Introduction into basic transgenic technologies

Professor Ralph Knöll
Chair in Myocardial Genetics

Some major steps in the development of recombinant DNA and transgenic technology

1869	Miescher first isolates DNA from white blood cells harvested from pus-soaked bandages obtained from a nearby hospital.
1944	Avery provides evidence that DNA, rather than protein, carries the genetic information during bacterial transformation.
1953	Watson and Crick propose the double-helix model for DNA structure based on x-ray results of Franklin and Wilkins.
1955	Kornberg discovers DNA polymerase, the enzyme now used to produce labeled DNA probes.
1961	Marmur and Doty discover DNA renaturation, establishing the specificity and feasibility of nucleic acid hydridization reactions.
1962	Arber provides the first evidence for the existence of DNA restriction nucleases, leading to their purification and use in DNA sequence characterization by Nathans and H. Smith.
1966	Nirenberg, Ochoa, and Khorana elucidate the genetic code.
1967	Gellert discovers DNA ligase, the enzyme used to join DNA fragments together.
1972–1973	DNA cloning techniques are developed by the laboratories of Boyer, Cohen, Berg, and their colleagues at Stanford University and the University of California at San Francisco.
1975	Southern develops gel-transfer hybridization for the detection of specific DNA sequences.
1975–1977	Sanger and Barrell and Maxam and Gilbert develop rapid DNA-sequencing methods.
1981–1982	Palmiter and Brinster produce transgenic mice; Spradling and Rubin produce transgenic fruit flies.
1982	GenBank, NIH's public genetic sequence database, is established at Los Alamos National Laboratory.
1985	Mullis and co-workers invent the polymerase chain reaction (PCR).
1987	Capecchi and Smithies introduce methods for performing targeted gene replacement in mouse embryonic stem cells.
1989	Fields and Song develop the yeast two-hybrid system for identifying and studying protein interactions.
1989	Olson and colleagues describe sequence-tagged sites, unique stretches of DNA that are used to make physical maps of human chromosomes.
1990	Lipman and colleagues release BLAST, an algorithm used to search for homology between DNA and protein sequences.
1990	Simon and colleagues study how to efficiently use bacterial artificial chromosomes, BACs, to carry large pieces of cloned human DNA for sequencing.
1991	Hood and Hunkapillar introduce new automated DNA sequence technology.
1995	Venter and colleagues sequence the first complete genome, that of the bacterium *Haemophilus influenzae*.
1996	Goffeau and an international consortium of researchers announce the completion of the first genome sequence of a eucaryote, the yeast *Saccharomyces cerevisiae*.
1996–1997	Lockhart and colleagues and Brown and DeRisi produce DNA microarrays, which allow the simultaneous monitoring of thousands of genes.
1998	Sulston and Waterston and colleagues produce the first complete sequence of a multicellular organism, the nematode worm *Caenorhabditis elegans*.
2001	Consortia of researchers announce the completion of the draft human genome sequence.
2004	Publication of the "finished" human genome sequence.

Table 8-3 *Molecular Biology of the Cell* (© Garland Science 2008)

How to inactivate a gene?

- In vitro?
- In vivo?

Types of manipulation

1. Since 1981/82 it is possible to “over-express” a gene of interest in mice (or fruitfly) (“random insertion”)
2. Overexpression can be “abnormally high”, in the “wrong tissue”, or at the “wrong time” in development
3. By placing the gene under the control of an inducible promoter the gene can be switched on or off at any time

