc gene is frequently observed after integration of viral **DNA (Moroy *et.al* 1996 and Fourel *et.al*., 1990)**

X15myc-Transgenic construct

Regulatory elements in HBV genome

FIG. 1

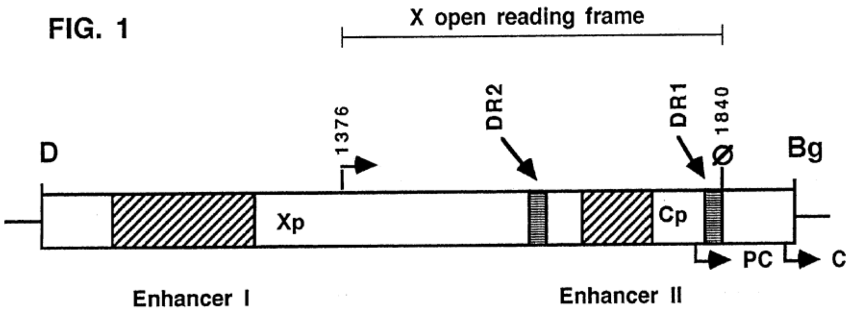
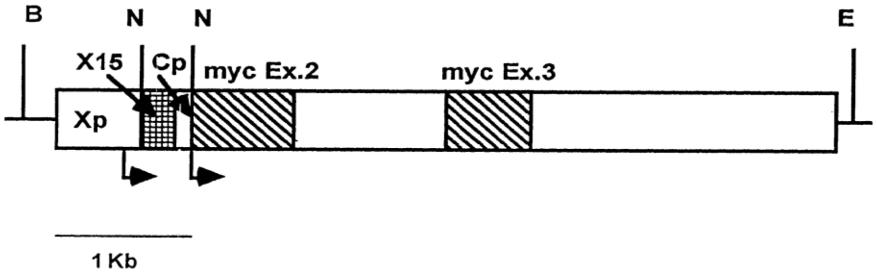


FIG. 2 X15-myc bicistronic construct



X15 region positioned to 5’ to the murine c-myc gene, and is operatively linked to under the regulatory control of its natural promoter and enhancer I element.

C-myc gene is operatively linked to under the regulatory control of core promoter and enhancer II elements

This compact present construct facilitate the the core promoter and and enhancer II regions are embodied in the X gene sequence

5.7Kb EcoR I and Bam HI fragment

D-Dra I, Bg-Bgl II, Xp-Natural X promoter, Cp-Core promoter, B-BamhH I, N-Nco I, E-EcoR I,

X 15- myc Transgenic Mouse Model for HCC

X15- myc Transgenic Mouse

- **Recombinant bicistronic construct (Singh *et al*, 2003) (Kumar *et al*, 2001) US patent no: 6274788 B1.**

- **X-15 myc transgenic mice appeared to be an ideal model to study the disease process and also screening drugs.**

Normal Liver X15- myc Tg Liver

Transgene	expressed Promoters	used Mouse strain	Pathology	Reference
HBx and c-myc	Xp and Cp	C57BL/6 x SJL	HCC in the first half of animals' life span (3 to 5 months)	1. Singh M,et.al., 1998 2. Kumar V,et.al., 2001(us patent)

X15-myc transgenic mouse model

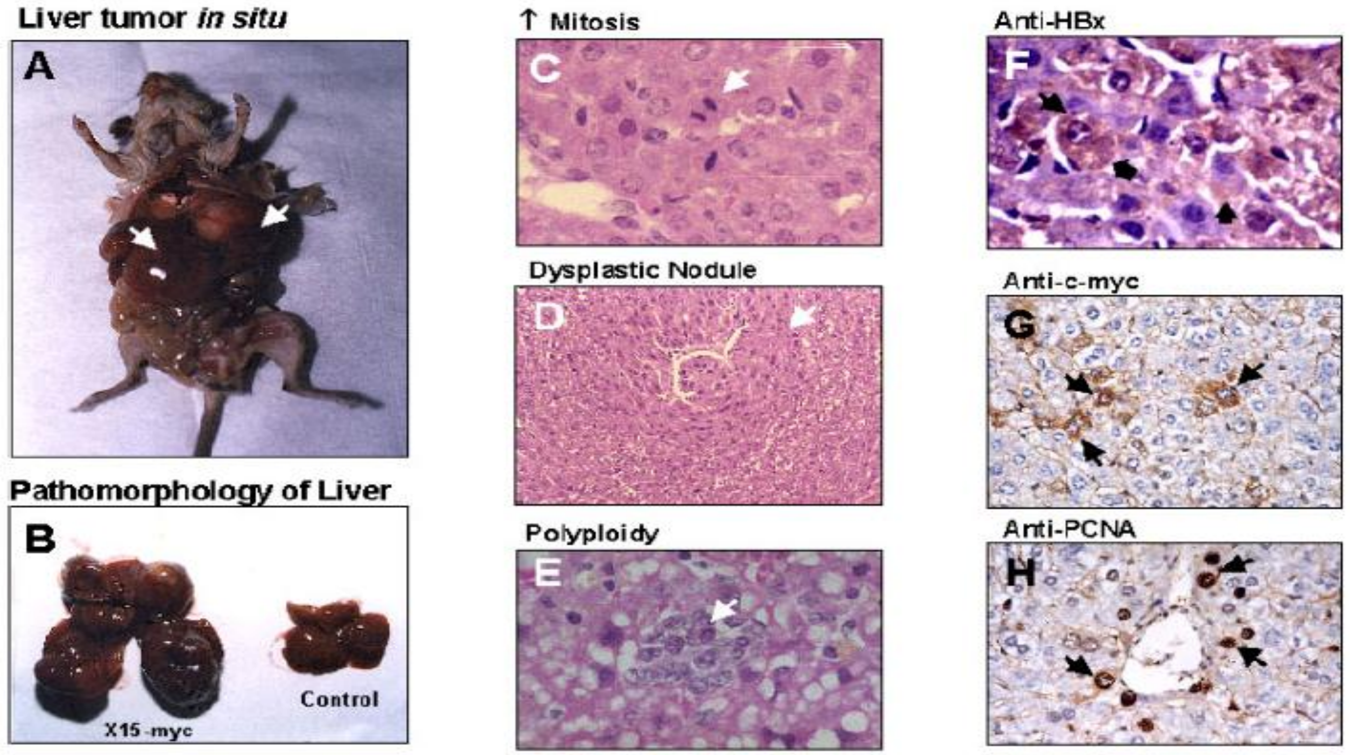
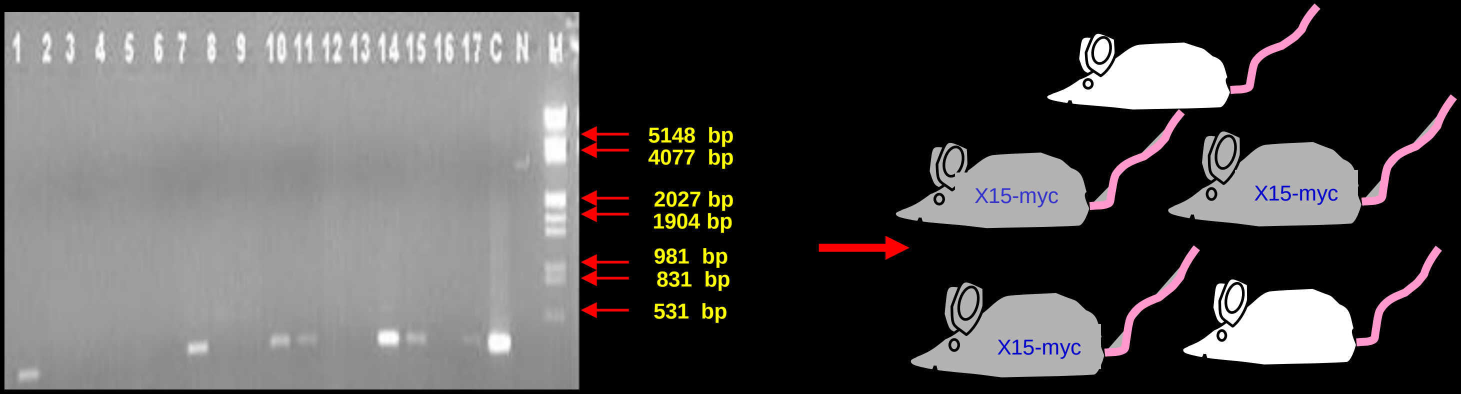


