The issue of cooperative mutations in cancer

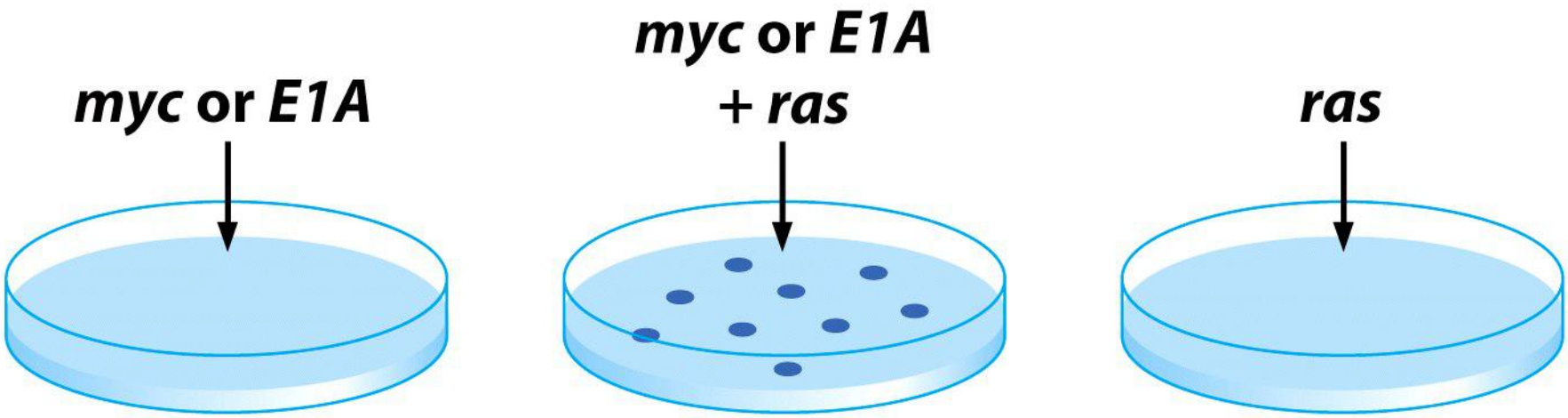
Eμ-Myc tumors
- Latency
- Mono-(oligo) clonality

→ Additional mutation?

More than one oncogene is needed to “transform” primary mouse fibroblasts

(Land and Weinberg, RAS and Myc cooperate in cellular transformation)

Tumorigenic conversion of primary MOUSE embryo fibroblasts requires at least two cooperating oncogenes.

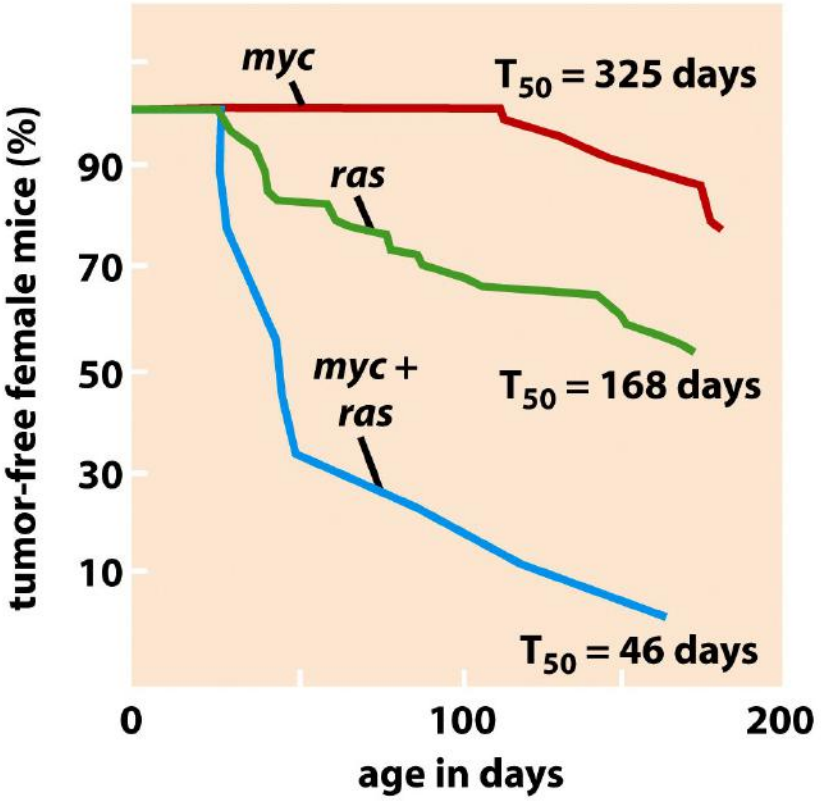


"(1) Transfection of embryo fibroblasts by a human ras oncogene does not convert them into tumour cells unless the fibroblasts are established and immortalized before transfection.
(2) The embryo fibroblasts become tumorigenic if a second oncogene such as a viral or cellular myc gene or the gene for the polyoma large-T antigen is introduced together with the ras gene."
Land H, Parada LF, Weinberg RA. Nature. 1983 Aug 18-24;304(5927):596-602.

Figure 11.23 *The Biology of Cancer* (© Garland Science 2007)

Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo.

Sinn E, Muller W, Pattengale P, Tepler I,
Wallace R, Leder P.
Cell. 1987 May 22;49(4):465-75.



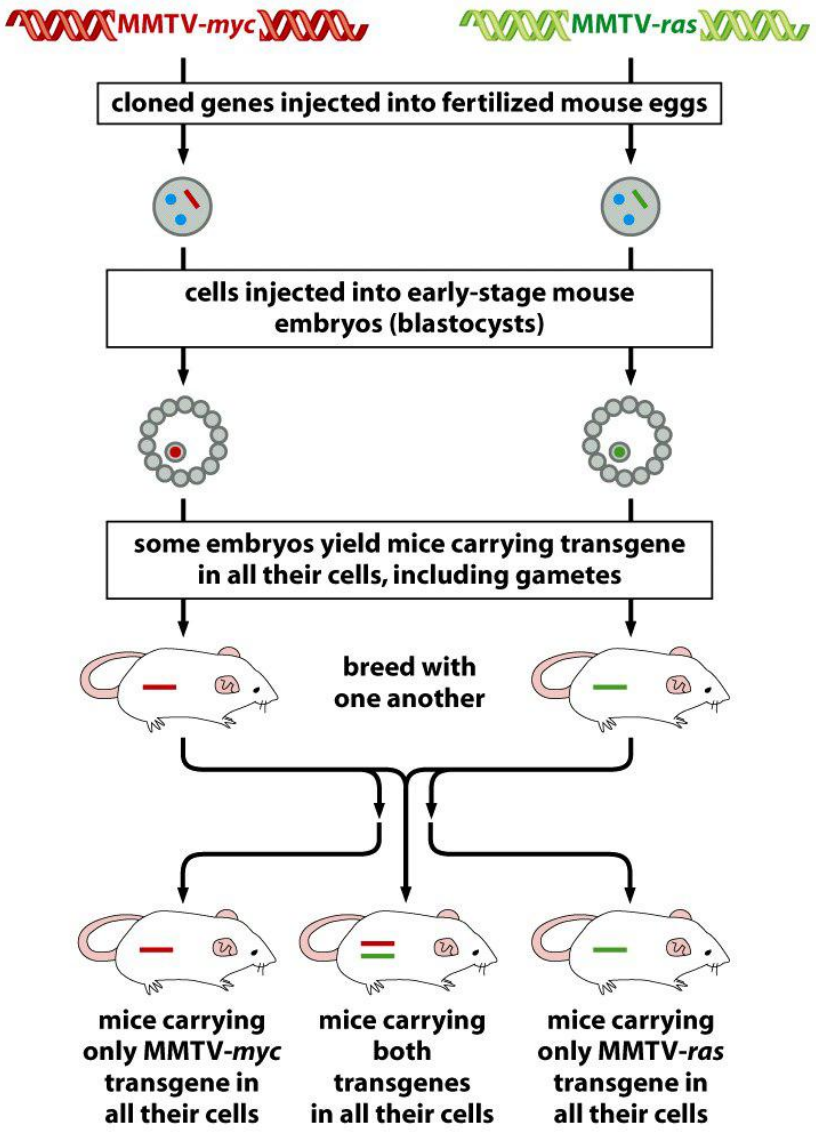
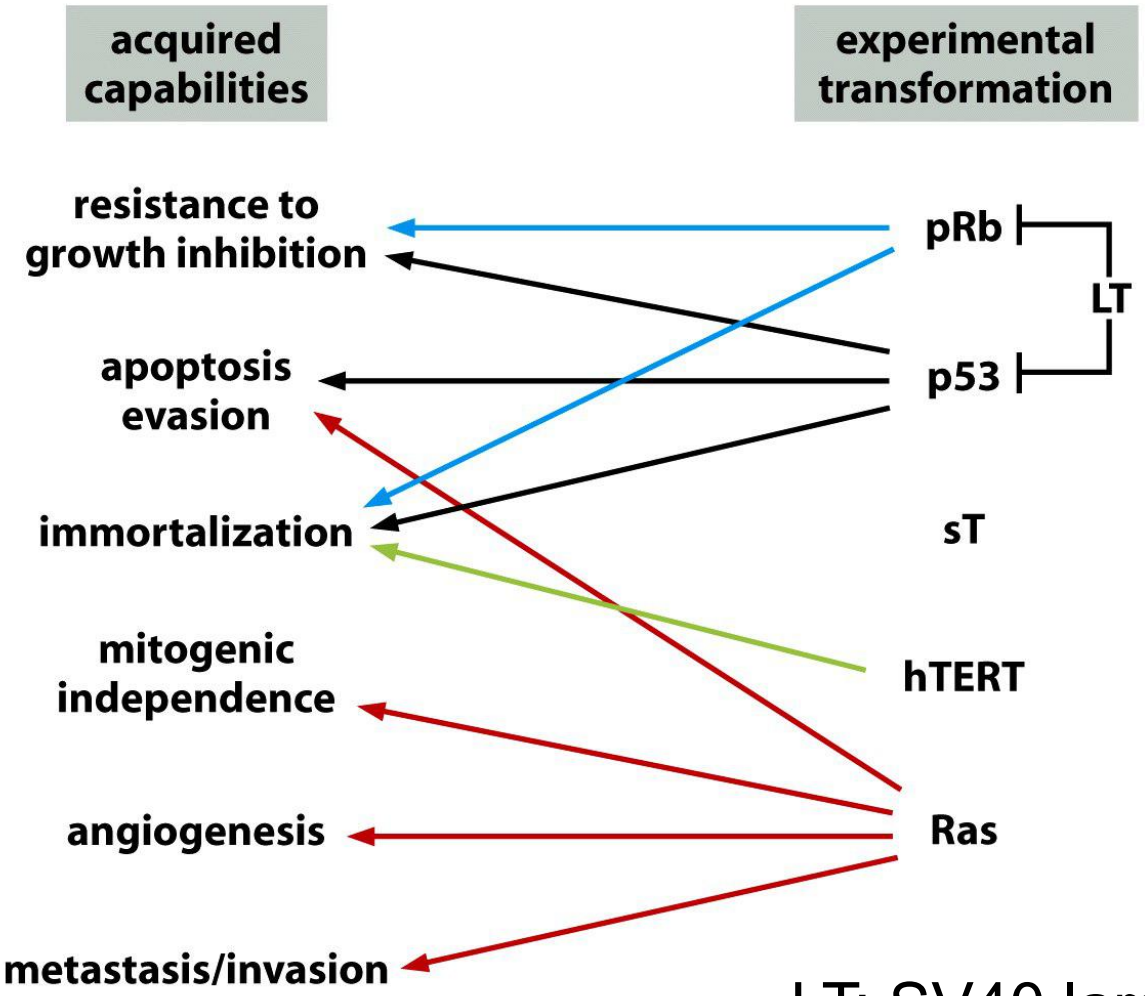


Table 11.2 Physiologic mechanisms of oncogene collaboration[a]

Oncogene pair	Cell type	Mechanisms of action
ras + SV40 *large T*	rat Schwann cells	*ras*: proliferation + proliferation arrest *large T*: prevents proliferation arrest and reduces mitogen requirement
ras + *E1A*	mouse embryo fibroblasts	*ras*: proliferation and senescence *E1A*: prevents senescence
erbB + *erbA*	chicken erythroblasts	*erbB*: induces GF-independent proliferation *erbA*: blocks differentiation
TGF-α + *myc*	mouse mammary epithelial cells	*TGF-α*: induces proliferation and blocks apoptosis *myc*: induces proliferation and apoptosis
v-*sea* + v-*ski*	avian erythroblasts	v-*sea*: induces proliferation v-*ski*: blocks differentiation
bcl-2 + *myc*	rat fibroblasts	*bcl-2*: blocks apoptosis *myc*: induces proliferation and apoptosis
ras + *myc*	rat fibroblasts	*ras*: induces anchorage independence *myc*: induces immortalization
raf + *myc*	chicken macrophages	*raf*: induces growth factor secretion *myc*: stimulates proliferation
src + *myc*	rat adrenocortical cells	*src*: induces anchorage and serum independence *myc*: prolongs proliferation

[a]In each pair, the first oncogene encodes a cytoplasmic oncoprotein while the second oncogene encodes a nuclear oncoprotein.

More than two events (mutations) are required to achieve full transformation **of human cells**



LT: SV40 large T antigen
ST: SV40 small T antigen

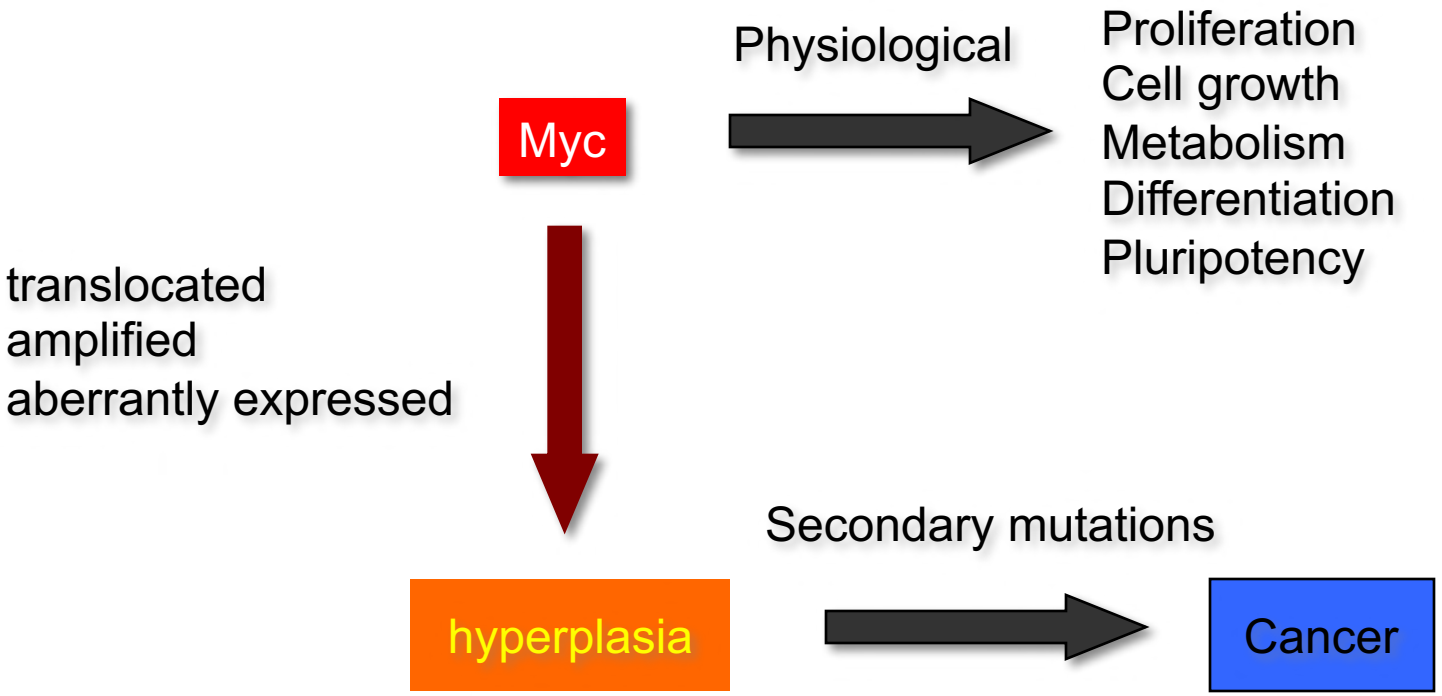
Figure 11.43 *The Biology of Cancer* (© Garland Science 2007)