Types of manipulation

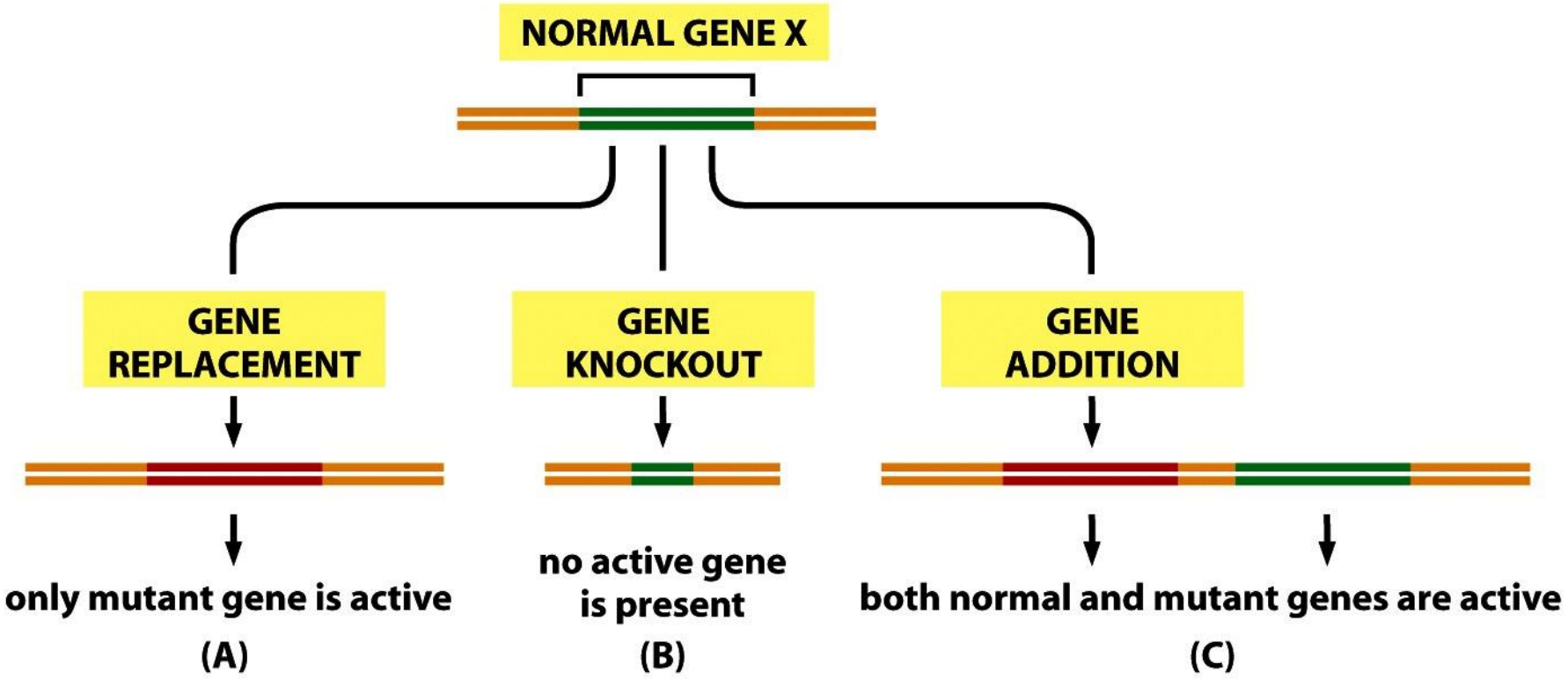


Figure 8-64 *Molecular Biology of the Cell* (© Garland Science 2008)

Gene modification

- Unlike higher eucaryotes (which are **multicellular and diploid**), bacteria, yeasts, and the cellular slime mold *Dictyostelium* generally exist as **haploid single cells**. In these organisms, an artificially introduced DNA molecule carrying a mutant gene can, with a relatively high frequency, replace the single copy of the normal gene by homologous recombination. It is therefore easy to produce cells in which the mutant gene has replaced the normal gene.

Gene modification

- In this way cells can be made in order to miss a particular protein or produce an altered form of it. The ability to perform direct gene replacements in lower eucaryotes, combined with the power of standard genetic analyses in these haploid organisms, explains in large part why studies in these types of cells have been so important for working out the details of those cell processes that are shared by all eucaryotes.

Ectopic misexpression of Wnt, a signaling protein that affects development of the body axis in the early Xenopus embryo.
In this experiment, mRNA coding for Wnt was injected into the ventral vegetal blastomere, inducing a second body axis (Sokol et al., Cell 1991)

Figure 8-61 *Molecular Biology of the Cell* (© Garland Science 2008)

Types of manipulation

4. Dominant negative mutations are often employed particularly in those organisms in which it is simpler to add an altered gene to the genome than to replace the endogenous genes with it. The dominant negative strategy exploits the fact that most proteins function as parts of larger protein complexes. The inclusion of just one nonfunctional component can often inactivate such complexes.

Types of manipulation

4. Therefore, by designing a gene that produces large quantities of a mutant protein that is inactive but still able to assemble into the complex, it is often possible to produce a cell in which all the complexes are inactivated despite the presence of the normal protein.