Figure 3. The X15-myc transgenic mouse model of HCC. Liver anatomy (A,B); histology of liver (C–E) and immuno-histochemistry for specific antigens (F–H)

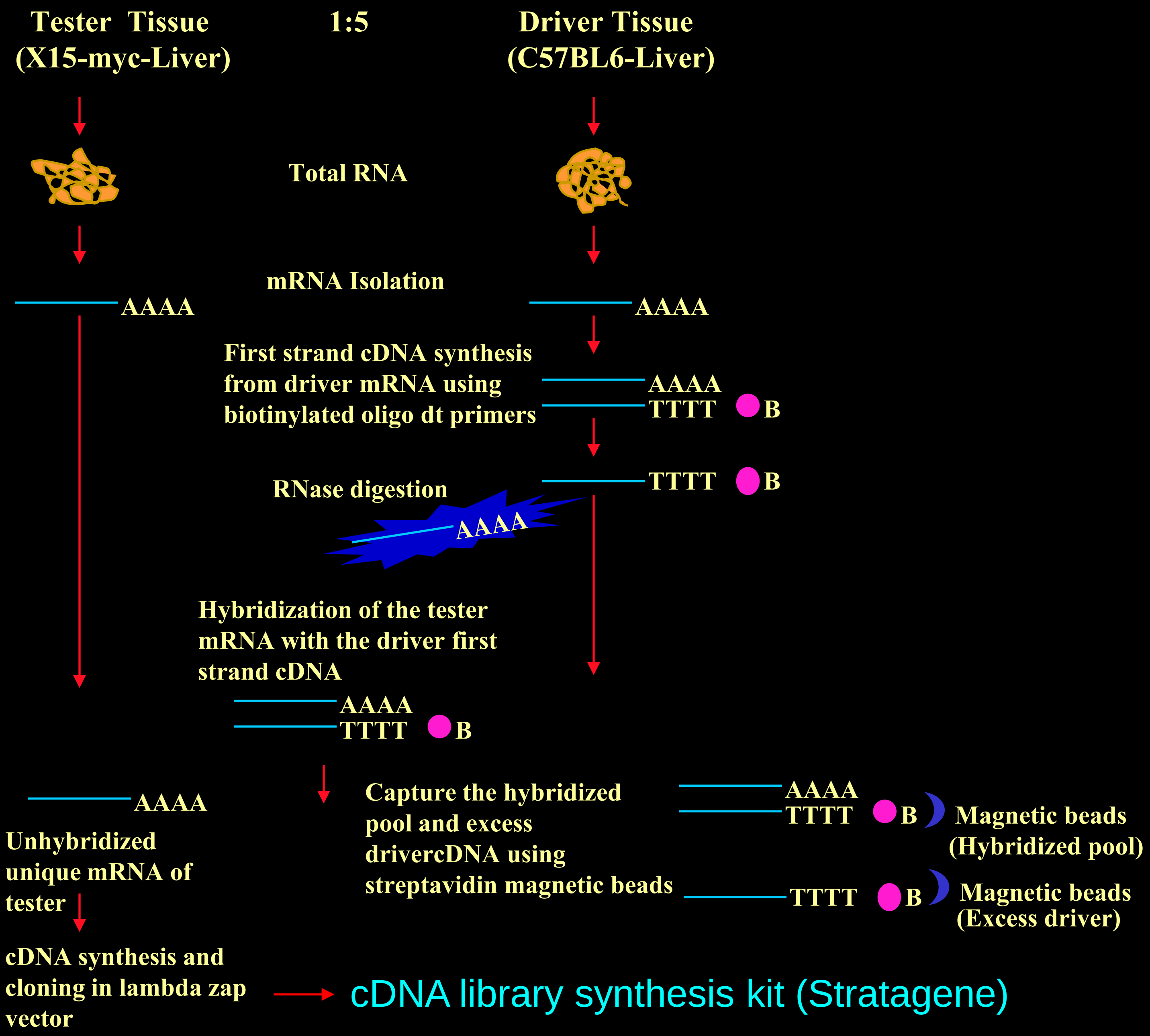
➢**Most of the pathomorphological and microscopic changes were similar to those observed with the HCC patients (Lakhtakia *et al*, 2003)**

Analysis of differential gene expression in the tumors of X15-myc oncomouse.



PCR analysis for X15-myc Transgenic positive animals
“ C “- Positive control; “ N” – Negative control; “ M “- λ DNA Marker

cDNA subtraction



Total RNA

mRNA Isolation

First strand cDNA synthesis from driver mRNA using biotinylated oligo dt primers

RNase digestion

Hybridization of the tester mRNA with the driver first strand cDNA

Capture the hybridized pool and excess drivercDNA using streptavidin magnetic beads

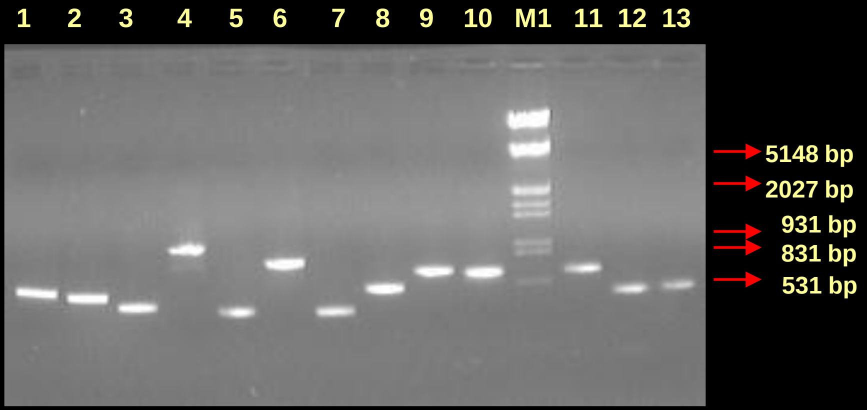
Unhybridized unique mRNA of tester

cDNA synthesis and cloning in lambda zap vector → cDNA library synthesis kit (Stratagene)