Pathways controlling cellular transformation in human cells

pathway	Ras	pRb	p53	telomeres	PP2A
genes/agents used to deregulate pathway	*ras*	*CDK4 + D1* SV40 *LT* HPV *E7*	DN *p53* SV40 *LT* HPV *E6*	*hTERT* *myc + SV40 LT*	SV40 *sT* sometimes: *myc* *Akt/PKB+Rac1* *PI3K* *B56* shRNA

Figure 11.25 *The Biology of Cancer* (© Garland Science 2007)

The c-Myc (proto)-oncogene



Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl-2*

Andreas Strasser, Alan W. Harris, Mary L. Bath & Suzanne Cory

The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

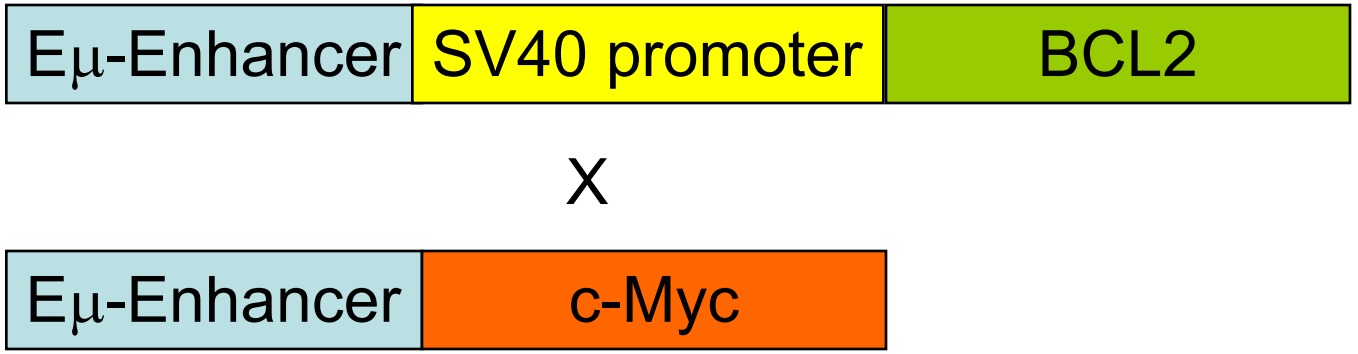
THE putative oncogene *bcl*-2 is juxtaposed to the immunoglobulin heavy chain (*Igh*) locus[1–3] by the t(14;18) chromosomal translocation typical of human follicular B-cell lymphomas[4]. The *bcl*-2

Bcl2:
- enhances viability of cultured lymphoid cells
- confers resistance to stress
- but no tumors in transgenic mice!

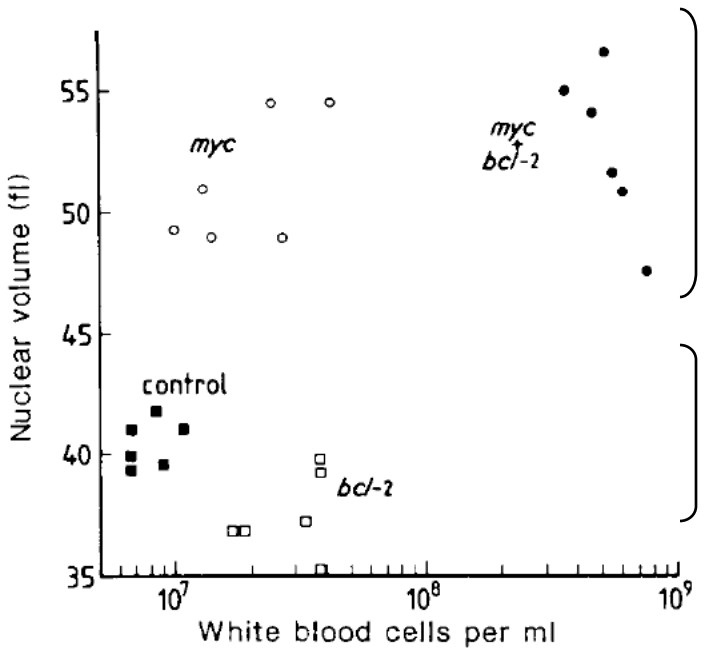
Ref 15-17 in Strasser et al.

Is BCL2 a co-operator?

Exp: breed BCL2-Tg mice with Myc-Tg



Cooperation at the pre-tumoral stage



Large nuclei: proliferating leukocytes

Small nuclei: non-proliferating leukocytes

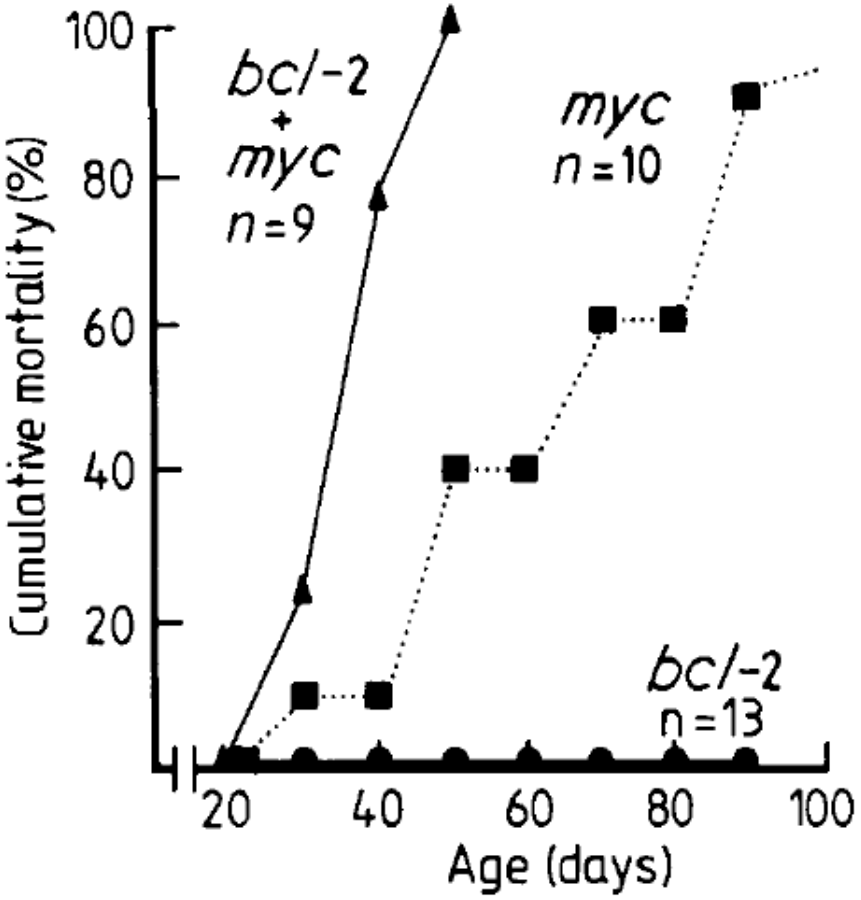
FIG. 1 Distinctive blood profiles induced by *bcl*-2 and *myc* transgenes. Leukocytes from 3-week-old mice were counted and sized in a ZM Coulter counter and channelizer as nuclei which were obtained by lysing diluted blood with Zaponin (Coulter) as described[21].

Results

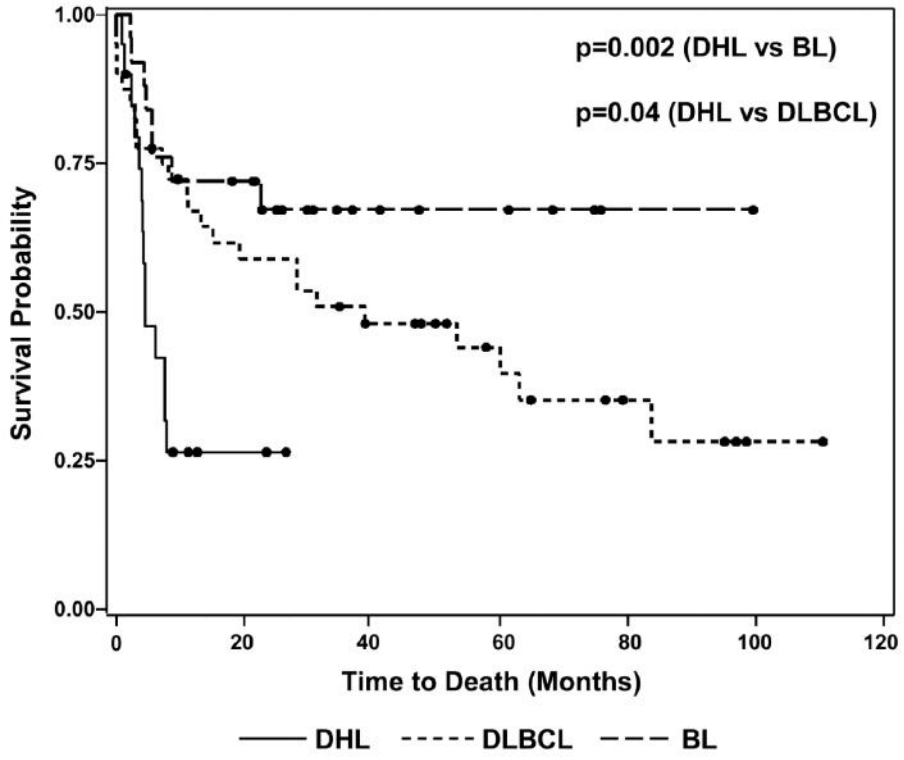
#	leuk n*	proliferation
Wild type		no
Eμ-Myc	+	yes
Eμ-BCL2	+	no
Eμ-Myc/BCL2	++++	yes

Hypothesis	
Eμ-Myc	proliferation
Eμ-BCL2	survival(?)

Cooperation in tumorigenesis (monitoring lifespan)



Clinical evidence of the Myc BCL2 co-operation



Double Hit lymphoma (DHL)
B-cell lymphomas with concurrent
IGH-BCL2 and *MYC* rearrangements

DLBCL: Diffuse Large B-cell lymphoma
BL: Burkitt's Lymphoma

Figure 1.
Kaplan-Meier Overall Survival Distributions for Double-Hit Lymphoma, Burkitt Lymphoma and IPI-Matched Diffuse Large B-cell Lymphoma Patients
Black circles denote patients who were alive at the time of last follow-up.
DHL: double-hit lymphoma, BL: Burkitt lymphoma, DLBCL: diffuse large B-cell lymphoma.

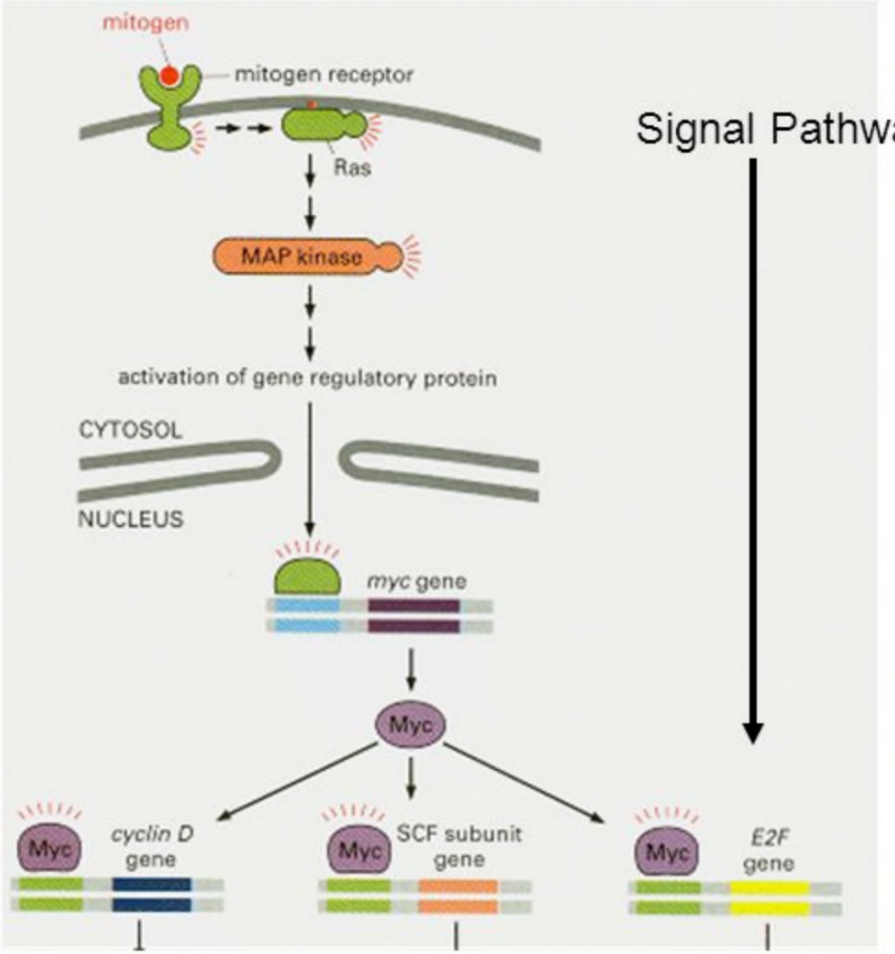
B-cell Lymphomas with Concurrent IGH-BCL2 and MYC Rearrangements Are Aggressive Neoplasms with Clinical and Pathologic Features Distinct from Burkitt Lymphoma and Diffuse Large B-cell Lymphoma
Matija Snuderl, M.D.1,5,, Am J Surg Pathol. 2010 March ; 34(3): 327–340. doi:10.1097*

Why do c-Myc and BCL2 cooperate?

“Induction of apoptosis in fibroblasts by c-myc protein”

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC.

Cell. 1992 Apr 3;69(1):119-28.