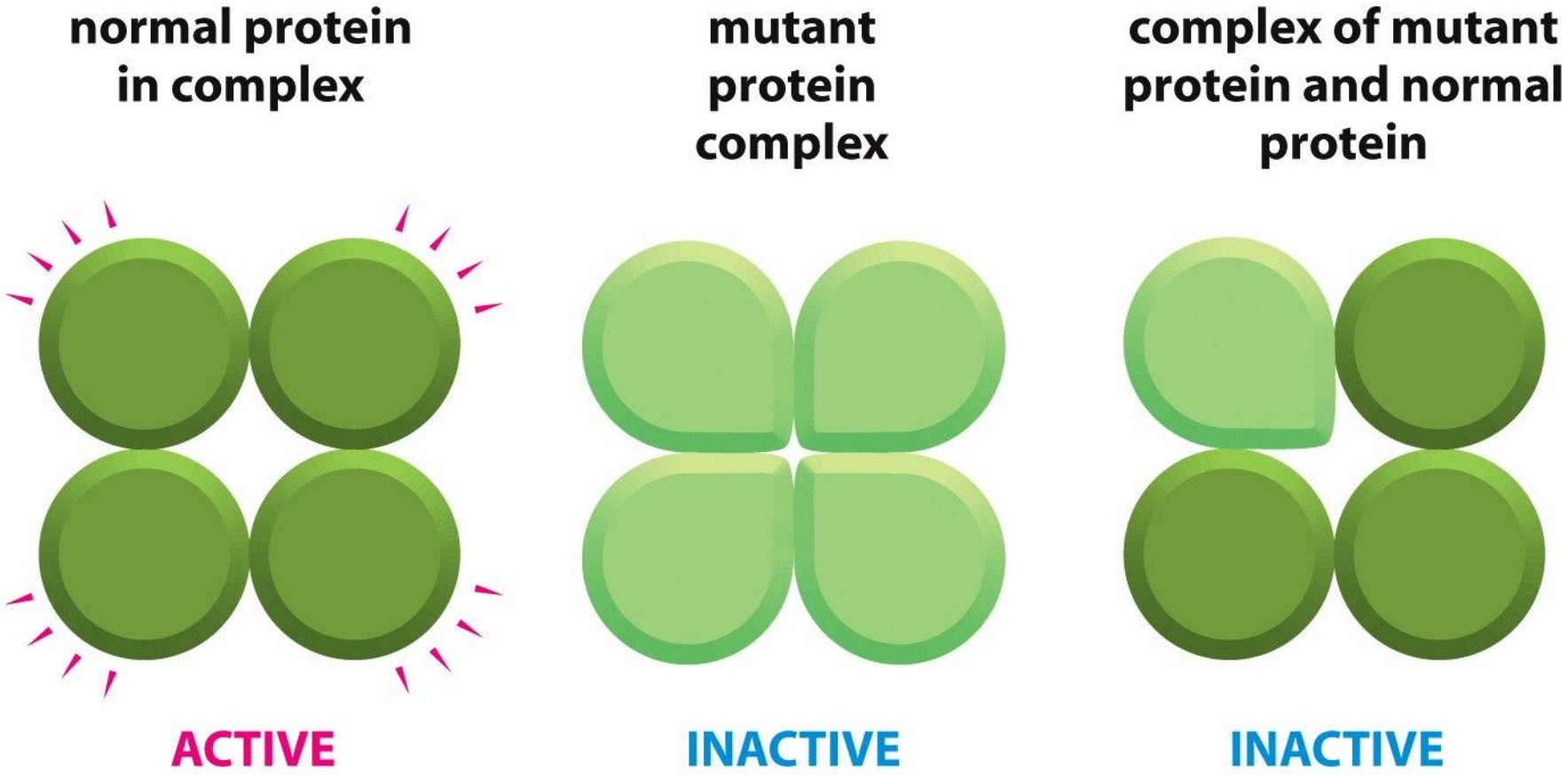


Figure 8-62 *Molecular Biology of the Cell* (© Garland Science 2008)

Site directed mutagenesis

- In studying the action of a gene and the protein it encodes, one does not always whish to make drastic changes. It is sometimes useful to make slight changes in a protein's structure so that one can begin to dissect which portions of a protein are important for its function. The activity of an enzyme, for example, can be studied by changing a single amino acid in its active site.