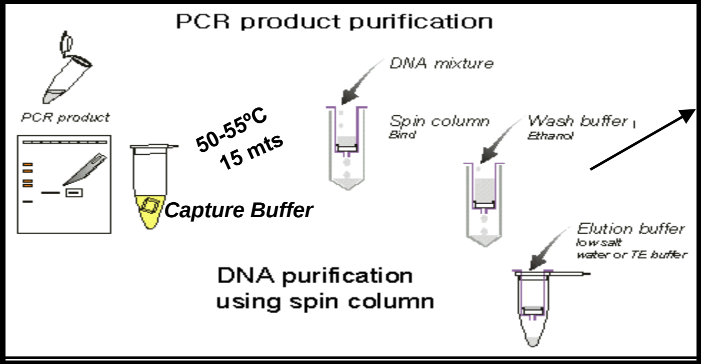
Primers

M13 F: 5'-CGTTGTAAAACGACGGCCAGTG -3'
M13 R: 5'-CACAGGAAACAGCTATGACCATG-3'

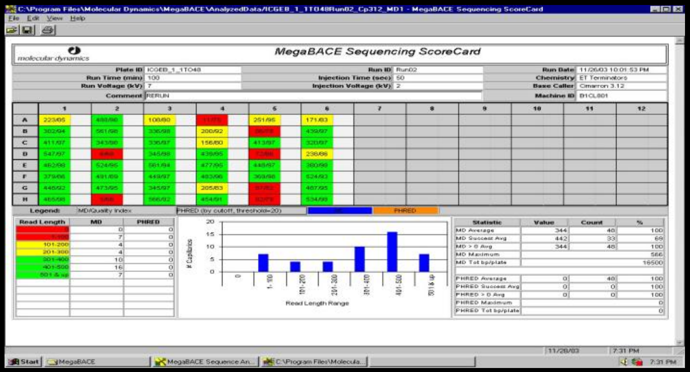
A.



B.



C.



MegaBACE Sequencing ScoreCard

Plate ID: ICGEB_1_1TO48
Run Time (min): 100
Run Voltage (kV): 7
Comment: RERUN
Run ID: Run02
Injection Time (sec): 50
Injection Voltage (kV): 2
Run Date: 11/26/03 10:01:53 PM
Chemistry: ET Terminators
Base Caller: Cimarron 3.12
Machine ID: B1CL801

	1	2	3	4	5	6	7	8	9	10	11	12
A	223/95	488/90	108/80	[illegible]	251/95	171/83						
B	302/94	561/98	336/98	200/92	[illegible]	439/97						
C	411/97	343/96	336/97	156/80	413/97	320/97						
D	547/97	[illegible]	345/98	439/95	[illegible]	238/88						
E	462/98	524/95	561/94	477/95	448/97	380/98						
F	379/96	491/89	449/97	483/96	369/96	524/93						
G	446/92	473/95	345/97	205/83	[illegible]	467/95						
H	465/98	[illegible]	566/92	454/91	[illegible]	534/98						

Legend: MD/Quality Index PHRED (by cutoff, threshold=20) MD PHRED

Read Length	MD	PHRED
0	0	0
1-100	7	0
101-200	4	0
201-300	4	0
301-400	10	0
401-500	16	0
501 & up	7	0

Statistic	Value	Count	%
MD Average	344	48	100
MD Success Avg	442	33	69
MD > 0 Avg	344	48	100
MD Maximum			566
MD Tot bp/plate			16500
PHRED Average	0	48	100
PHRED Success Avg	0	0	100
PHRED > 0 Avg	0	0	100
PHRED Maximum			0
PHRED Tot bp/plate			0

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A. Agarose gel analysis of PCR amplified cDNA inserts from phagemids. M1, DNA Marker, B. DNA band purification steps by GFX PCR, C. Chromotogram of MegaBACE sequencing score card.

Vector Screened & Changed in to FASTA format sequences

↓

BLAST Analysis

http://www.ncbi.nlm.nih.gov/blast/blast.cgi

List of differentially expressed genes.

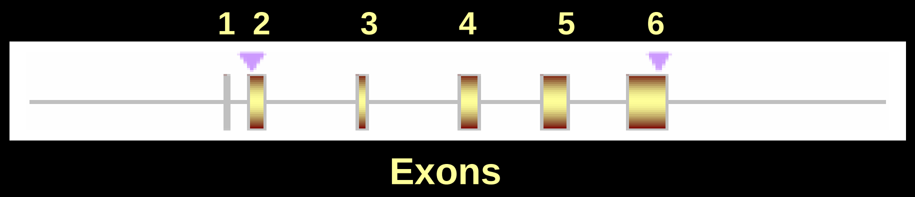
Sequence analysis of transcripts revealed 19 discreet categories

Protein biosynthesis genes	100
Electron transport genes	81
Metabolism related genes	75
Transport molecule genes	36
Ubiquitin proteasome pathway genes	25
Protein metabolism genes	23
Signal transduction genes	22
RNA processing genes	18
Calcium/sugar/carbohydrate/copper binding genes	14
Cytoskeletal prganisation/ cell adhesion genes	17
Endocytosis /protein transport genes	13
Oxidative stress genes	13
Transcription genes	13
Cell cycle and growth differentiation genes	12
Immune response genes	11
Apoptosis and anti apoptosis genes	9
Blood cogulation genes	9
Molecular chaperone genes	8
Replication gene	1

List of top five differentially expressed genes obtained from X15-myc Transgenic mouse liver cDNA subtraction library.

SYMBOL	**GENE NAME**	**FREQUENCY**
Rps27a	Ribosomal Protein s27a	20
COX3	Cytochrome C Oxidase, Subunit III	16
ATP6	ATP Synthase F0 Subunit 6	14
COX2	Cytochrome C Oxidase, Subunit II	11
ND1	NADH dehydragenase, Subunit, type 1	11