The Proteins From These Genes Stimulate Entry Into S phase

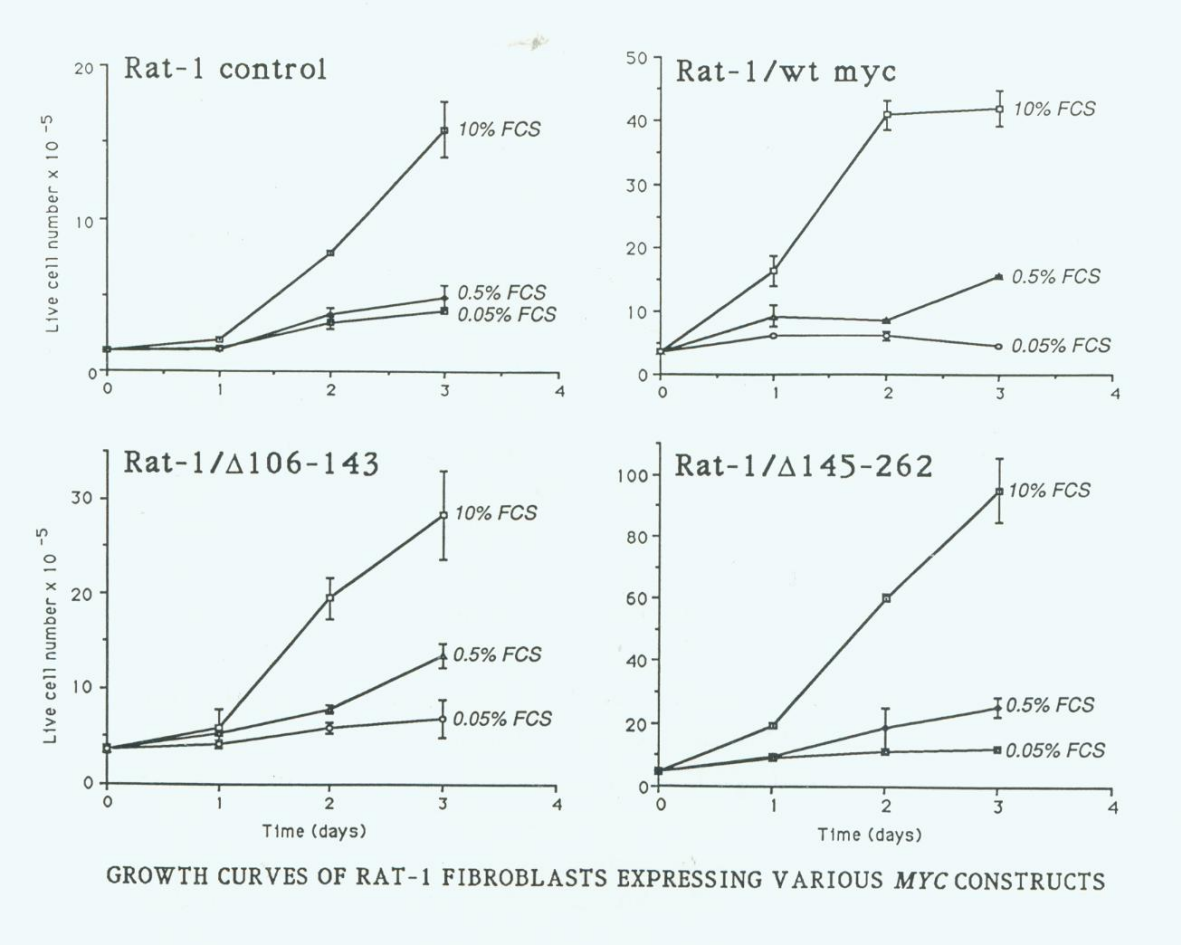
Background
-Myc is expressed at Go-G1 transition and during cell cycle

-Myc is repressed when cells undergo cell cycle arrest

Q1: Will Myc confer proliferative advantage to cell growth?

Q2: Will Myc prevent growth arrest in low serum?

Fig.1: rat-1 cells w/ MSCV-Myc (MSCV= viral constitutive promoter to escape self-inhibition)
Myc-Del (106-143)=non-transforming/not tumorigenic
Myc-Del(145-262)=inactive



GROWTH CURVES OF RAT-1 FIBROBLASTS EXPRESSING VARIOUS *MYC* CONSTRUCTS

Q1: Will <u>over-expression</u> of myc confer proliferative or cell growth advantage?

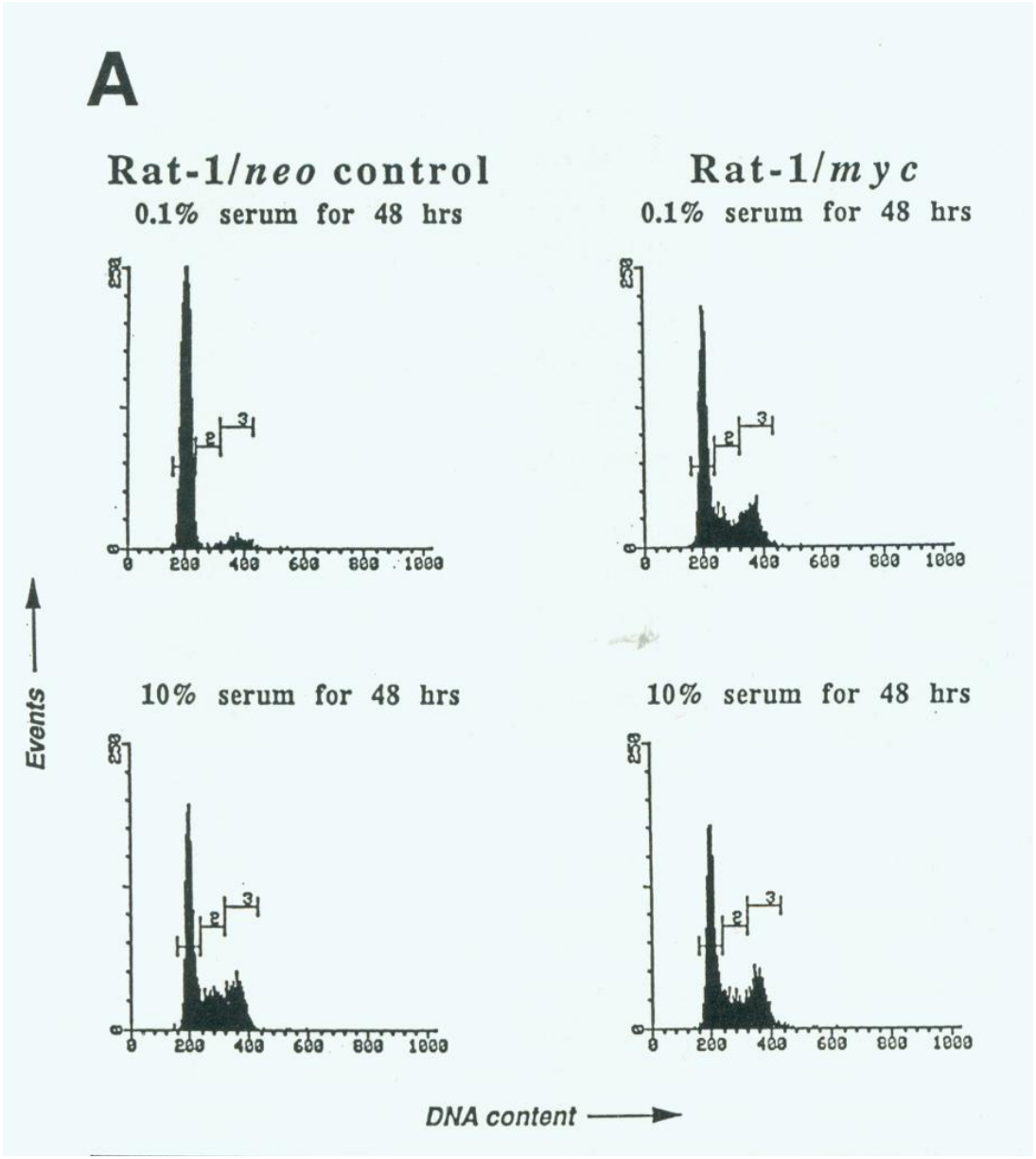
Exp: cell growth in 10%FCS (Serum)

Q2: Will <u>over-expression</u> of myc prevent growth arrest in low serum?

Exp:cell growth in low serum

Result: Myc does not alter cell growth!

Q: Will myc prevent cell cycle arrest in low serum?
NB: there is a difference between cell growth and cell cycle

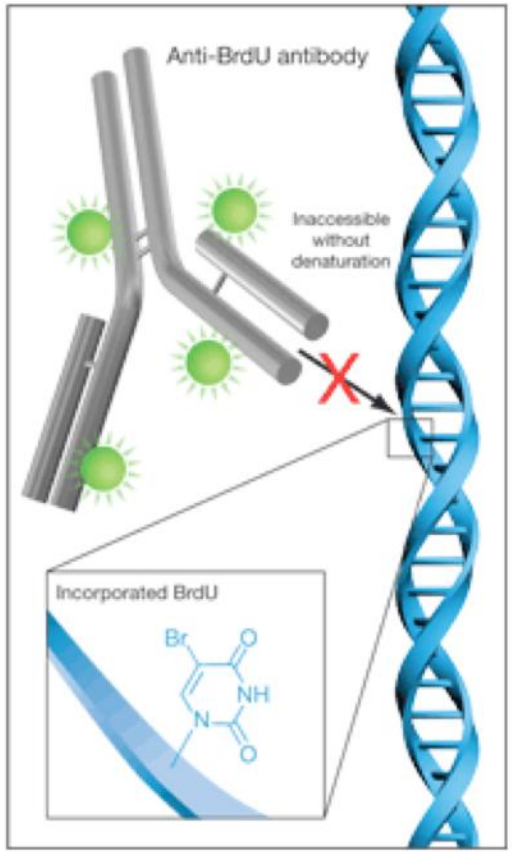


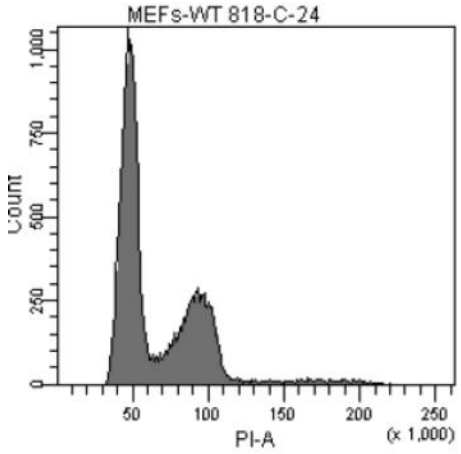
PI (Propidium Iodide staining): Fluorescent dye, stains DNA. Cells are analysed by FACS. Steady-state description of the cell cycle

From cell cycle analysis:

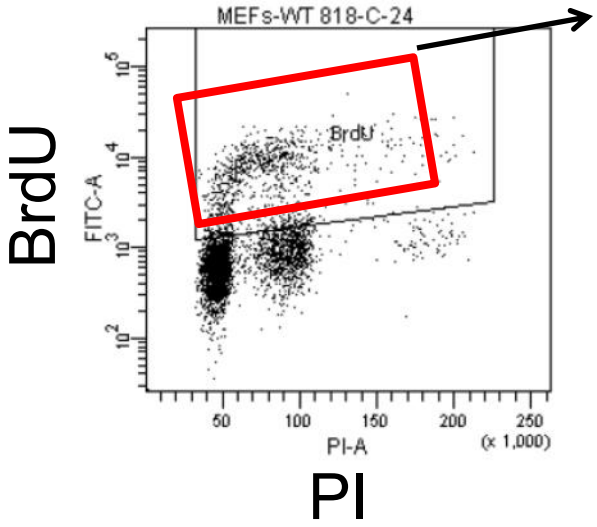
1. Myc prevents arrest in Go
2. Yet, are all the cells in S and G2/M cycling?
3. Or are they arrested at cell cycle phases different from Go?

BrdU incorporation: nucleotide (analogue of T), incorporated in DNA during S-phase (BrdU^{+}cells stained with an Anti-BrdU antibody)
(dynamic view of the cell cycle)





BrdU positive cells



BrdU

PI

Cell cycle by BrDU incorporation:

B

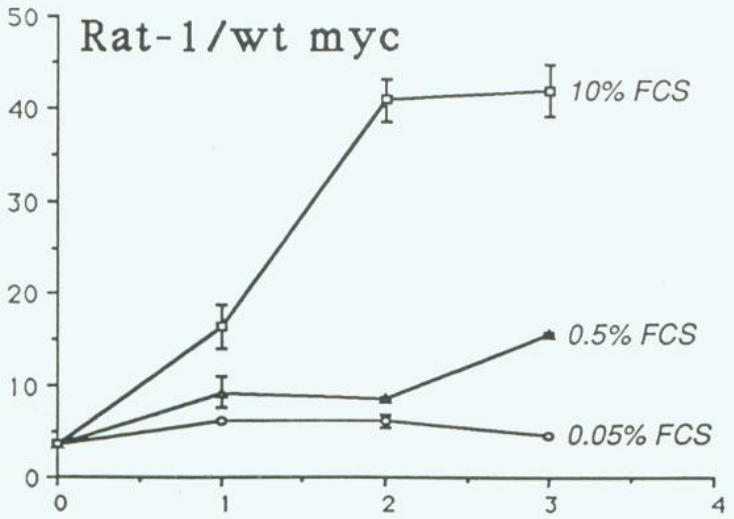
Cell Line	Treatment (48 hrs)	%age in S-phase/hr
Rat-1/*neo* control	10% serum	50
	0.1% serum	3
Rat-1/*myc*	10% serum	41
	0.1% serum	45

RESULTS: Myc keeps cells in cycle even in serum deprived cultures

PARADOX: why there is no increase in cell numbers?

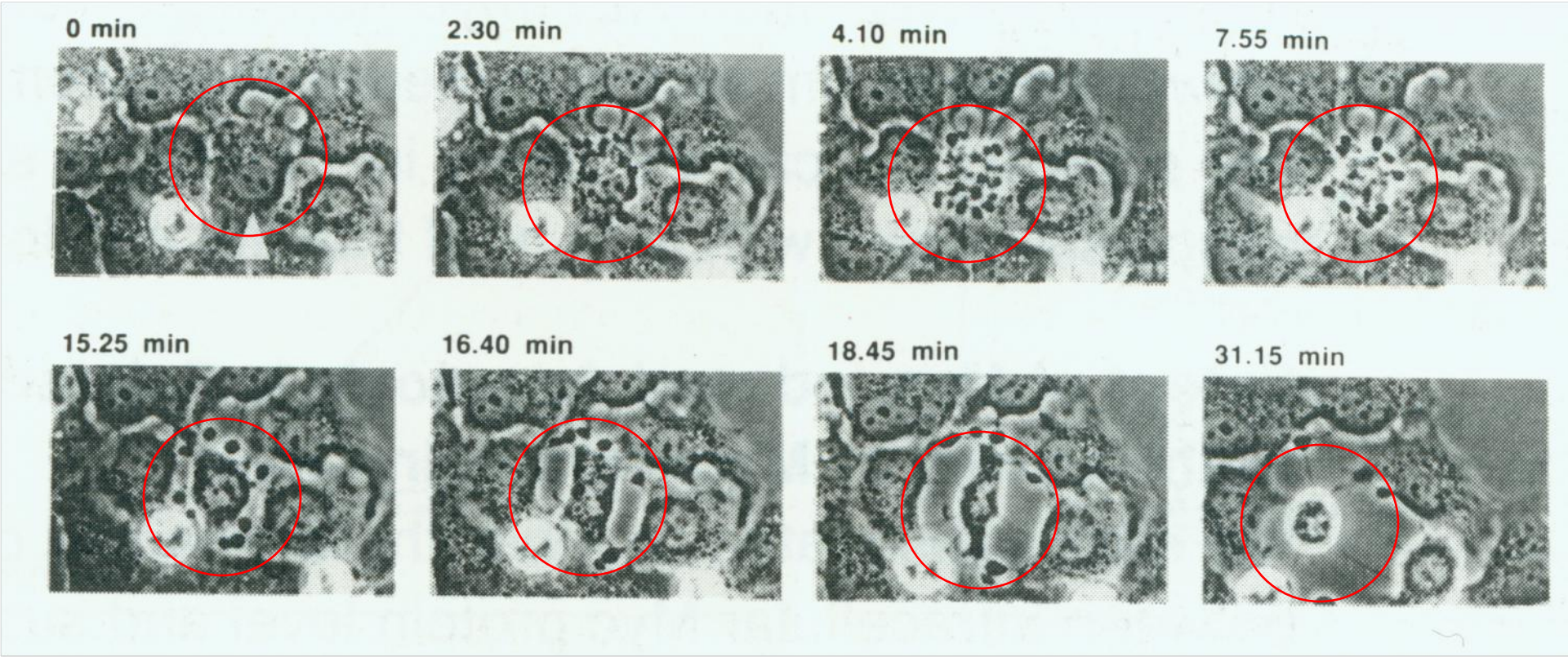
B

Cell Line	Treatment (48 hrs)	%age in S-phase/hr
Rat-1/*neo* control	10% serum	50
	0.1% serum	3
Rat-1/*myc*	10% serum	41
	0.1% serum	45