Site directed mutagenesis

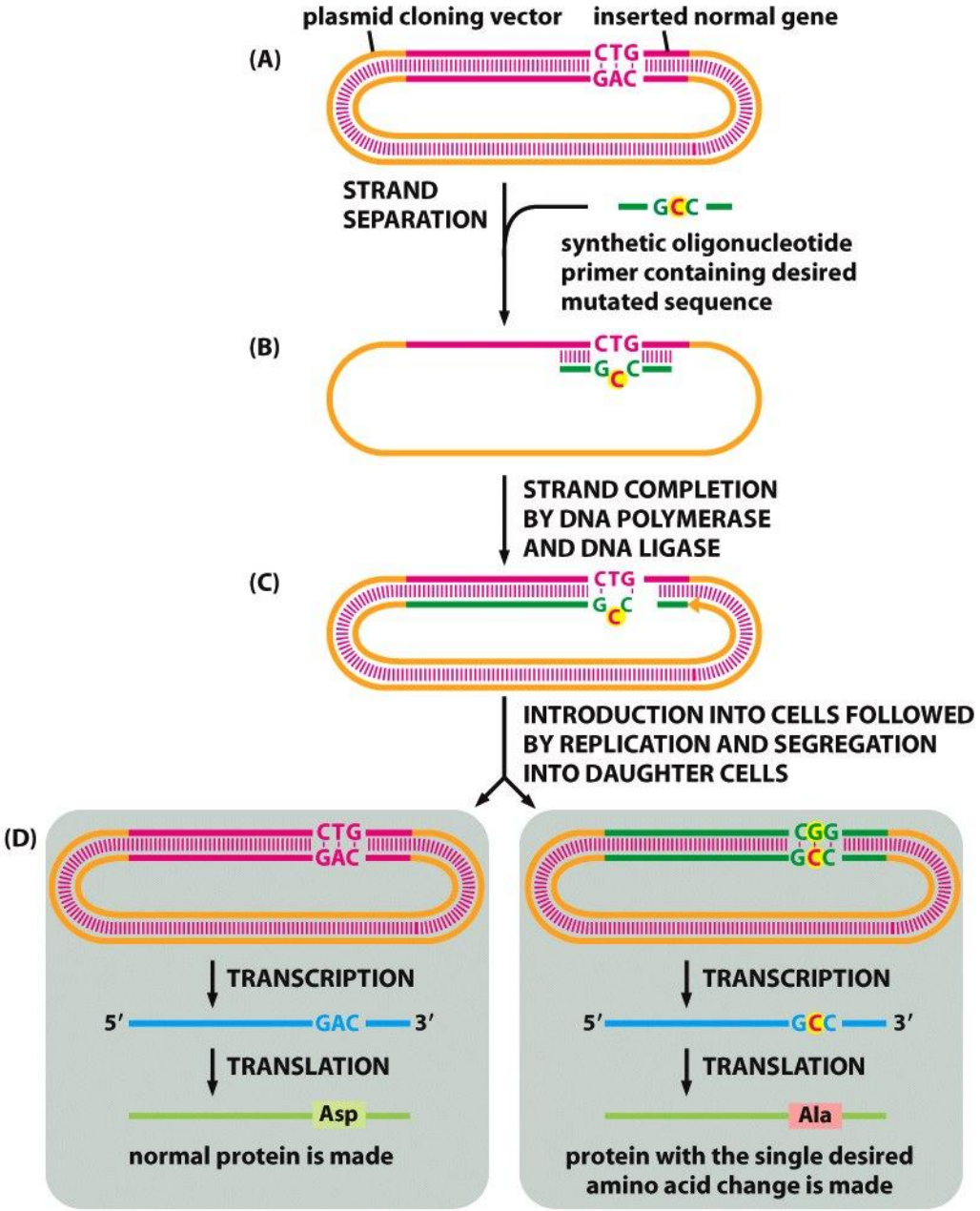


Figure 8-63 *Molecular Biology o*