A



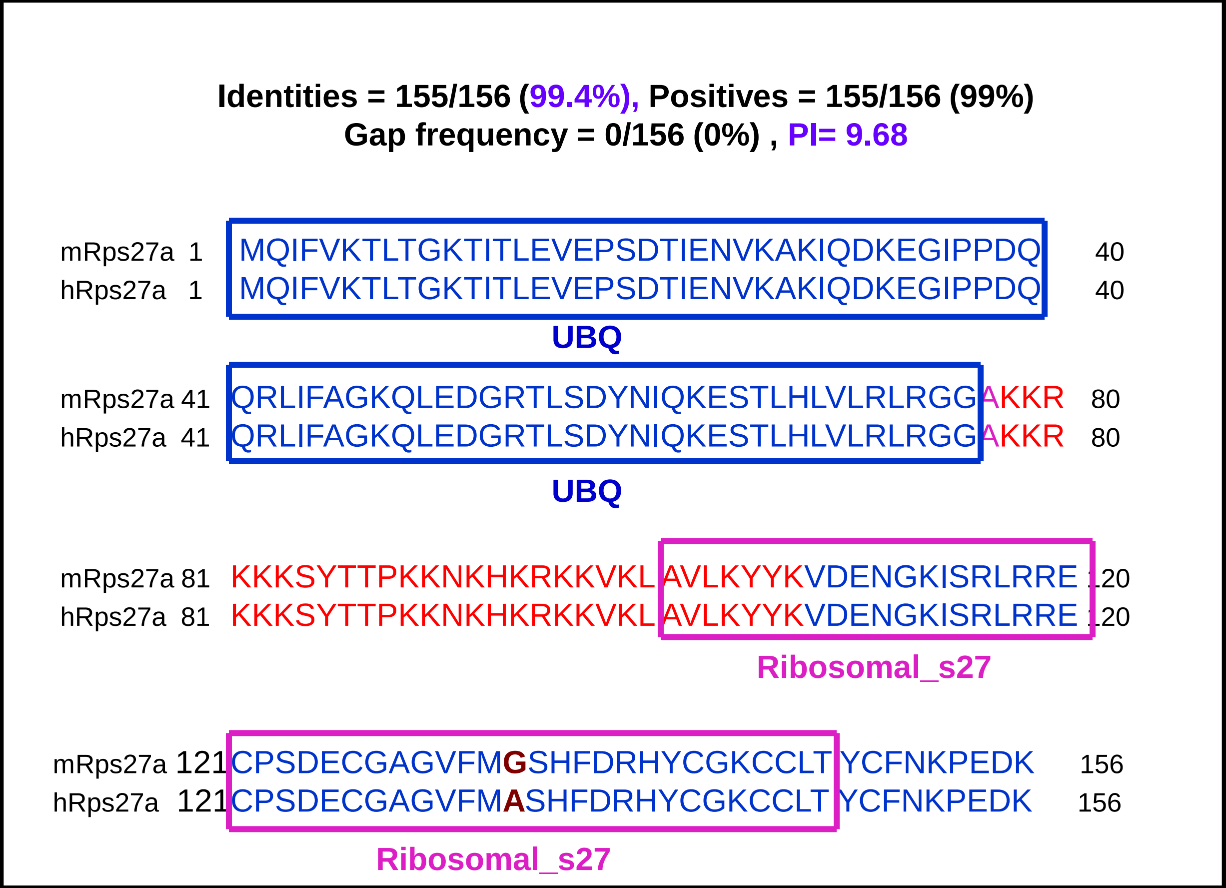
B

Chromosome: 11, Location 11 A3.3

Filename : *Mus musculus* Rps27a-CDS
Sequence Size : 471
Translation Position : 1 - 471; Genetic Code : Universal

```
         10        20        30        40        50        60
ATGCAGATCTTTGTGAAGACCCTTACGGGGAAAACCATCACGCTCGAGGTTGAACCCTCG
M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  P  S   20
         70        80        90       100       110       120
GACACTATAGAAAATGTAAAGGCCAAGATCCAGGATAAGGAAGGAATTCCTCCTGATCAG
D  T  I  E  N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q   40
        130       140       150       160       170       180
CAGAGGCTGATCTTTGCTGGTAAGCAGCTGGAAGATGGCCGGACTTTGTCTGACTACAAC
Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  S  D  Y  N   60
        190       200       210       220       230       240
ATTCAAAAGGAGTCCACCCTTCATCTGGTGTTGAGACTTCGGGGTGGTGCTAAGAAAAGG
I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G↑ A  K  K  R   80
        250       260       270       280       290       300
AAGAAGAAGTCTTACACCACTCCCAAGAAGAACAAGCATAAGAGGAAGAAGGTTAAGTTG
K  K  K  S  Y  T  T  P  K  K  N  K  H  K  R  K  K  V  K  L  100
        310       320       330       340       350       360
GCTGTGCTGAAATACTATAAGGTGGATGAAAATGGCAAAATTAGCCGACTTCGTCGAGAG
A  V  L  K  Y  Y  K  V  D  E  N  G  K  I  S  R  L  R  R  E  120
        370       380       390       400       410       420
TGTCCTTCTGATGAATGTGGTGCTGGAGTTTTCATGGGAAGCCACTTTGACAGGCATTAC
C  P  S  D  E  C  G  A  G  V  F  M  G  S  H  F  D  R  H  Y  140
        430       440       450       460       471
TGTGGCAAGTGTTGTCTGACTTACTGCTTCAACAAACCAGAAGACAAGTAG
C  G  K  C  C  L  T  Y  C  F  N  K  P  E  D  K  *        156-AA
```

Structure of the Rps27a gene of *Mus musculus* and cDNAs. **A**, Organization and chromosomal location. In total, 6 exons are shown in boxes. Closed boxes designate the coding sequences. **B**, ORF of the Rps27a gene showing 156 amino acids in single letter code (An red aerrowmark indicates the site of proteolytic cleavage that would be required to generate mature ubiquitn from the primary translation product).



Identities = 155/156 (99.4%), Positives = 155/156 (99%)
Gap frequency = 0/156 (0%) , PI= 9.68

mRps27a 1 MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ 40
hRps27a 1 MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ 40

UBQ

mRps27a 41 QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGAKKR 80
hRps27a 41 QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGAKKR 80

UBQ

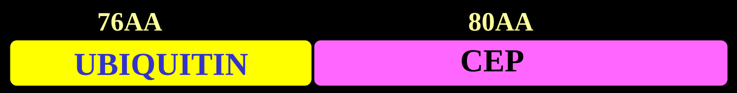
mRps27a 81 KKKSYTTPKKNKHKRKKVKLAVLKYYKVDENGKISRLRRE 120
hRps27a 81 KKKSYTTPKKNKHKRKKVKLAVLKYYKVDENGKISRLRRE 120

Ribosomal_s27

mRps27a 121 CPSDECGAGVFM**G**SHFDRHYCGKCCLTYCFNKPEDK 156
hRps27a 121 CPSDECGAGVFM**A**SHFDRHYCGKCCLTYCFNKPEDK 156

Ribosomal_s27

Alignment of the primary sequences of human and mouse Rps27a Identical residues are shown in same color, while the non-identical regions are marked in different colors. Blue and rose boxes denote the regions of ubiquitin and Ribosomal_s27 respectively.



RPS27a

- The Rps27a Highly conserved very basic protein due to presence of 80 basic AA in the Carboxyl terminal (CEP) of Ubiquitin.