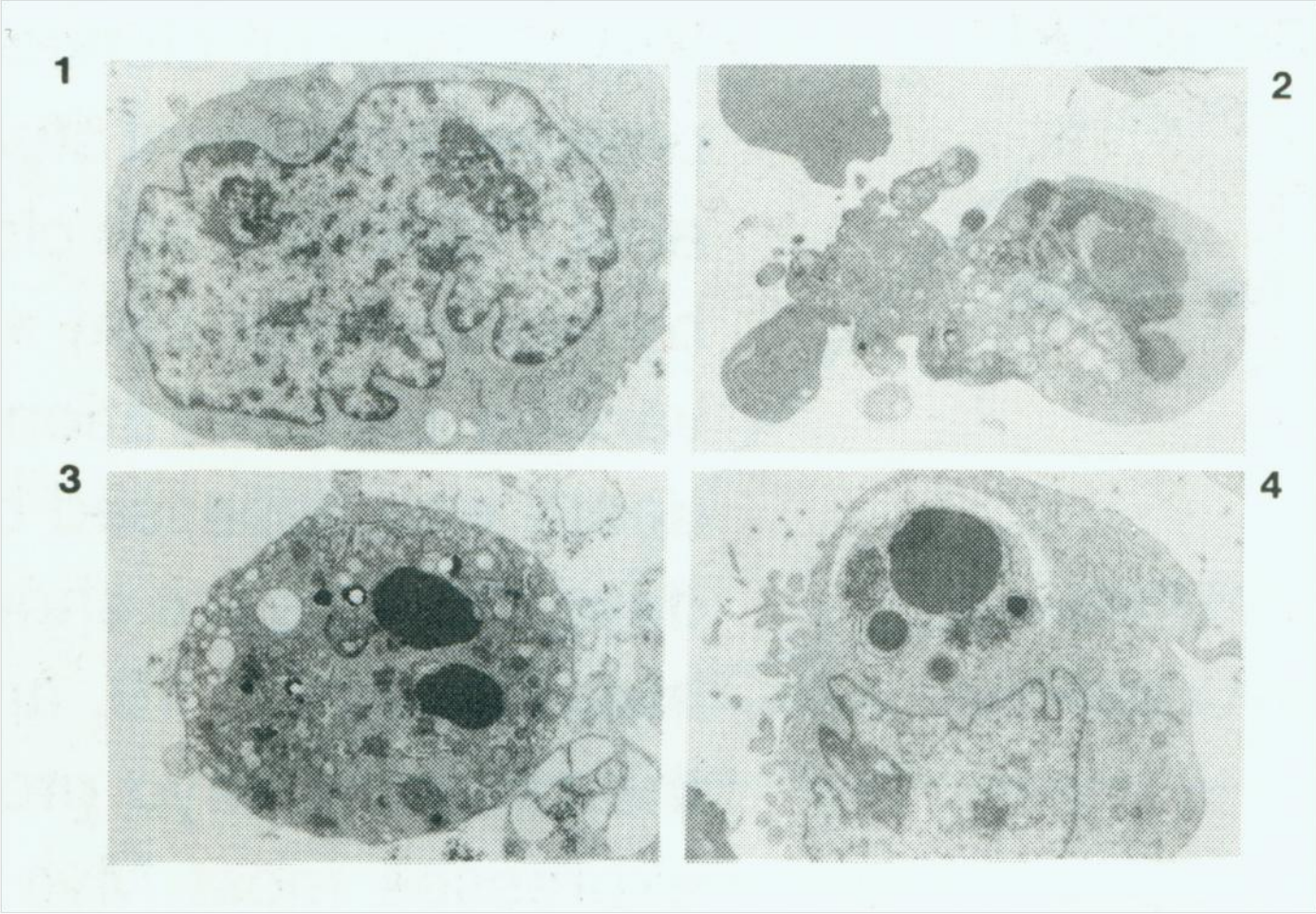


Hyp: is myc dependent proliferation balanced by increased cell death?

Fig.3: video-microscopy, Myc-Rat-1 cells shifted in low serum

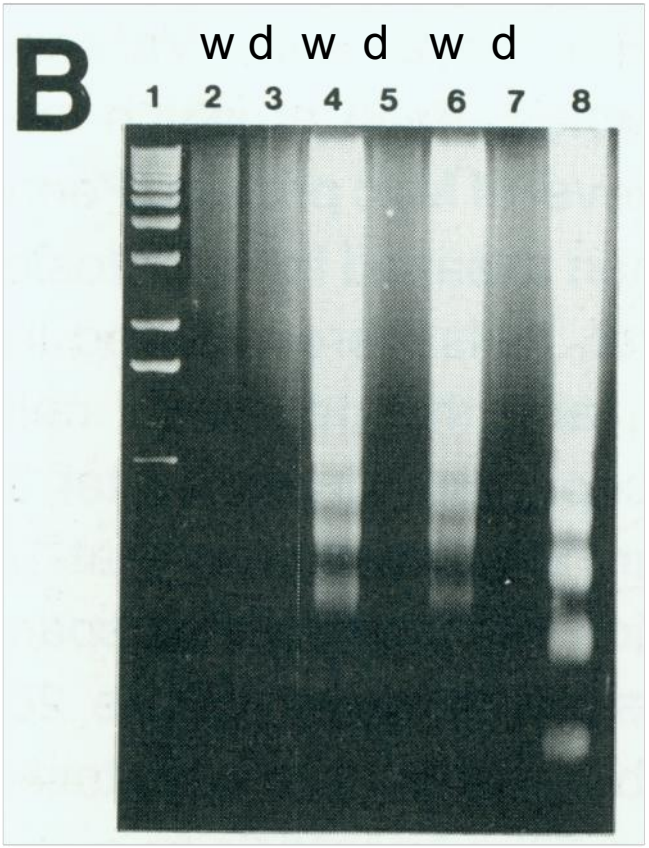


EM indicates nuclear fragmentation and cytoplasmic vescicles



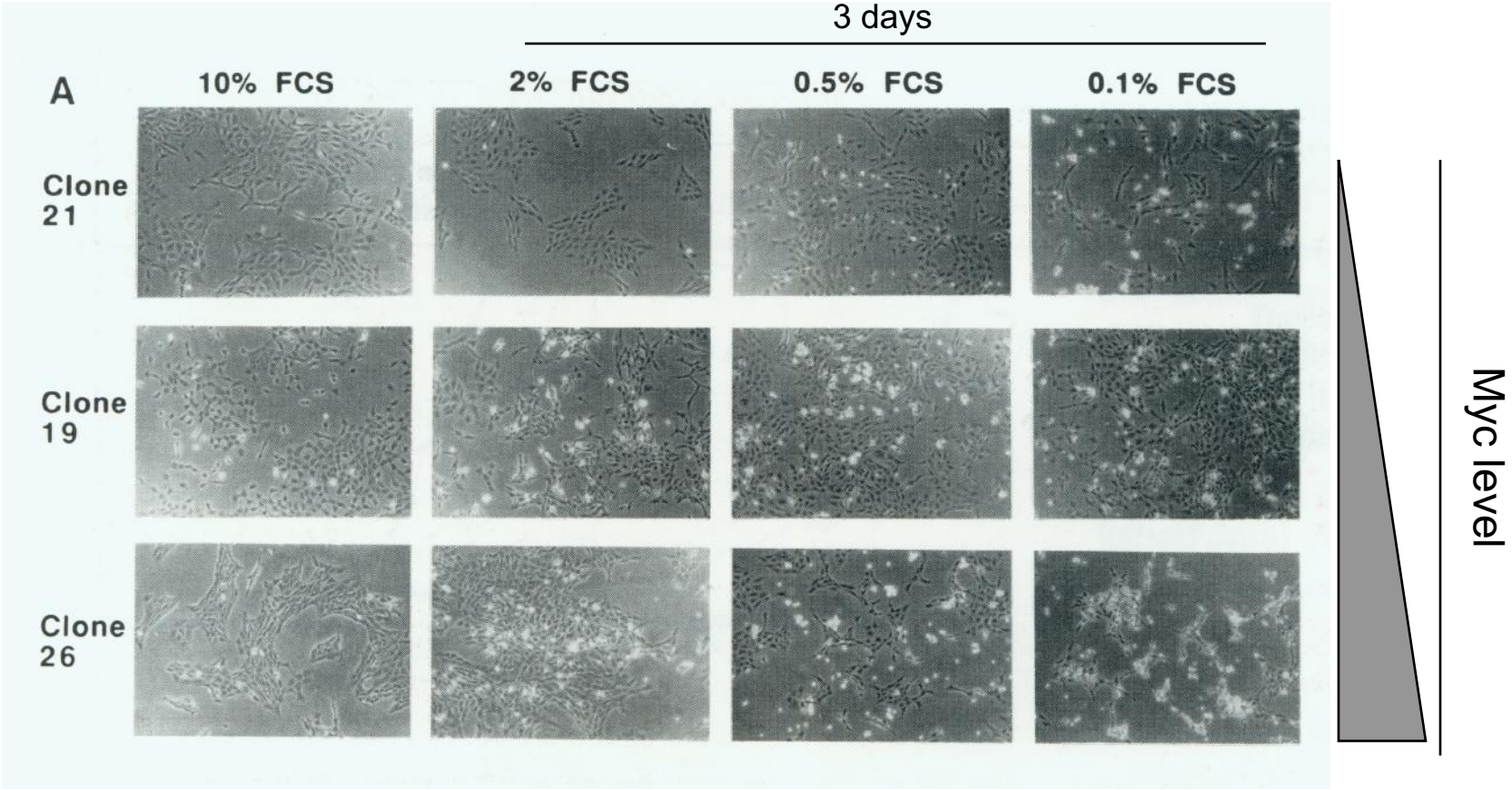
Genomic DNA fragmentation (apoptosis)

hours in low serum: 0 30 40



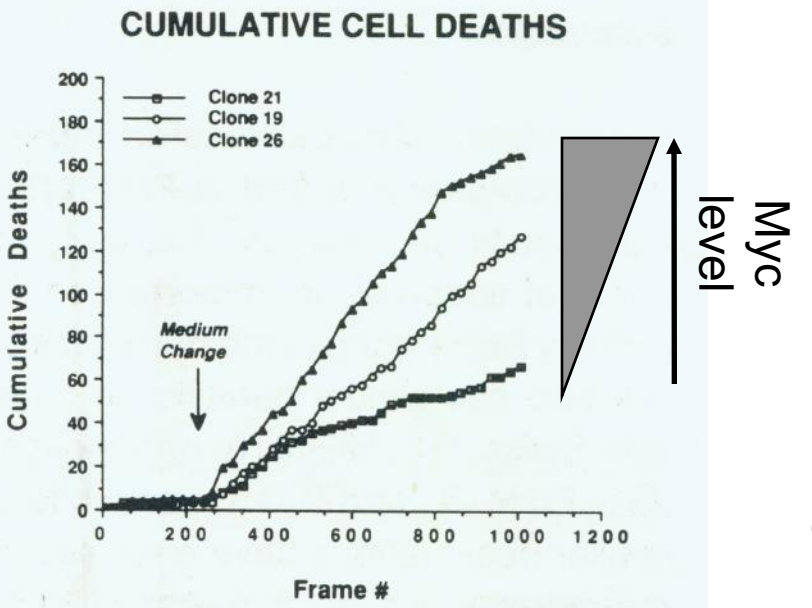
w:wild-type Myc
d:Del106-143 Myc(inactive)

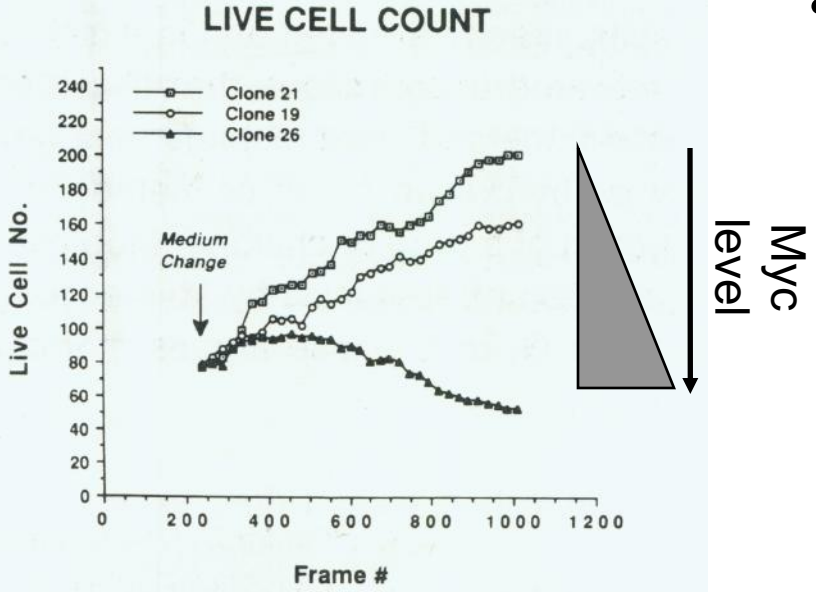
Fig 6.: Apoptosis depends on Myc levels and % of Serum (survival signals)



NOTE: apoptosis depends on Myc level and strength of survival signals (FCS%)

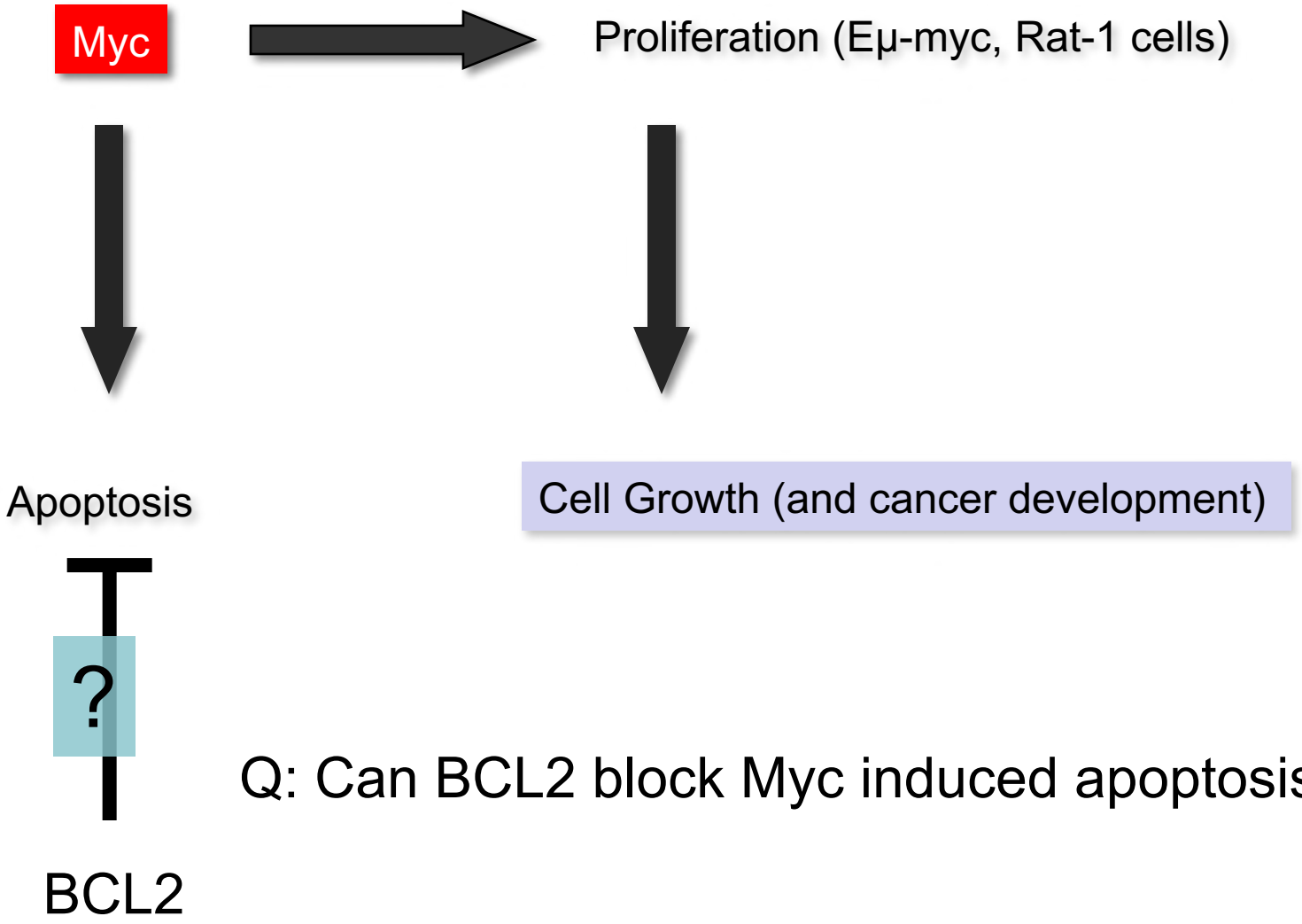
B





Quantitative view:
higher Myc level associates
w/ high apoptosis
and low cell growth

Conclusions & perspectives



Q: Can BCL2 block Myc induced apoptosis?