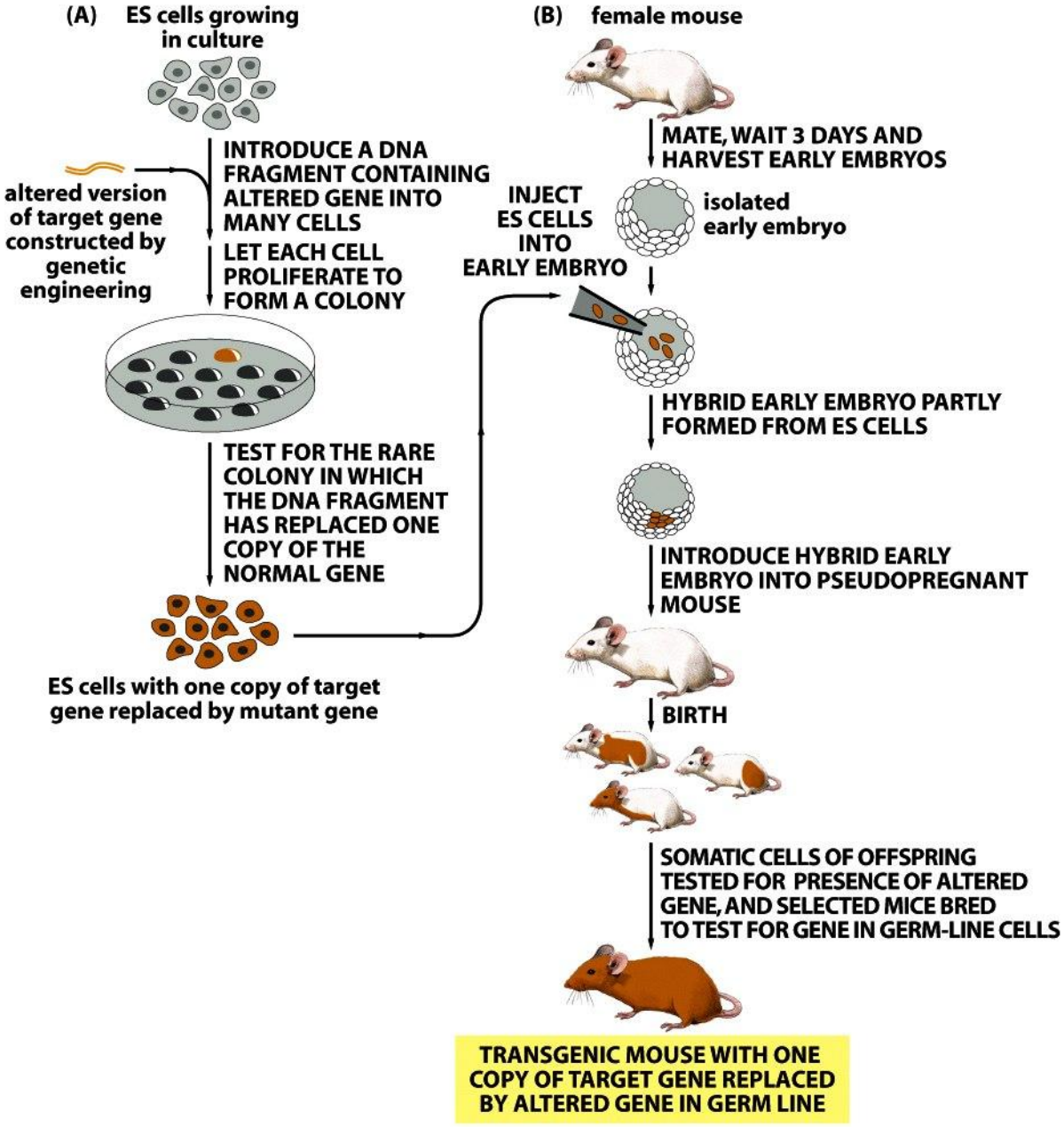
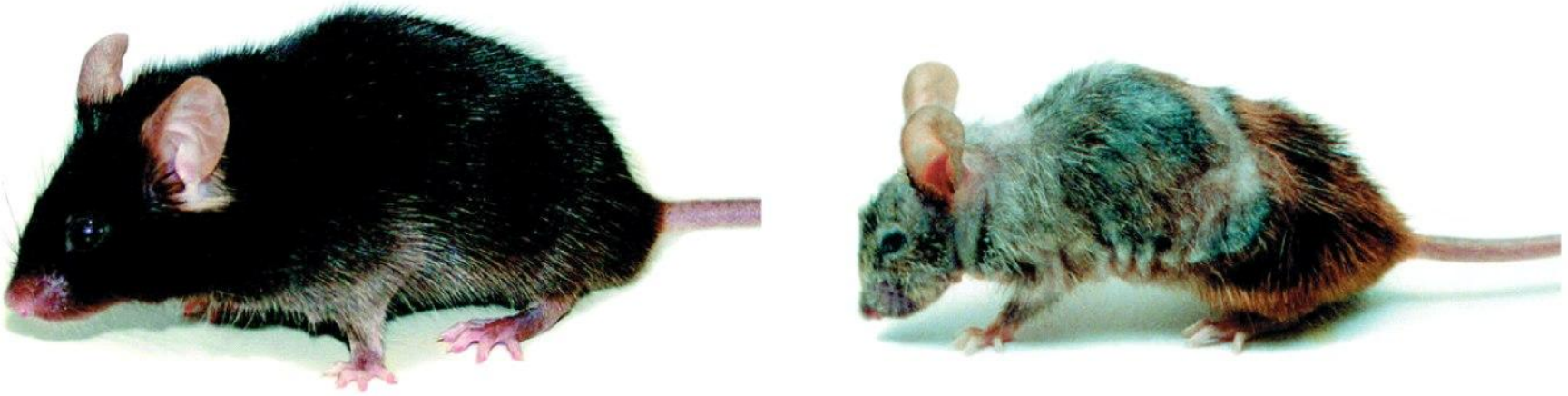