- UBQ-76 AA conserved eukaryotic protein- Diverse cellular functions

- UBQ-Poly ubiquitin chain (UBb, UBc) or Fused to unrelated protein (UBA)

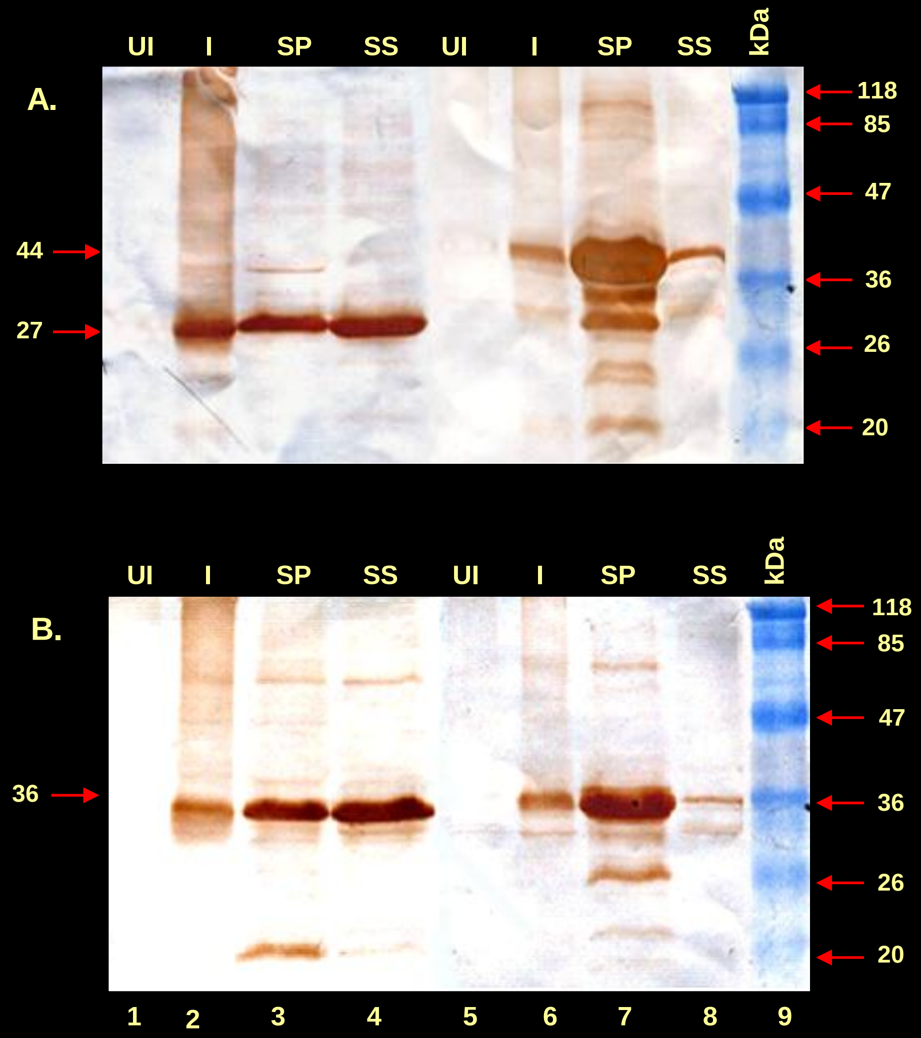
- UBA → UBA80 (Rps27a)or UBA52 (RpL40). Conserved in man, yeast and plants

[Salvensan et al, Nucleic Acid Research (1987), Ozkaynak et al, EMBO J (1987), Lund et al, J. Bio. Chem (1985)]

- CEP analysis shows strong homology at the AA level with Zinc finger protein.

- CEP can associate with ribosome binding specifically ribosome or messenger RNA.

- Function of UBQ-CEP fusion genes has not been fully elucidated

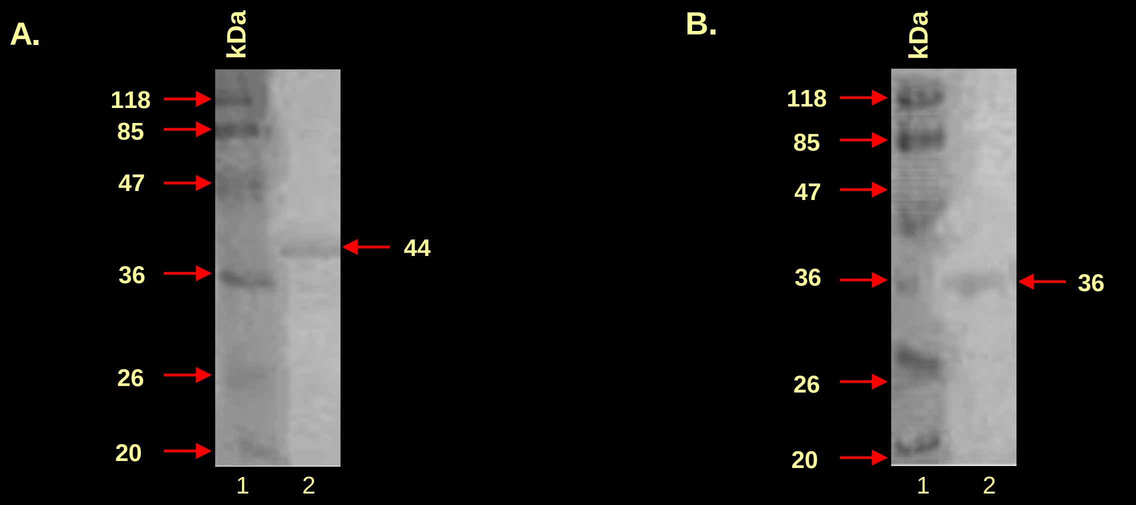


Western blot analysis of GST-Rps27a and GST-CEP recombinant protein expression in *E.coli.*

(A) IPTG (1mM) induced both crude and sonicated GST (27 kDa) and recombinant GST-Rps27a (44 kDa) protein

(B) GST-CEP1 (36 kDa) protein extracts were probed with GST antibody. UI, Un-induced; I, Induced; SP, Sonicated Pellet; SS, Sonicated Supernatnt.