Can BCL2 block Myc-induced apoptosis?

LETTERS TO NATURE

Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2

Reid P. Bissonnette, Fernando Echeverri, Artin Mahboubi & Douglas R. Green

Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 11149 North Torrey Pines Road, La Jolla, California 92037, USA

The experimental system

Conditional expression of Myc (heat shock promoter)
And constitutive expression of BCL2 in cell lines
(clones derived by stable transfection)

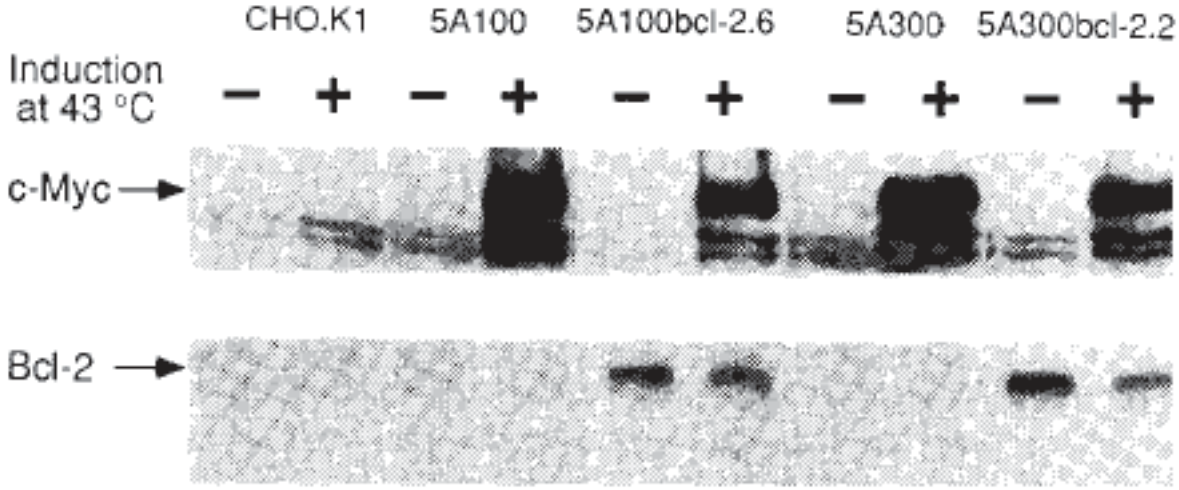


FIG. 1 Western blot analyses of c-Myc and Bcl-2 expression in parental and pSFFV bcl-2nl transfected 5AHS*myc* cells before (−) and after (+) induction of c-*myc* expression by heat shock.

NB: conditional expression prevents cell adaptation

BCL2 improves short-term viability following Myc induction

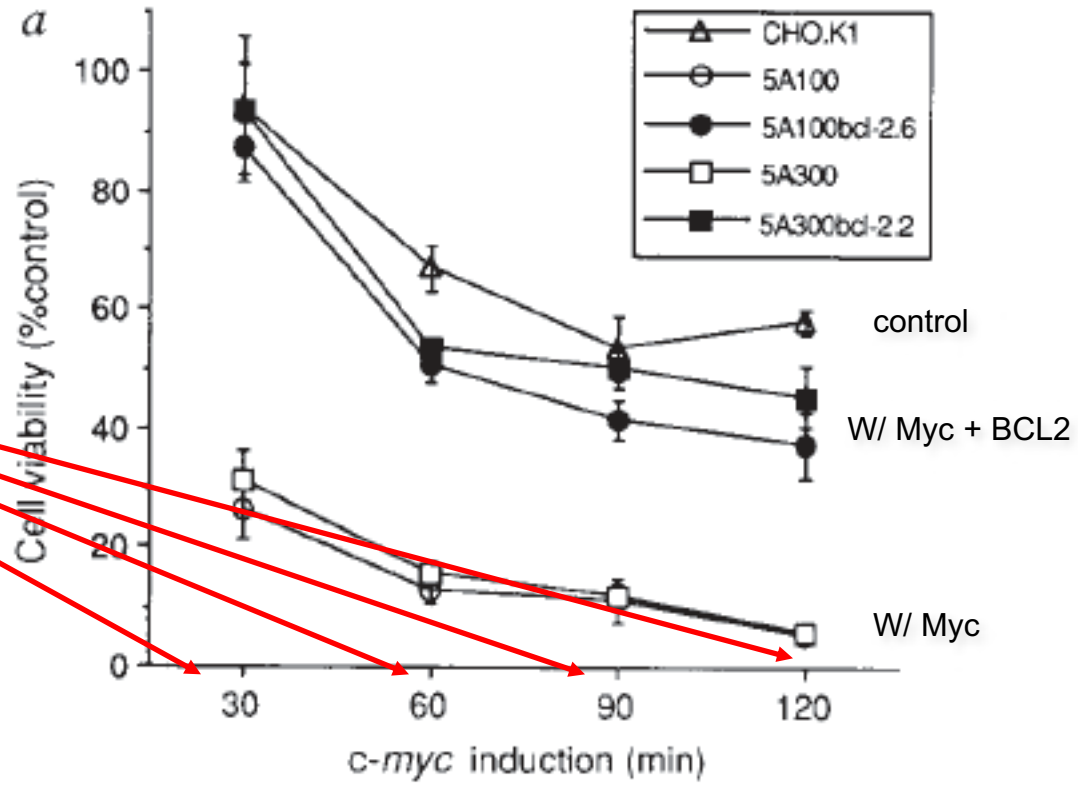
Experimental design

CHO cells

Heat shock (different times)
(Myc ON)

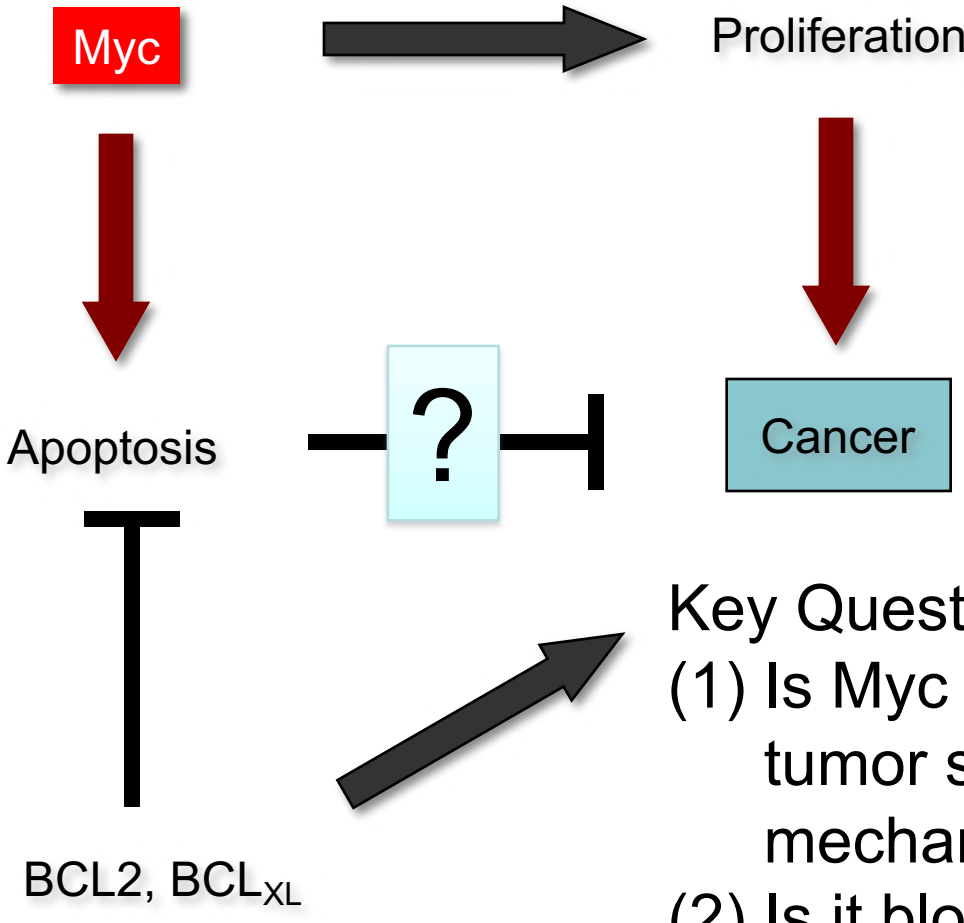
24h at 37C

Live cell count



METHODS. The effects of c-*myc* induction by heat shock were determined as follows. On the previous day, the cells were trypsinized, seeded into either 25-cm^2 flasks (1×10^6) or 24-well plates (1×10^5). The cells were heat shocked to induce c-Myc expression by immersion in a 43 °C water bath for the times indicated on the *x*-axis. After heat shock, the media was changed and the cells incubated at 37 °C. Cell viability was determined 24 h later by trypan-blue exclusion. For the clonogenicity assay, the cells were

Based on the experimental evidences we can draw the following scheme:



Key Questions:

(1) Is Myc induced apoptosis a tumor suppressive mechanism?

(2) Is it blocked by BCL2 (or Bcl2 like proteins)

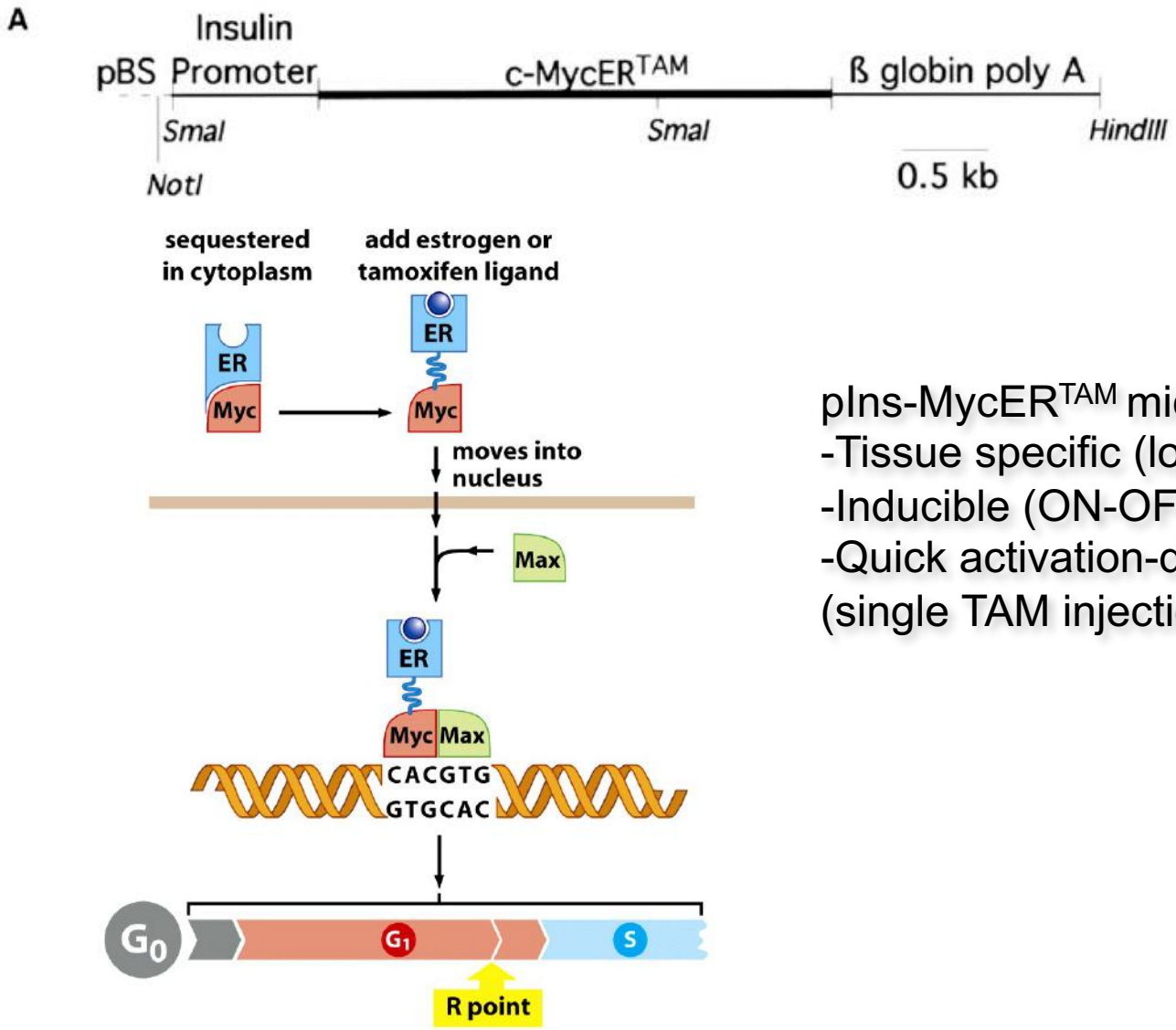
Cell, Vol. 109, 321–334, May 3, 2002, Copyright ©2002 by Cell Press

Suppression of Myc-Induced Apoptosis in β Cells Exposes Multiple Oncogenic Properties of Myc and Triggers Carcinogenic Progression

Question: Will the blocking of apoptosis promote Myc dependent tumorigenesis?