Figure 8-65 *Molecular Biology of the Cell* (© Garland Science 2008)

Transgenic mice engineered to express a mutant DNA helicase show premature aging



(A) (B)

The helicase, encoded by the Xpd gene, is involved in both transcription and DNA repair. Compared with a wild-type mouse of the same age (A), a transgenic mouse that expresse a defective version of Xpd (B) exhibits many of the symptoms of premature aging, including osteropososis, emaciation (cachexia), early graying, infertility, and reduced life-span. The mutation in Xpd used here impairs the activity of the helicase and mimics a mutation that in humans causes “trichodystrophy”, a disorder characterized by brittle hair, skeletal abnormalities, and a very reduced live expectancy. These results indicate that an accumulation of DNA damage can contribute to the aging process in both humans and mice (de Boer et al., Science 2002)

Phospholamban – the first "cardiovascular" gene knockout model

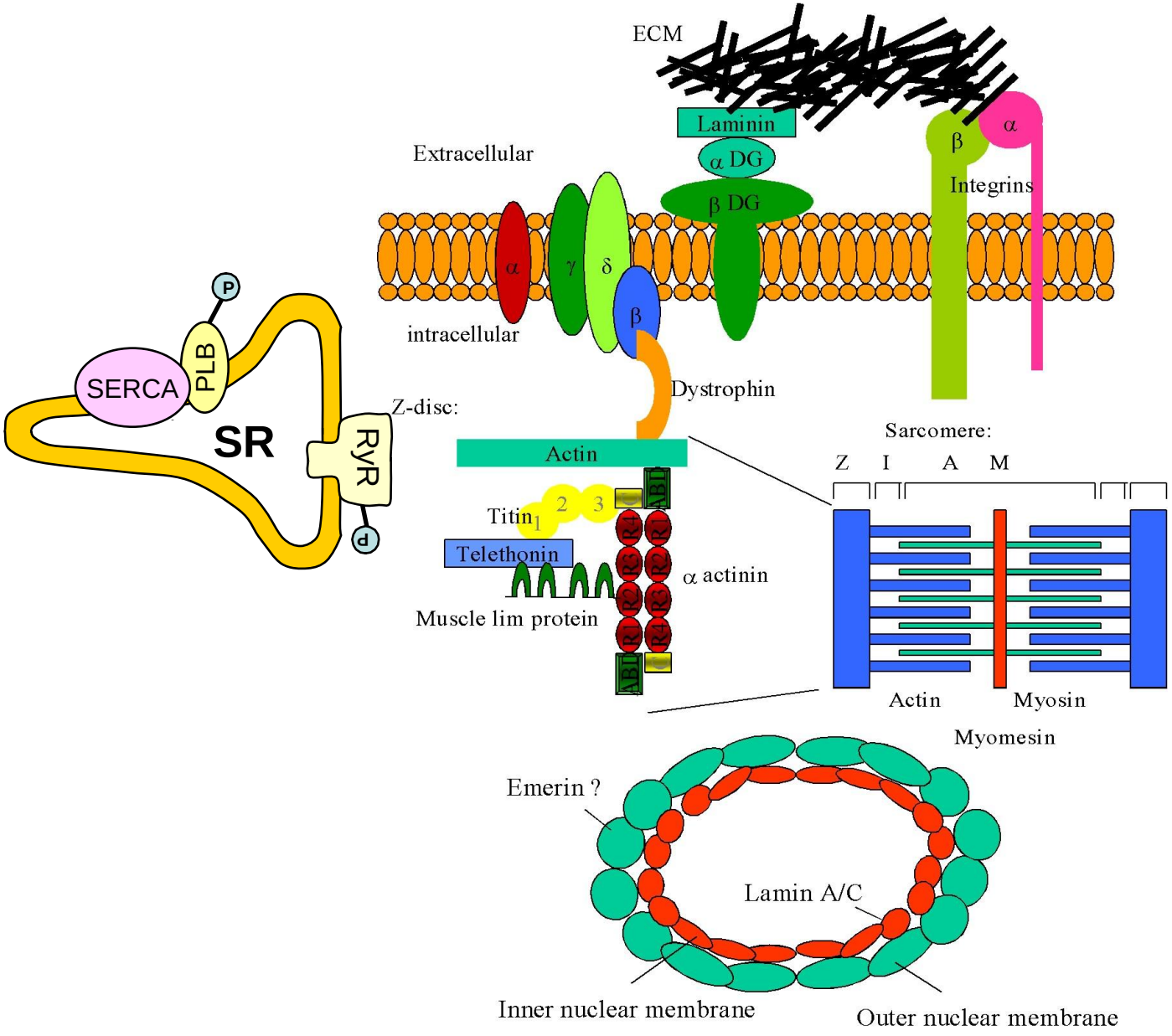
Targeted Ablation of the Phospholamban Gene Is Associated With Markedly Enhanced Myocardial Contractility and Loss of β-Agonist Stimulation

Wusheng Luo, Ingrid L. Grupp, Judy Harrer, Sathivel Ponniah, Gunter Grupp, John J. Duffy, Thomas Doetschman, Evangelia G. Kranias

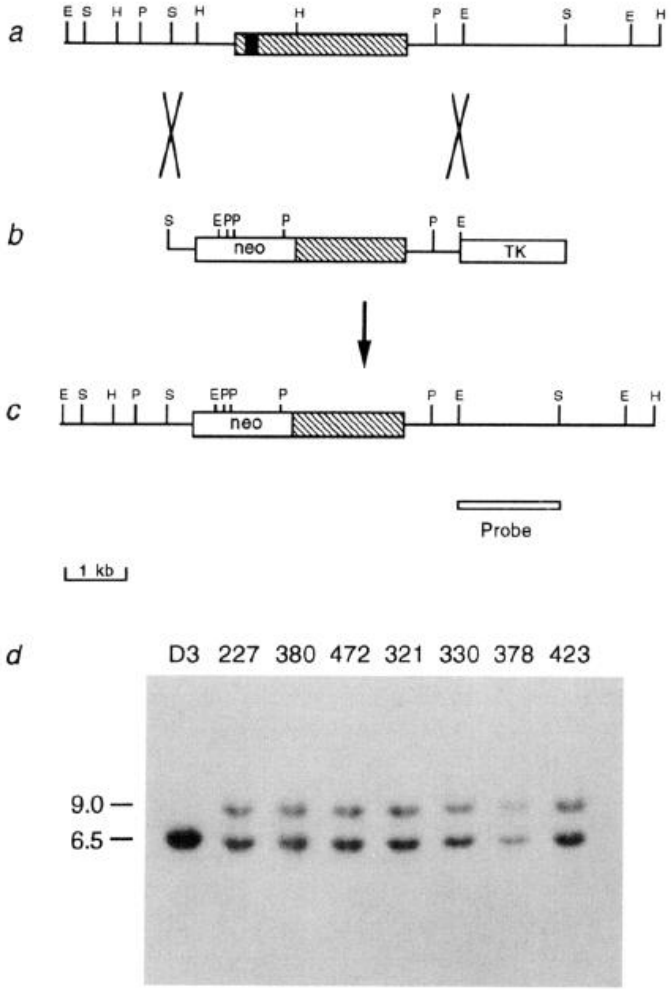