Authentication of Recombinant Rps27a and CEP proteins

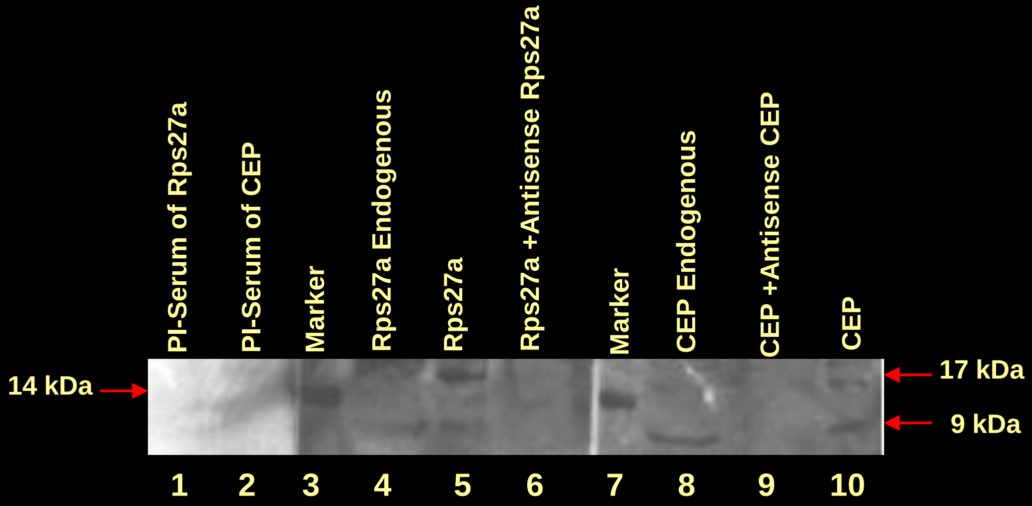


Polyclonal Rps27a Ab°

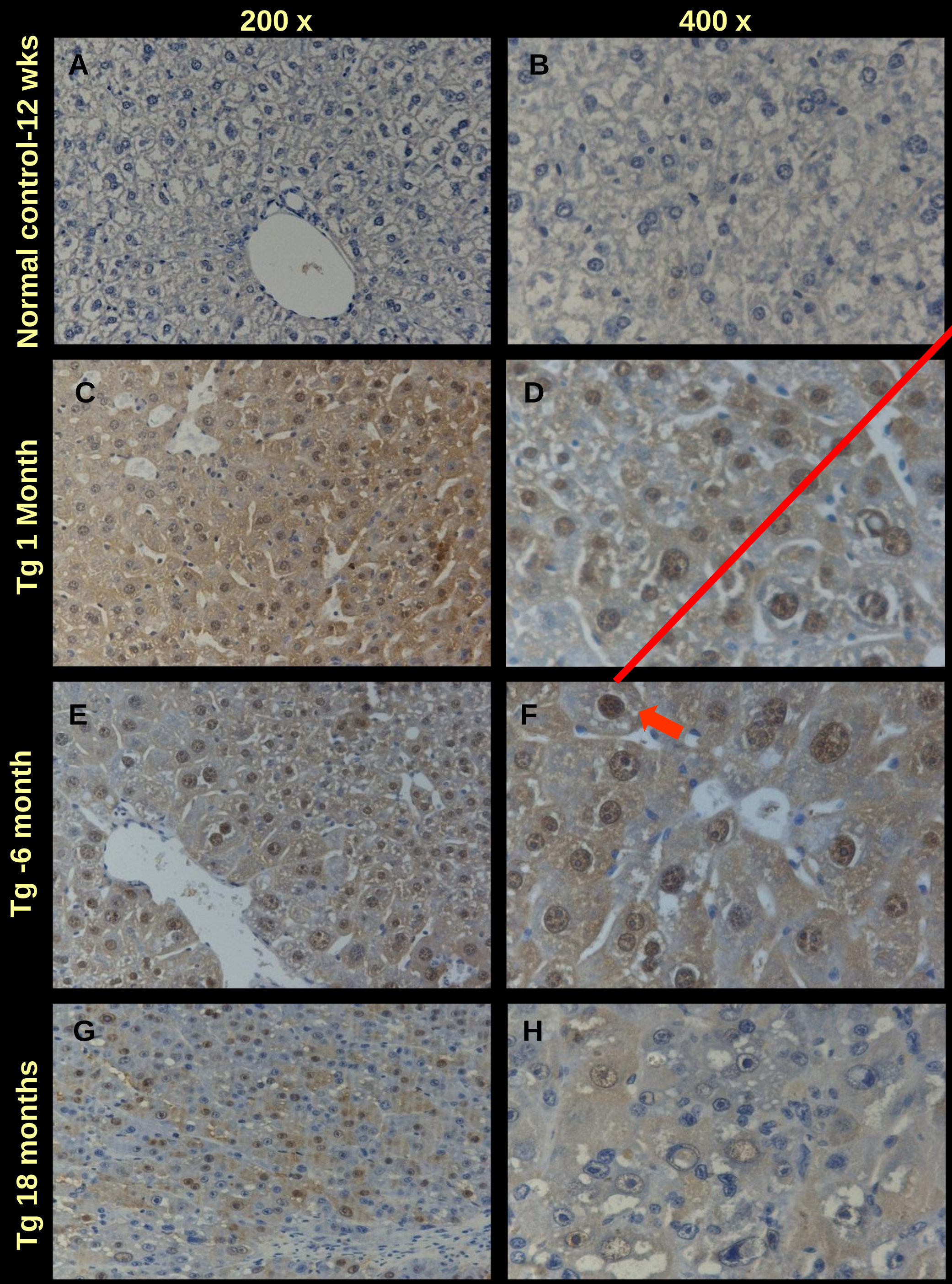
Polyclonal CEP Ab°

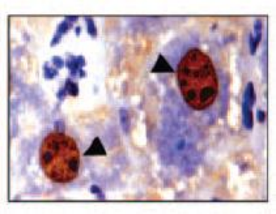
Western blot analysis GST-Rps27a and GST-CEP

E.coli expression of recombinant GST-Rps27a (A) and GST-CEP (B) proteins were transferred on to nitrocellulose strips and probed with anti-Rps27a (1:200) (A. Lane 2) and anti-CEP polyclonal sera (B. Lane 2). Antigen- antibody reaction was shown on RH side arrow mark.
kDa, Prestain marker.

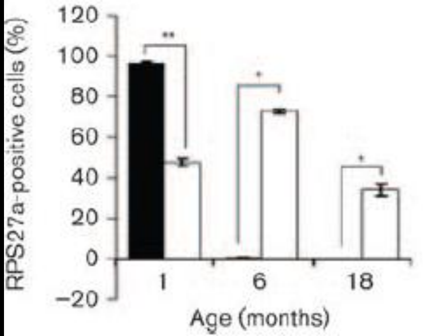


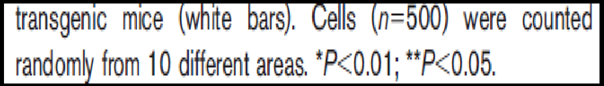
Detection of Rps27a and CEP expression in Huh7 cell lines. Total cells were lysed after 48hr post transfection with Rps27a (lanes 5), anti-Rps27a (lanes 6), CEP (lanes10) and anti-CEP (lanes 9).





Positive staining is noted more in nuclear and perinuclear **(red arrow**)