We need an *in vivo* system to study Myc induced apoptosis

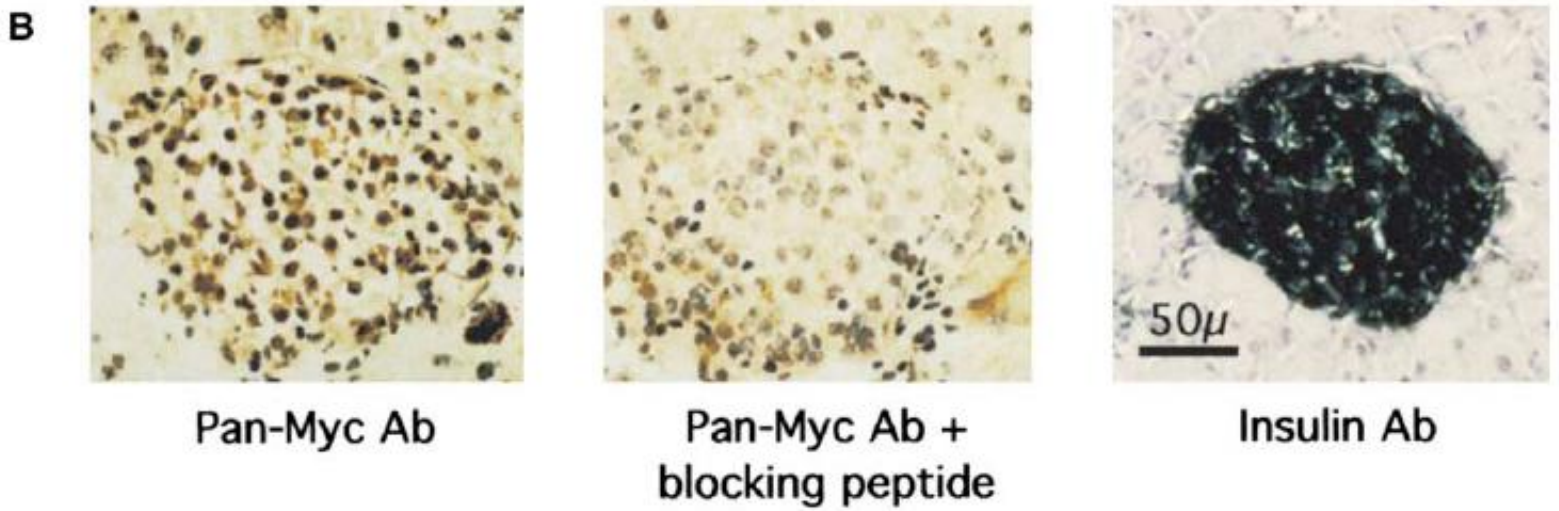
The experimental system:



pIns-MycER^{TAM} mice:
-Tissue specific (low proliferating tissue)
-Inducible (ON-OFF)
-Quick activation-deactivation
(single TAM injection: 24h Myc activation)

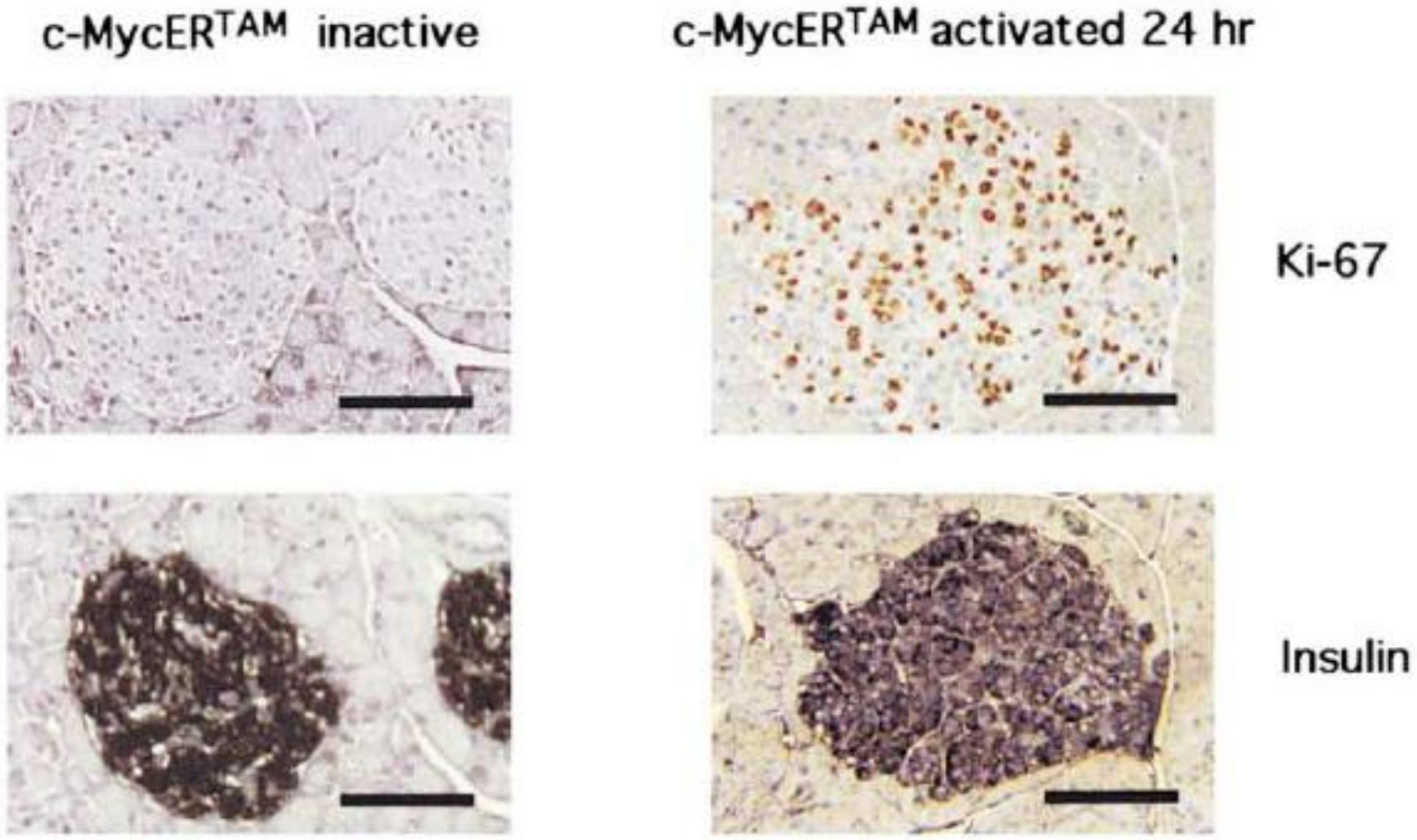
Figure 8.29 *The Biology of Cancer* (© Garland Science 2007)

MycER is expressed in every β-cell



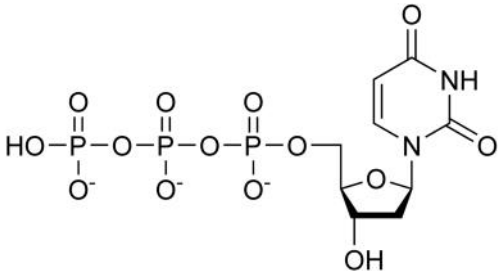
…at *near* physiological levels

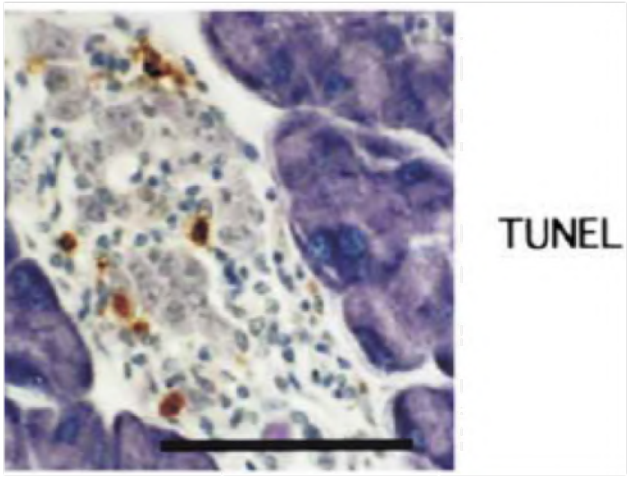
Myc activation is sufficient to induce cellular proliferation



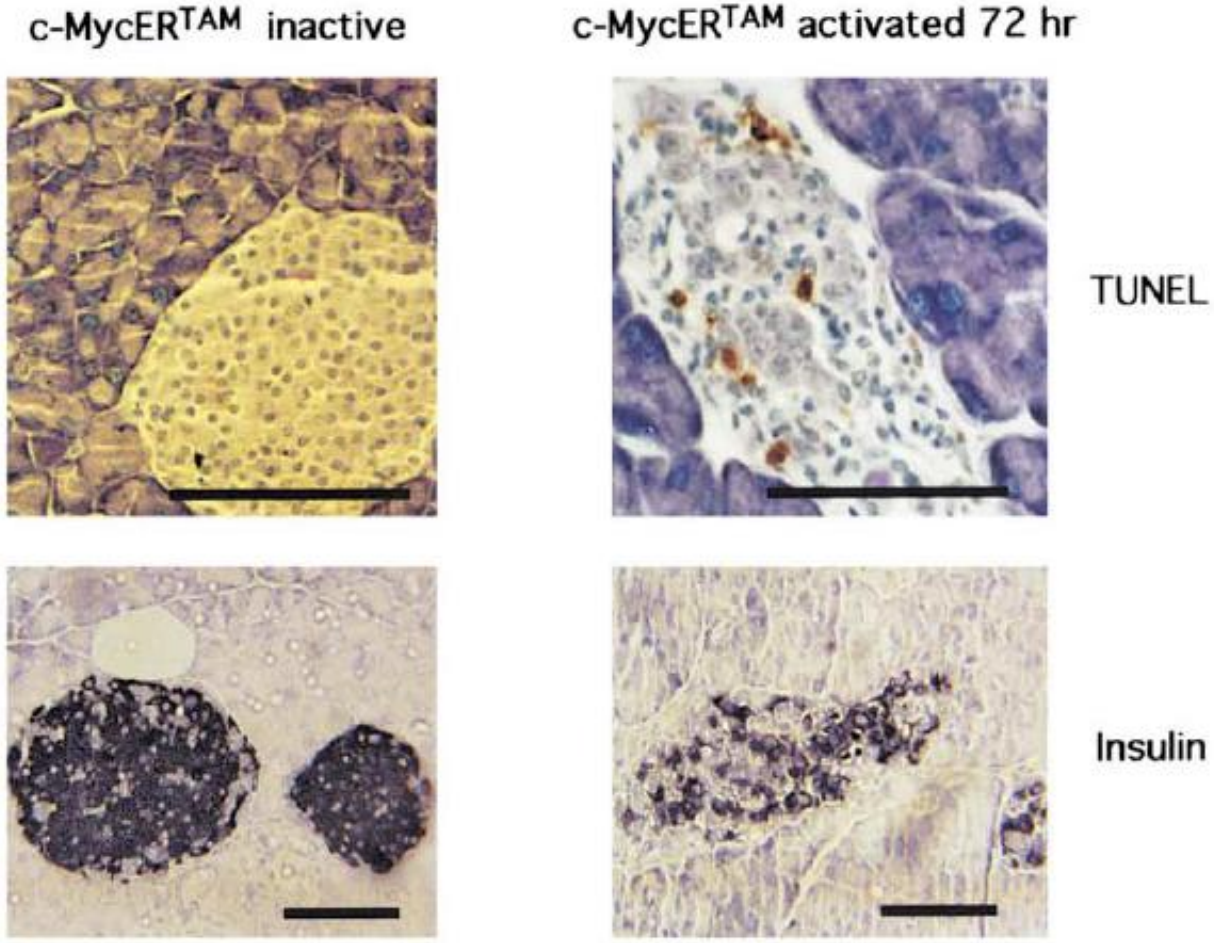
How we can detect apoptosis in vivo?
Tunel assay

TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

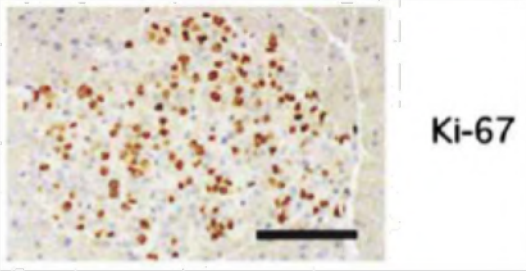


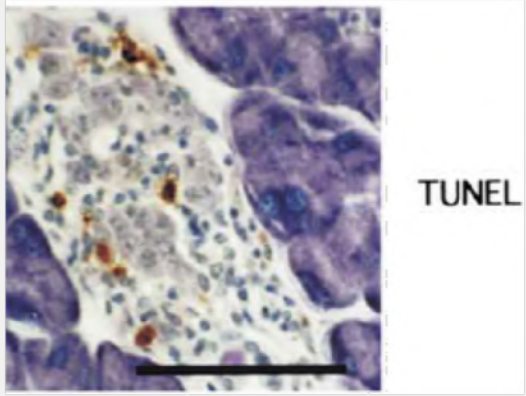


1. **Prolonged** Myc activation results in apoptotic response (4-7% positive cells)
2. Lower insulin following Myc activation suggests de-differentiation



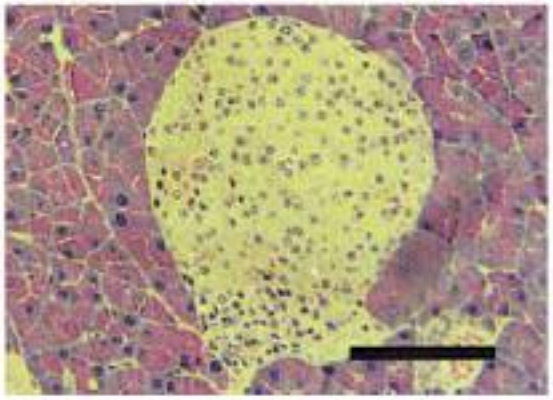
Myc proliferation (70% cells in islet) vs Myc apoptosis (4-7% cells in islet): Apoptosis wins!



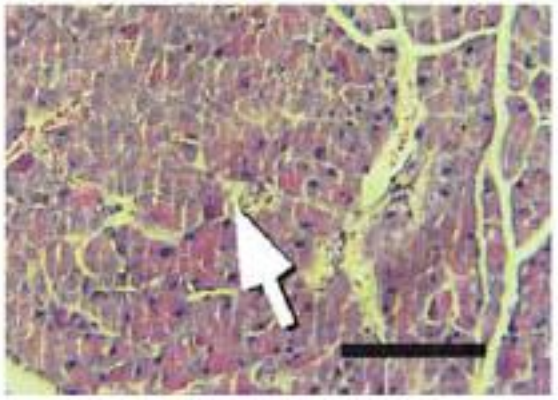


Involution of islet following 6 days of Myc activation

c-MycER^{TAM} inactive



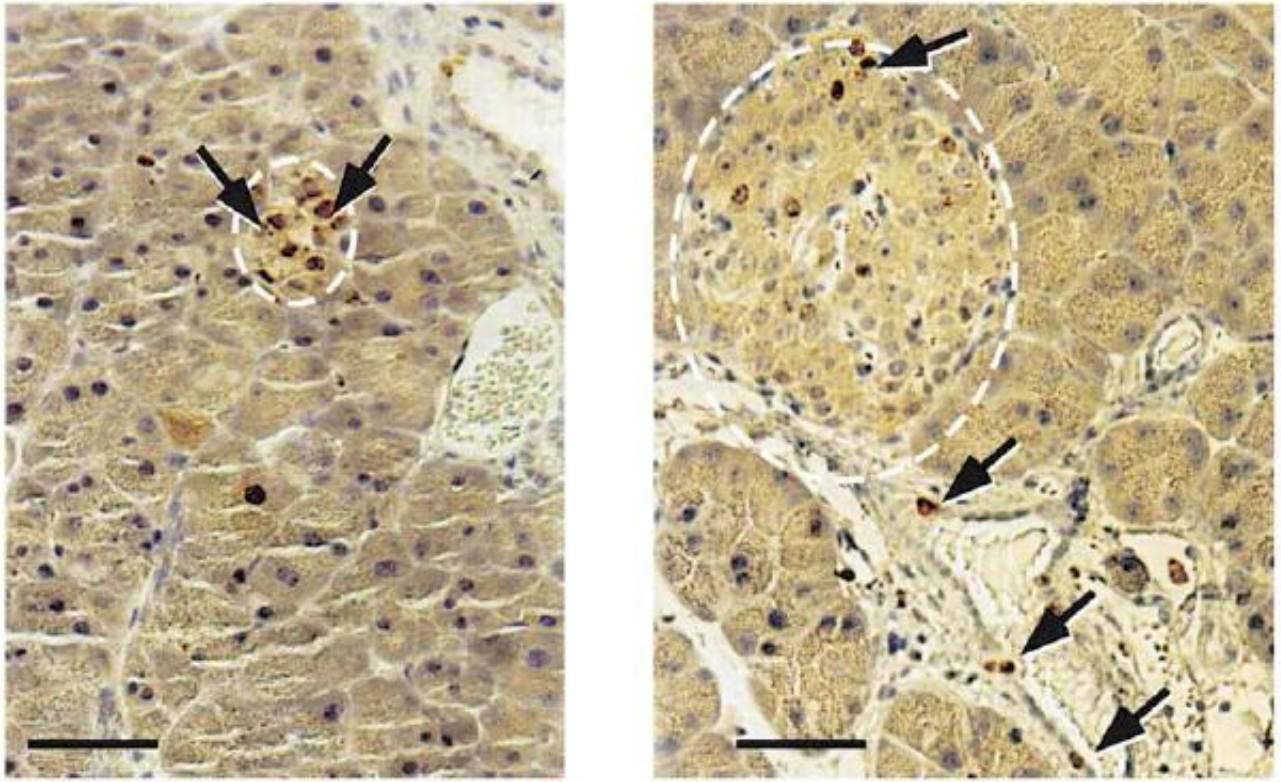
c-MycER^{TAM} activated 6 days



Switching OFF Myc: islets come back!