Abstract Phospholamban is the regulator of the Ca^{2+}-ATPase in cardiac sarcoplasmic reticulum (SR), and it has been suggested to be an important determinant in the inotropic responses of the heart to β-adrenergic stimulation. To determine the role of phospholamban in vivo, the gene coding for this protein was targeted in murine embryonic stem cells, and mice deficient in phospholamban were generated. The phospholamban-deficient mice showed no gross developmental abnormalities but exhibited enhanced myocardial performance without changes in heart rate. The time to peak pressure and the time to half-relaxation were significantly shorter in phospholamban-deficient mice compared with their wild-type homozygous littermates as assessed in work-performing mouse heart preparations under identical venous returns, afterloads, and heart rates. The first derivatives of intraventricular pressure (±dP/dt) were also significantly elevated, and this was associated with an increase in the affinity of the SR Ca^{2+}-ATPase for Ca^{2+} in the phospholamban-deficient hearts. Baseline levels of these parameters in the phospholamban-deficient hearts were equal to those observed in hearts of wild-type littermates maximally stimulated with the β-agonist isoproterenol. These findings indicate that phospholamban acts as a critical repressor of basal myocardial contractility and may be the key phosphoprotein in mediating the heart's contractile responses to β-adrenergic agonists. (***Circ Res.*** **1994; 75:401-409.)**