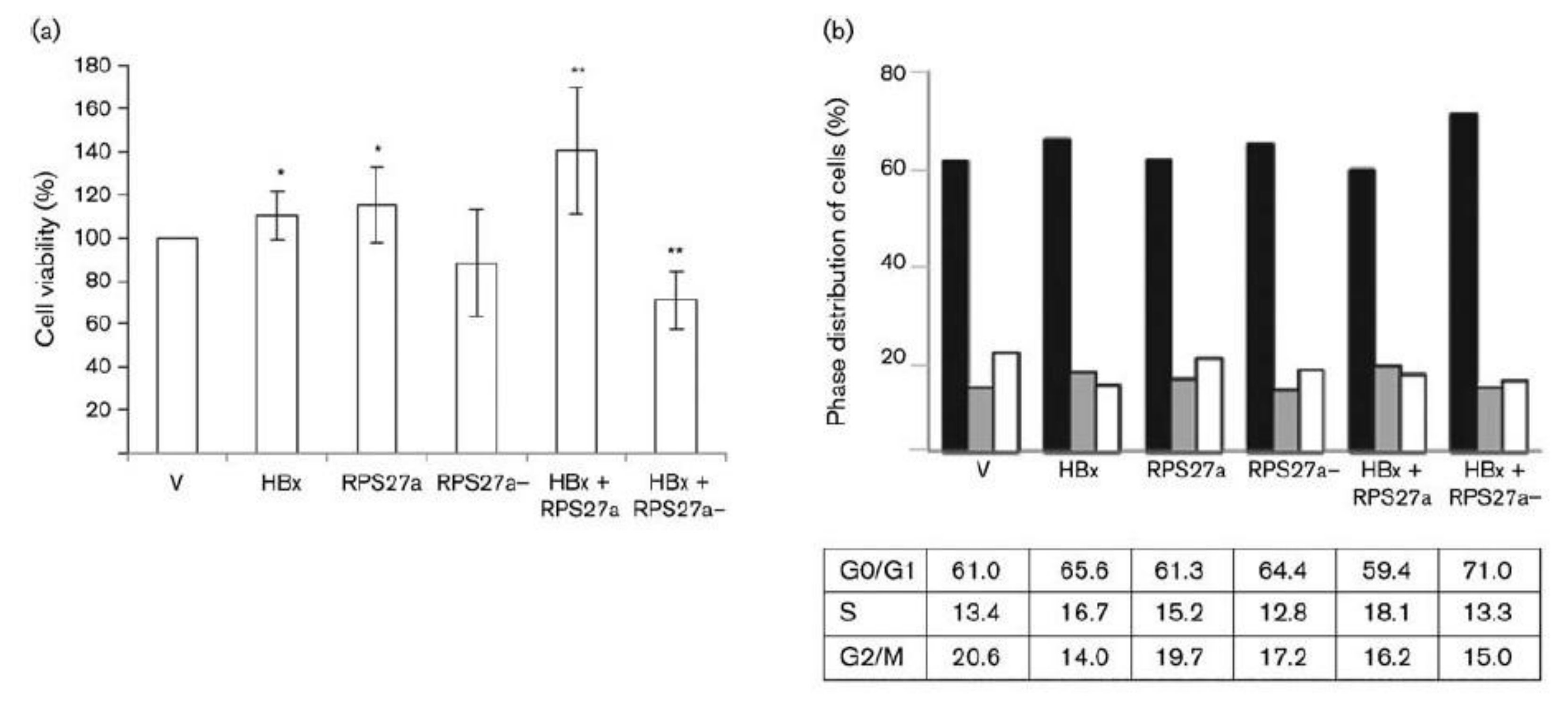




transgenic mice (white bars). Cells (n=500) were counted randomly from 10 different areas. *P<0.01; **P<0.05.

Rps27a expression in X15-myc Tg mice liver tissues.

Co-expression of Rps27a and HBx leads to improved survival and proliferation of cells



G0/G1	61.0	65.6	61.3	64.4	59.4	71.0
S	13.4	16.7	15.2	12.8	18.1	13.3
G2/M	20.6	14.0	19.7	17.2	16.2	15.0

*$P<0.01$ **$P<0.001$, compared with controls.

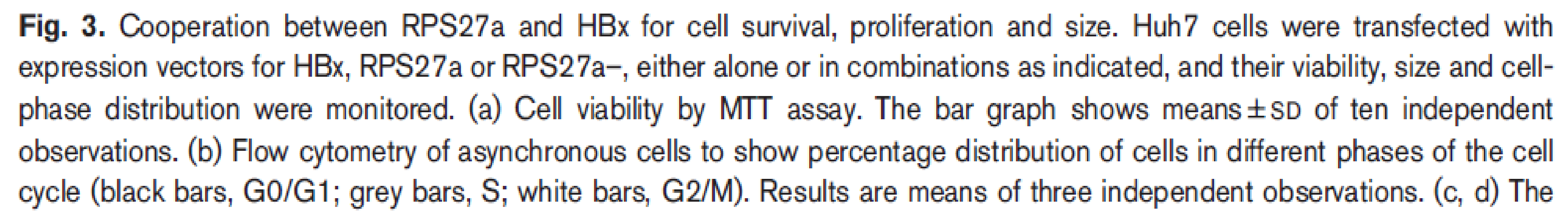
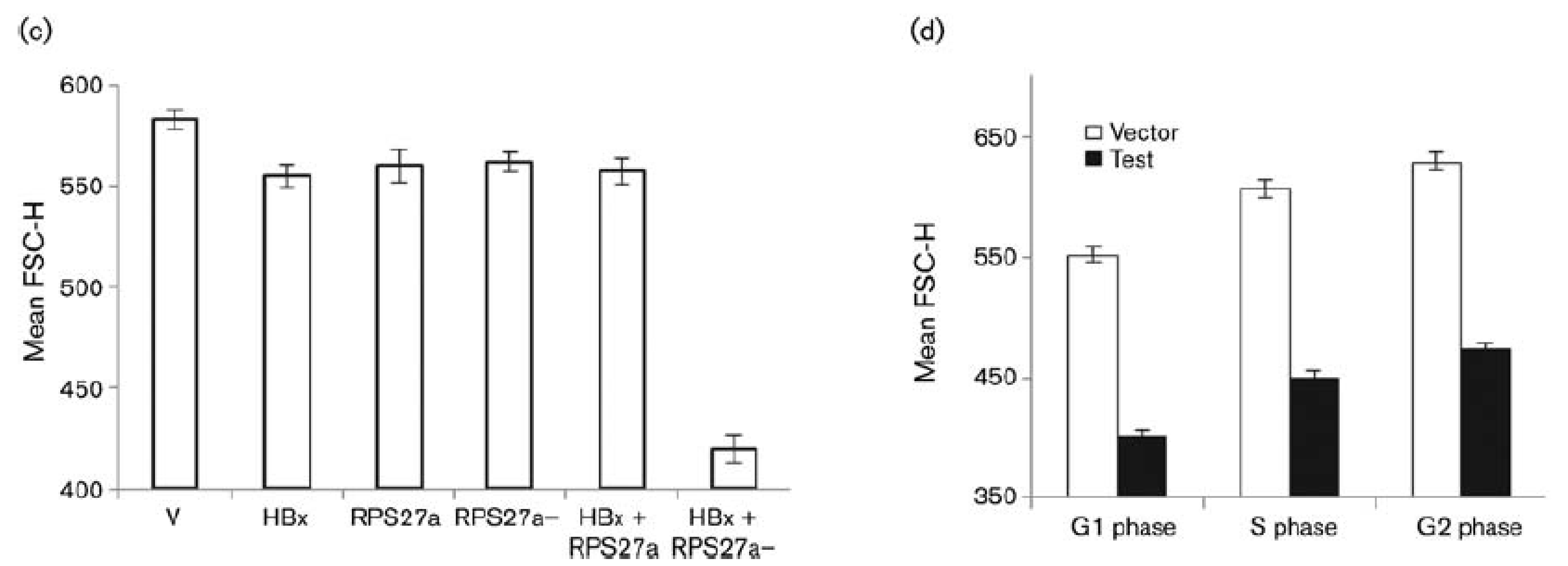


Fig. 3. Cooperation between RPS27a and HBx for cell survival, proliferation and size. Huh7 cells were transfected with expression vectors for HBx, RPS27a or RPS27a−, either alone or in combinations as indicated, and their viability, size and cell-phase distribution were monitored. (a) Cell viability by MTT assay. The bar graph shows means ± SD of ten independent observations. (b) Flow cytometry of asynchronous cells to show percentage distribution of cells in different phases of the cell cycle (black bars, G0/G1; grey bars, S; white bars, G2/M). Results are means of three independent observations. (c, d) The