c-MycER^{TAM} activated for 6 days

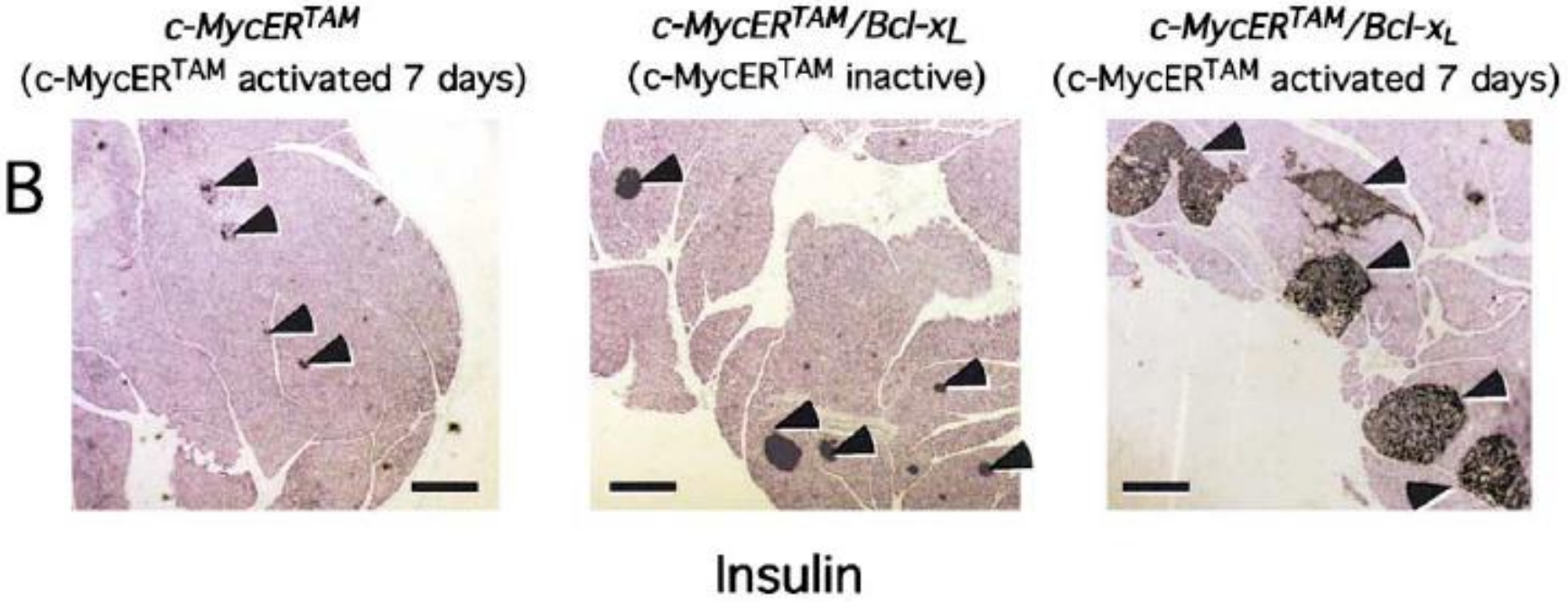
c-MycER^{TAM} activated for 6 days then de-activated for 9 days



Ki-67

What are the consequences of blocking Myc dependent apoptosis?

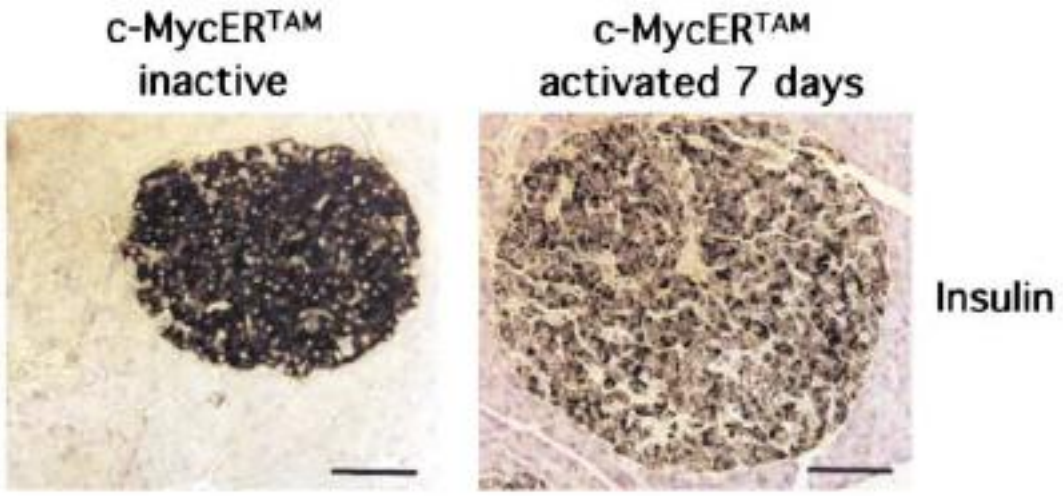
Exp: cross the Ins-MycER mice w/ mice Tg-BCL $_{XL}$



Result: Bcl-x_L prevents apoptosis and islets involution → mice develop <u>multifocal tumors</u>

Cellular consequences of long-term activation of Myc in pancreas:

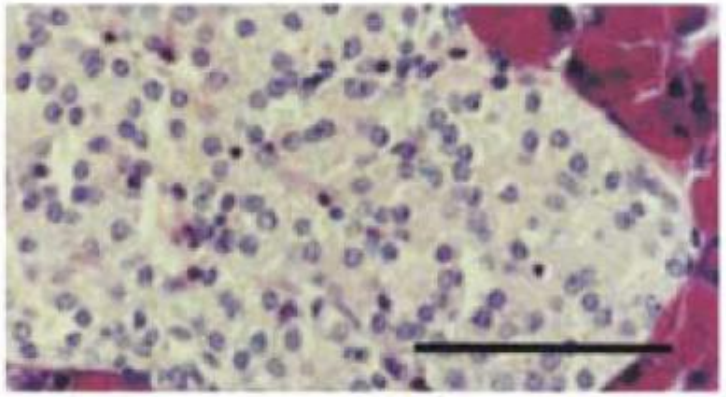
1. De-differentiation of tumors: less insulin
(marker of fully differentiated β-cells)

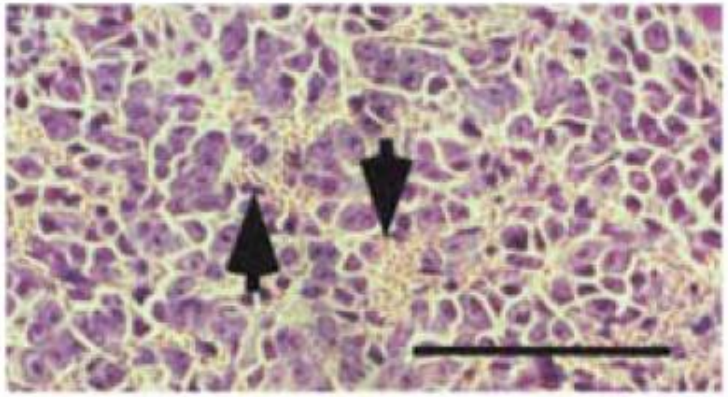


Extensive blood vessels: tumor angiogenesis

c-MycER^{TAM}
inactive

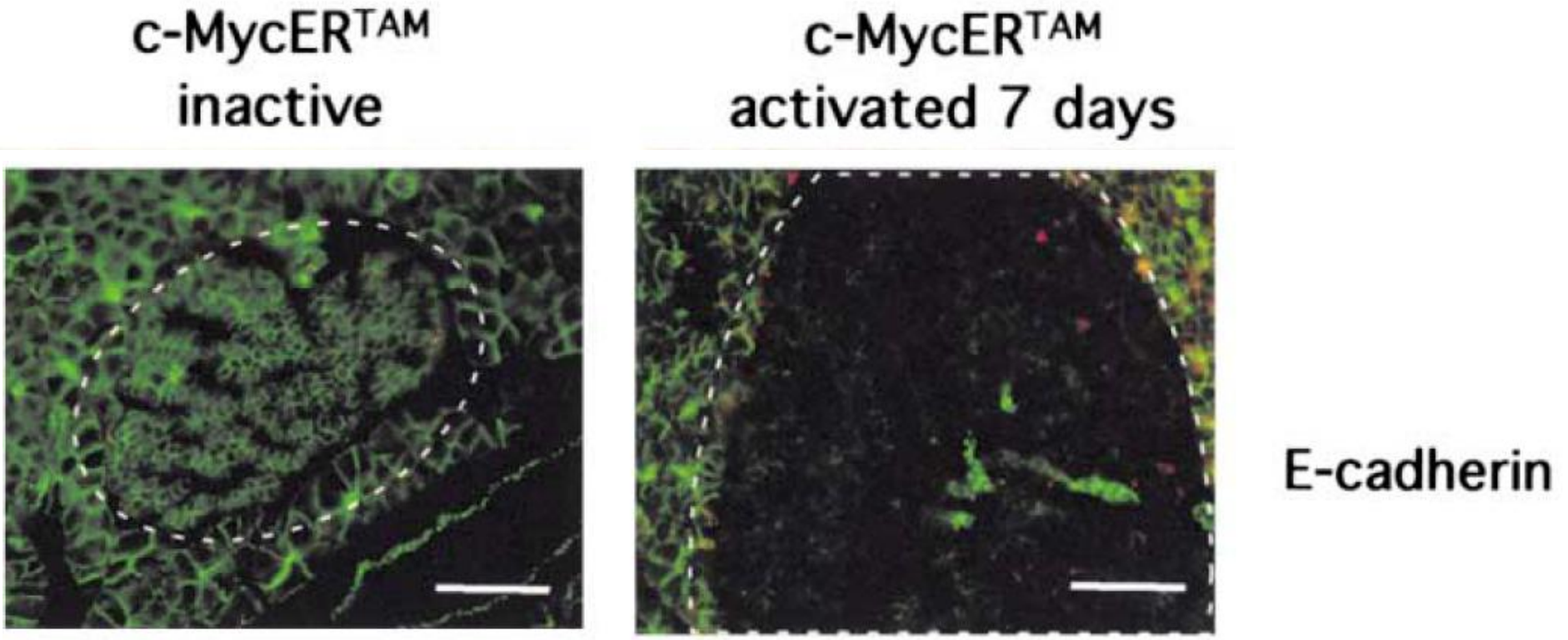
c-MycER^{TAM}
activated 7 days



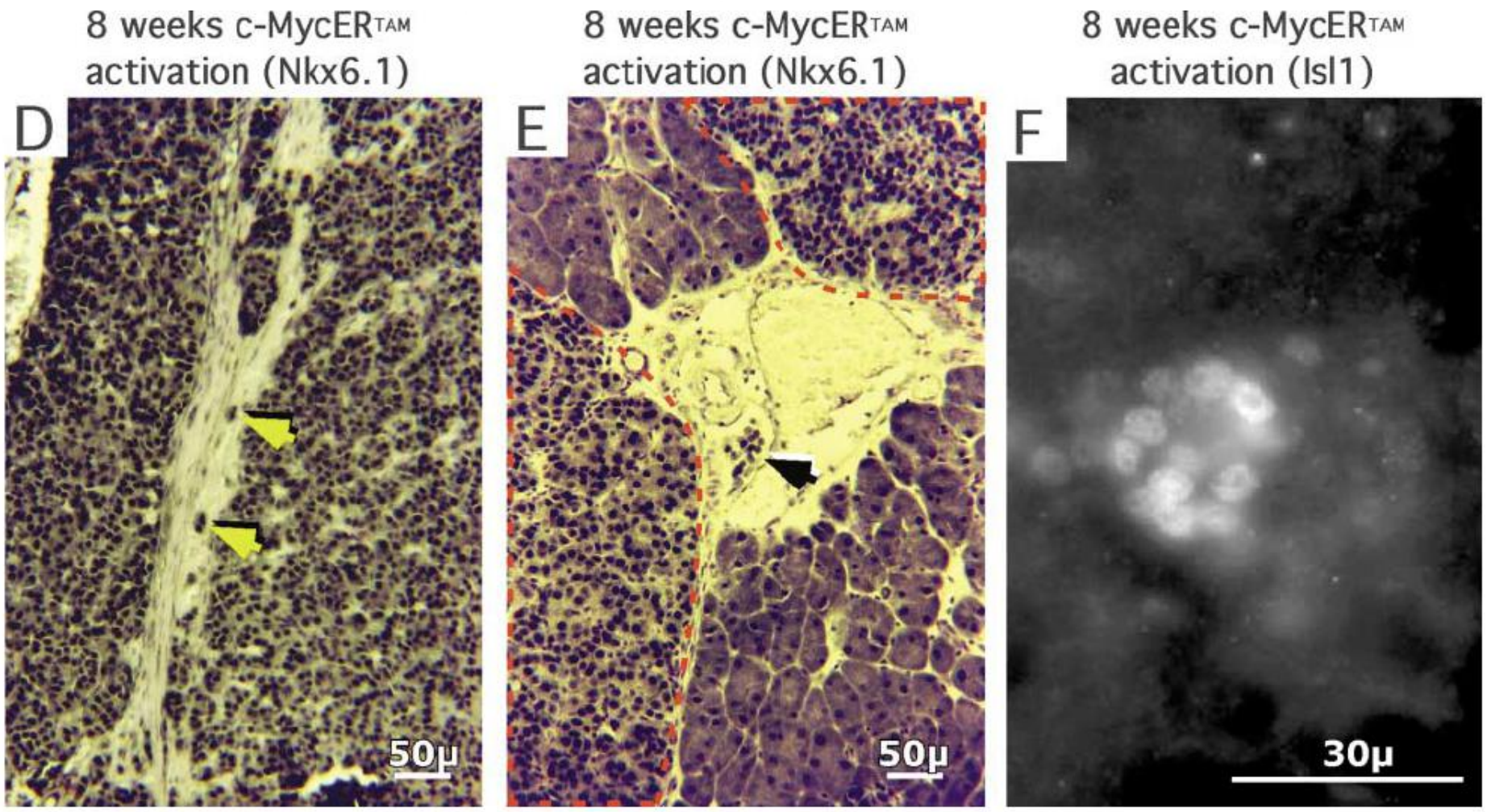


H&E

Loss of E-cadherin: as observed in highly metastatic cancers



dissemination of β-cells (Nkx6.1 or Isl1 positive)

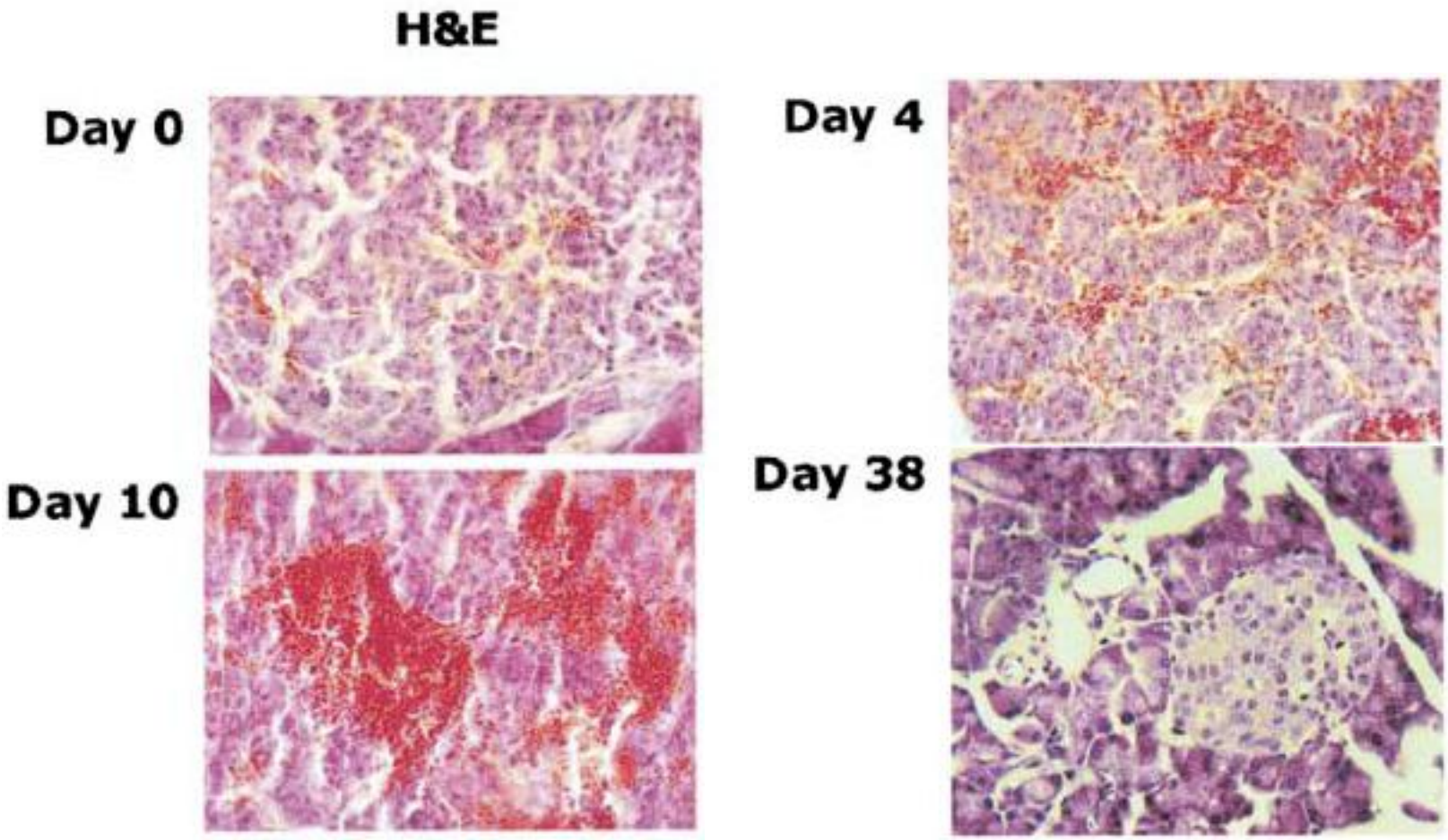


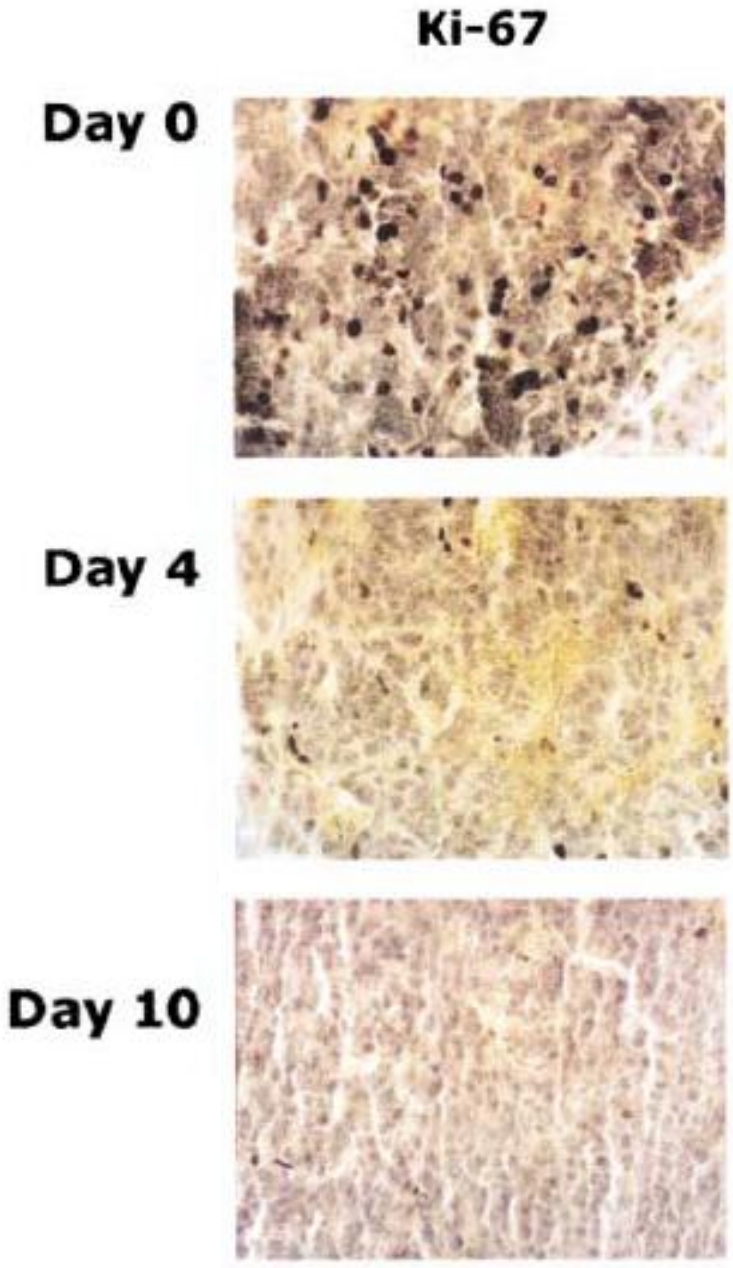
Blood vessels Pancreatic duct Lymph node

Invasive tumor

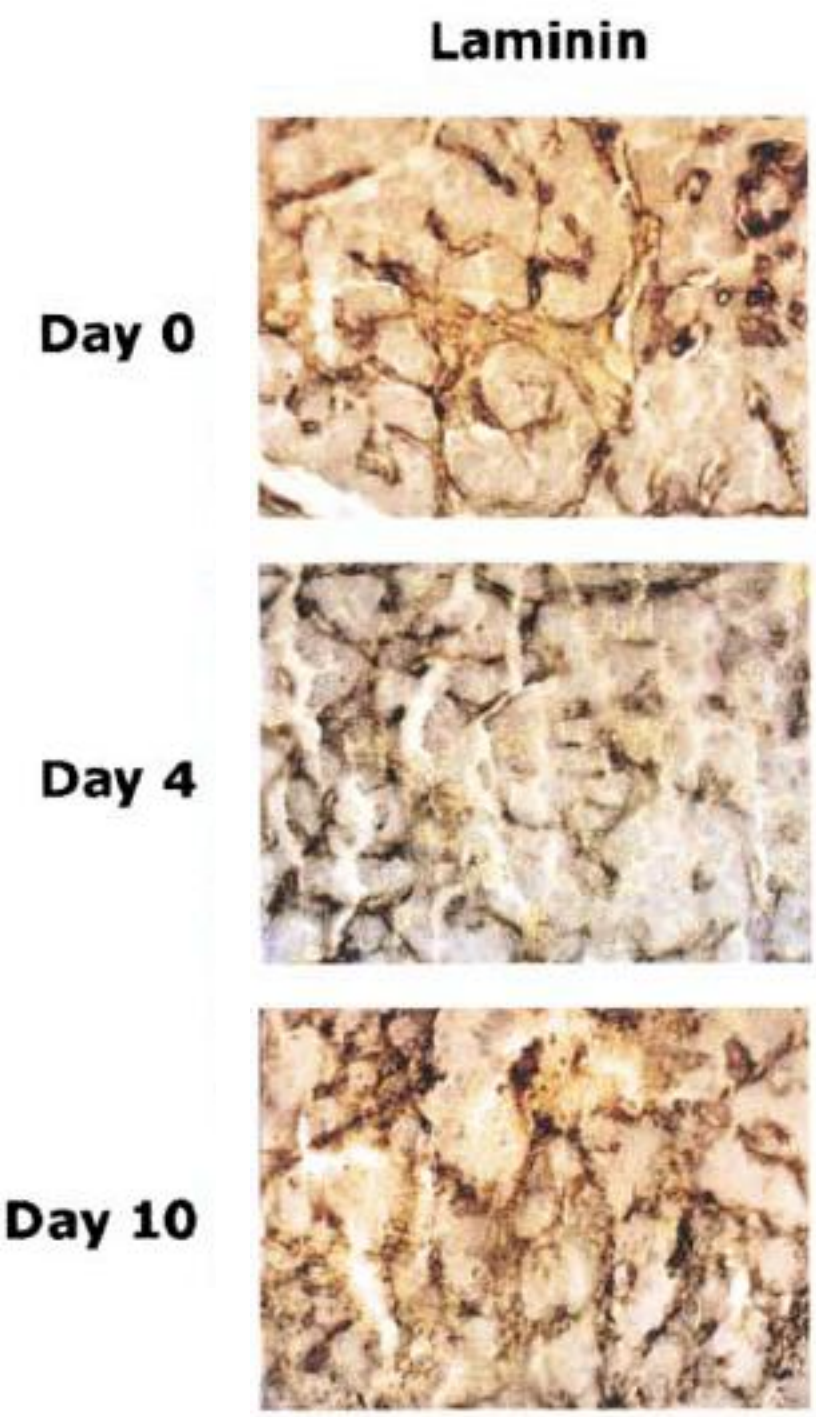
What would happen if we turn-off Myc in tumors?

Myc deactivation causes regression of the tumor (oncogene addiction) and Islet regeneration

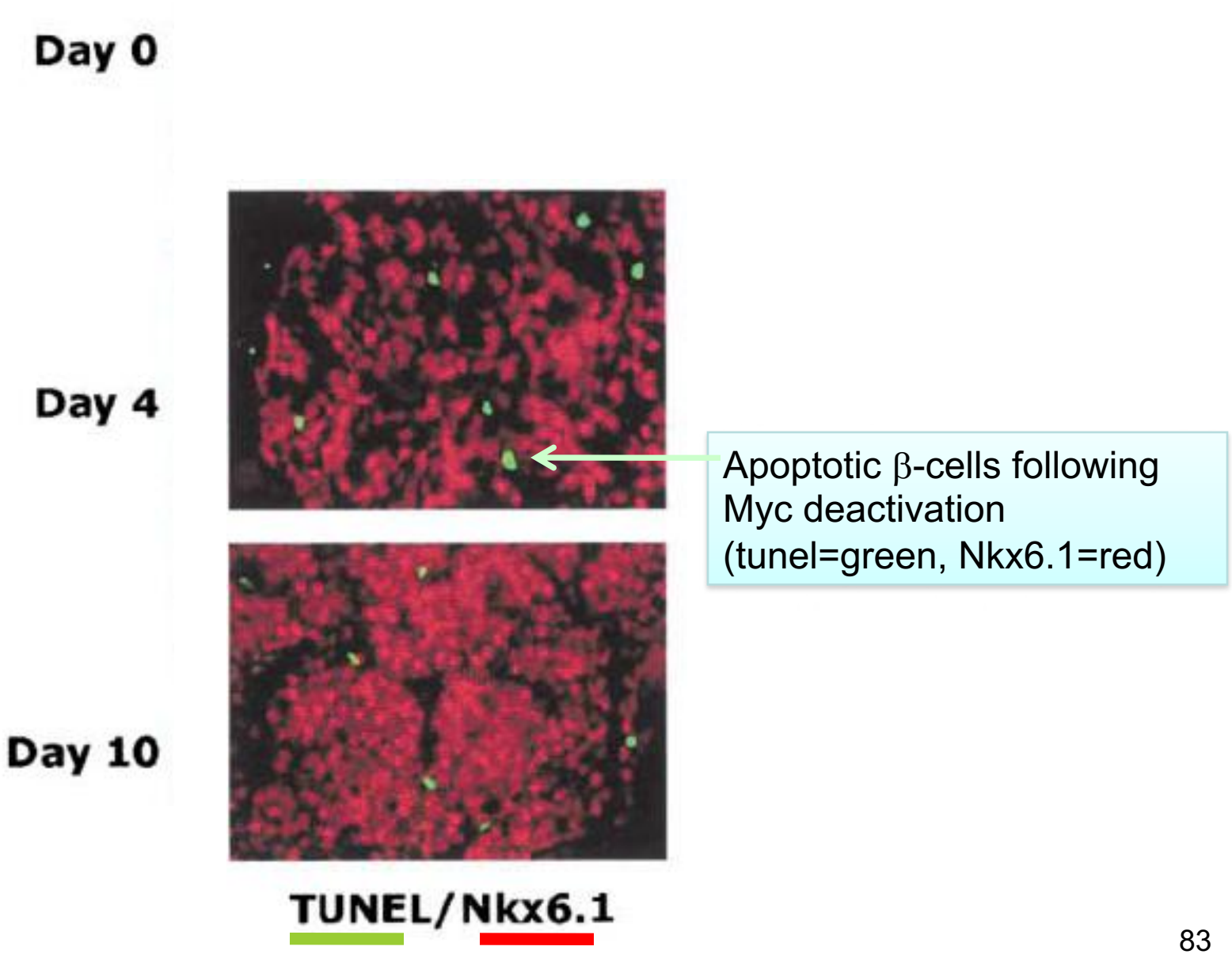




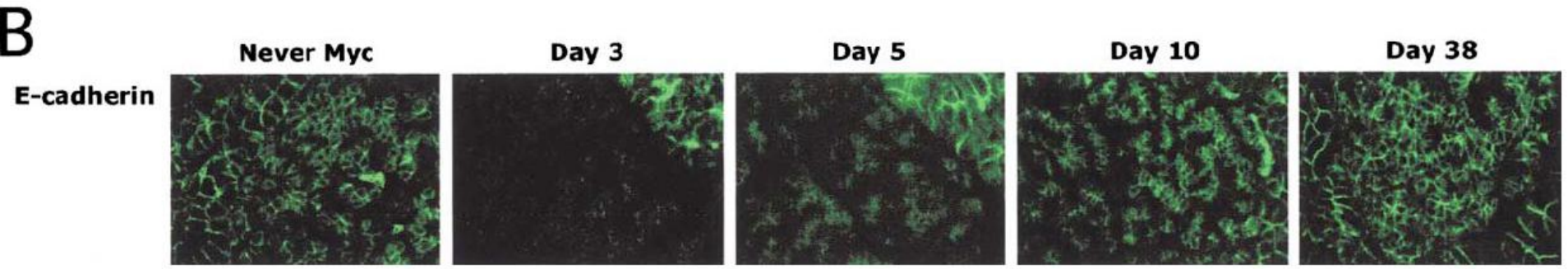
Loss of proliferation following Myc deactivation



Loss of blood vessels architecture following Myc deactivation


Day 0
Day 4
Day 10
Apoptotic β-cells following Myc deactivation (tunel=green, Nkx6.1=red)
TUNEL/Nkx6.1

β-cells reacquire normal cell to cell contacts following Myc deactivation



Myc deactivation

RELEVANT POINTS

Myc activation (*in vivo*) triggers proliferation and apoptosis

Myc dependent apoptosis is a potent tumor suppressor mechanism

BCL_{XL}: efficiently bypass apoptosis → leads to tumor

Myc is a potent oncogene with multiple roles during tumor development, apart from stimulating cell growth and cell division its activation supports:

-angiogenesis
-dedifferentiation
-invasion

<u>Oncogene addiction offers therapeutic options</u>