Rps27a regulates the cell size checkpoint in the presence of HBx

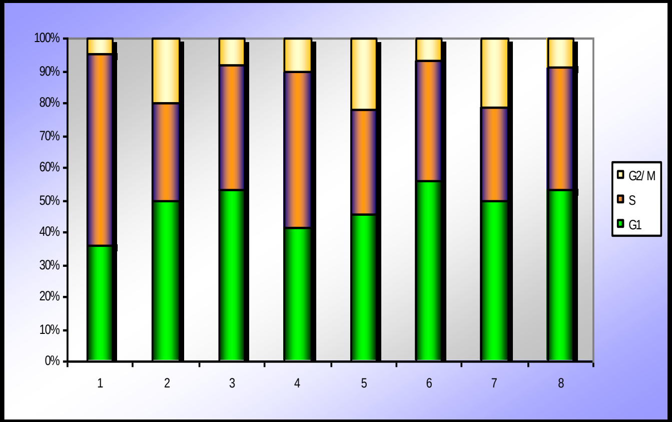


*$P<0.01$ **$P<0.001$, compared with controls.

Need for Rps27a in an HBx micro environment primarily for maintaining cell size, and thus regulating proliferation of cells

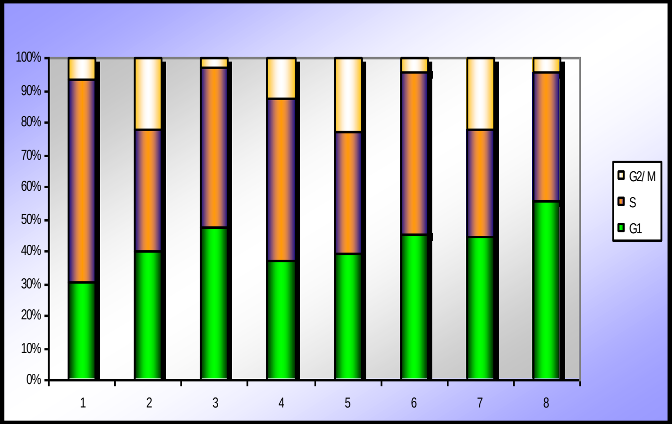
A

12hrs



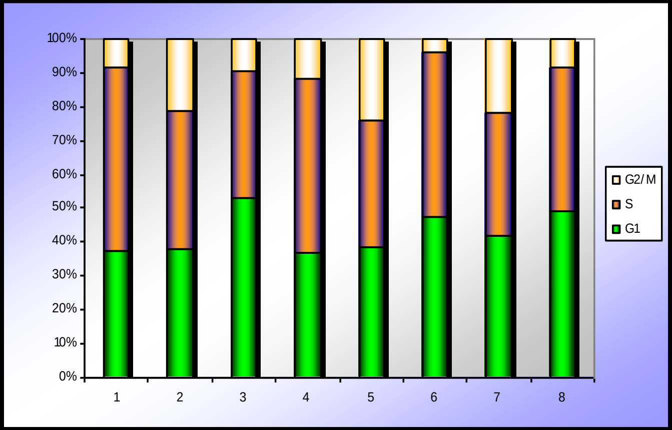
B

24hrs



C

42 hrs



Constructs

1. Rps27a
2. Antisense Rps27a
3. Rps27a + Antisense Rps27a
4. CEP
5. Antisense CEP
6. CEP + Antisense CEP
7. Rps27a + Antisense Rps27a +Antisense CEP
8. pSG5

Effect of Rps27a and CEP on cell cycle. Huh7 cells were transfected with Rps27a, CEP, anti-Rps27a and anti-CEP constructs at 60-70 % confluency and cells were serum syncronized and collected at various time points of 12hrs (A) , 24hrs (B) and 42 hrs (C). The percentages of cells at different phases of the cell cycle were analysed by FACS and values are shown on the left (Y-axis) bar diagram.

Report-Deletion Rps6 (40s RS)

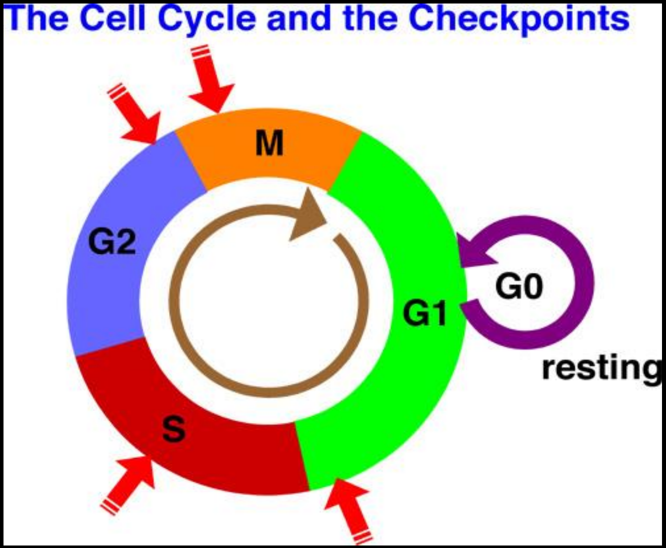
(Rps27a & CEP) ↓

Cell proliferation

G2/M phase arrest

Cyclin E (sherr et.al.,1999)

Cyclin B1
(Yu et.al., 2002)



Rps27a- early growth response gene G1 phase cell division

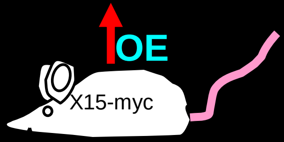
Induction of
Cyclin D-CDK4
Cyclin D-CDK2

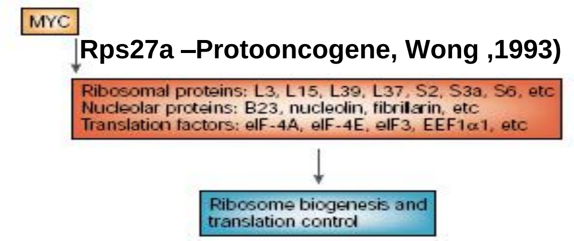
“S”PHASE ↑
DNA replication

(Rps27a & CEP) ↑

OE

OE





Rps27a –Protooncogene, Wong ,1993)

DeBenedetti *et al.,* 1990

In yeast, UB13 deletion-Slow growth of phenotype (1.6 hr-6.8hr) (Finley et.al., 1989)
Many small & Large Rib.Sub unit Protein ↑ variety of p.Tumor- Ex RPL36A. OE-Over Expression

Summary

- Rps27a, a ubiquitin precursor protein fused to the 80 amino acid carboxyl extension protein (CEP)

- The flow cytometry analysis reveled that Rps27a overexpressing cells accelerated cells to enter G1 phase to S phase with enhanced cell proliferation

- Immunohistochemical staining of Rps27a expression was moderately reduced from 3 months to 18 months old Transgenic liver tissues.

Overall, The sensitivity of over expressed Rps27a in HCC might be a general biomarker for tumor proliferation.

THANKS