Key Words • phospholamban • gene targeting • sarcoplasmic reticulum • cardiac contractility • β-agonists

Overview – Cardiomyocyte



Phospholamban gene targeting



Detection of specific RNA or DNA molecules by gel transfer hybridization (Northern (RNA) and Southern DNA blots)

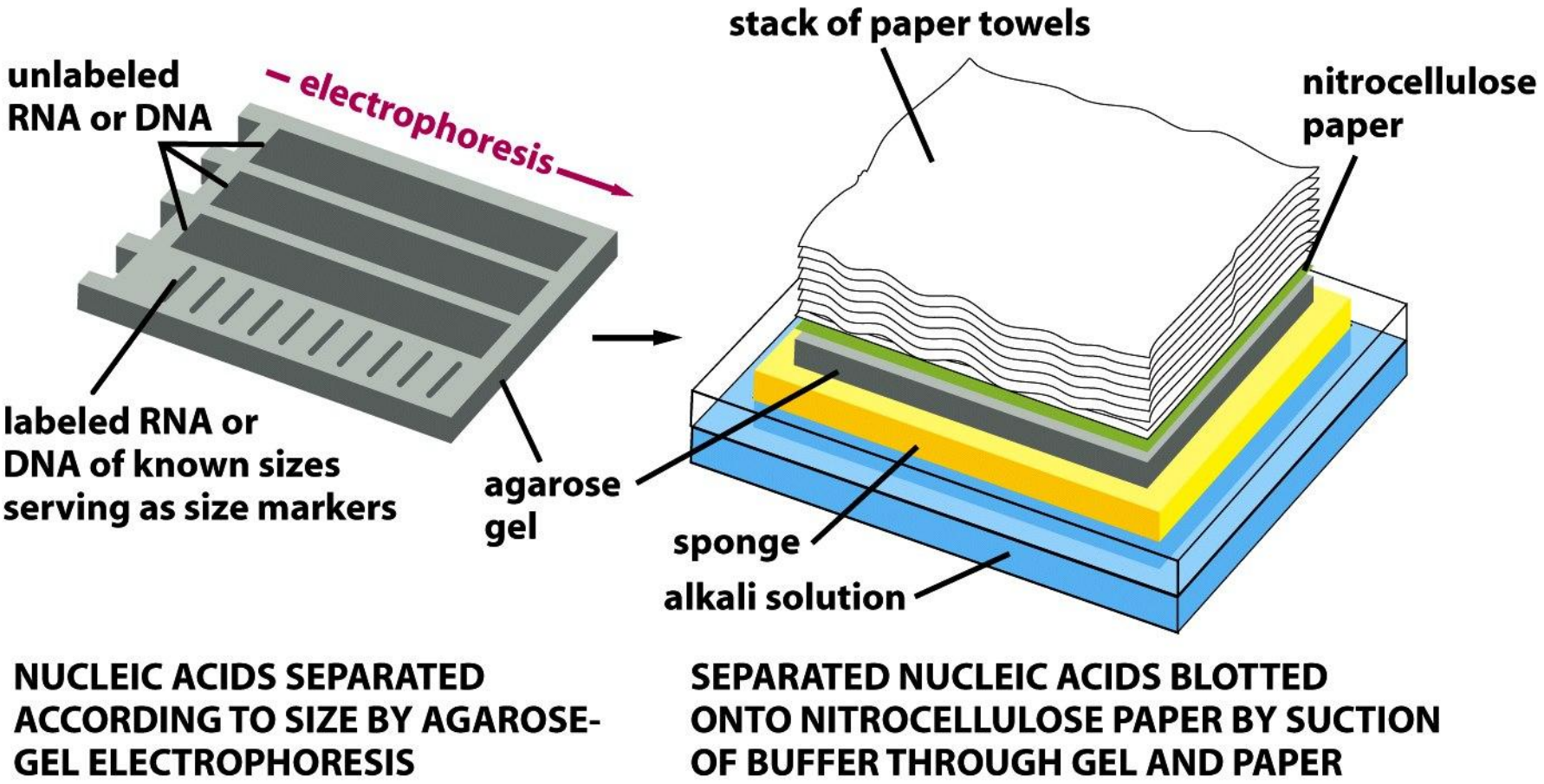


Figure 8-38 (part 1 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

Detection of specific RNA or DNA molecules by gel transfer hybridization (Northern (RNA) and Southern DNA blots)

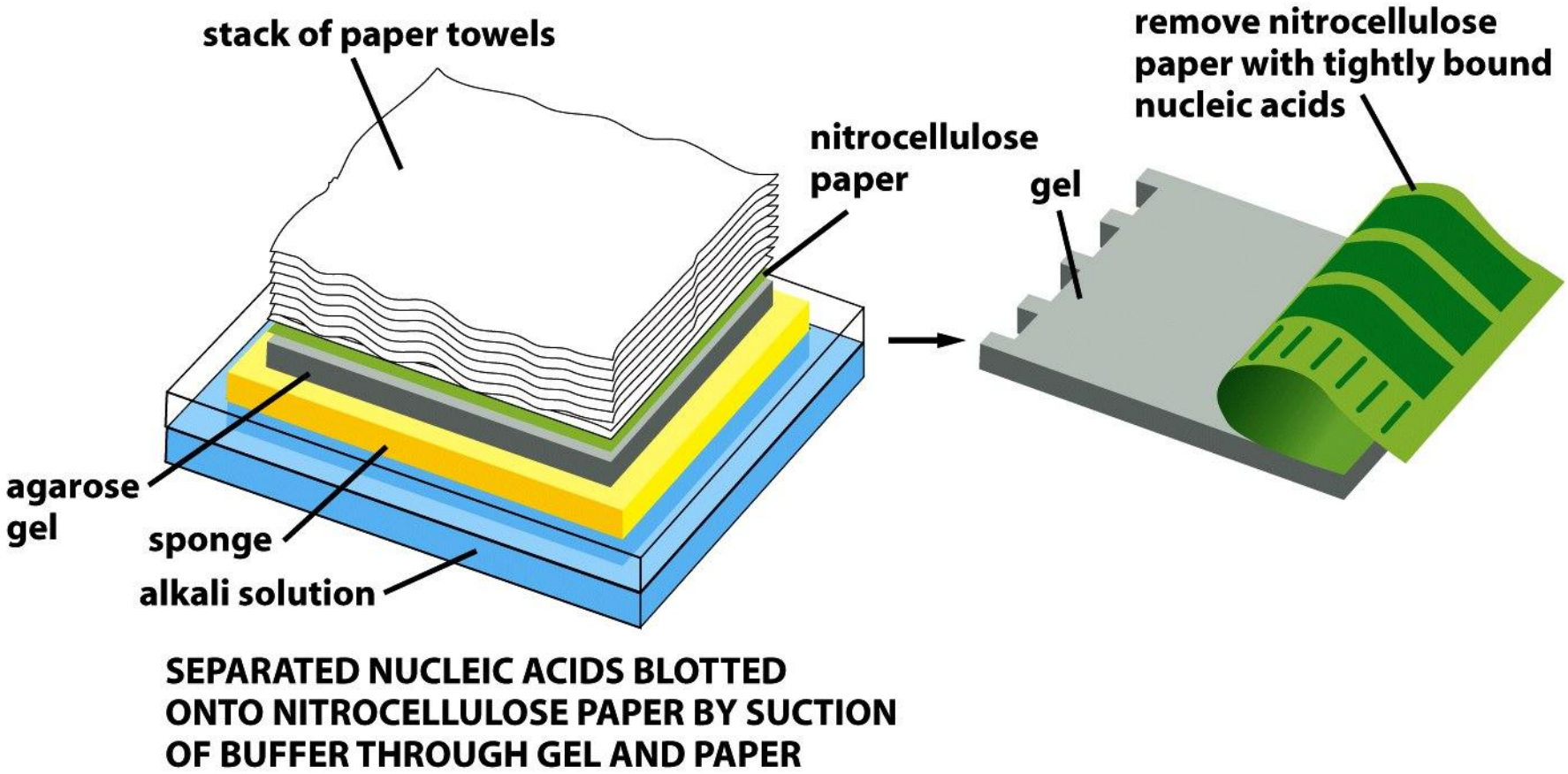


Figure 8-38 (part 2 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

Detection of specific RNA or DNA molecules by gel transfer hybridization (Northern (RNA) and Southern DNA blots)

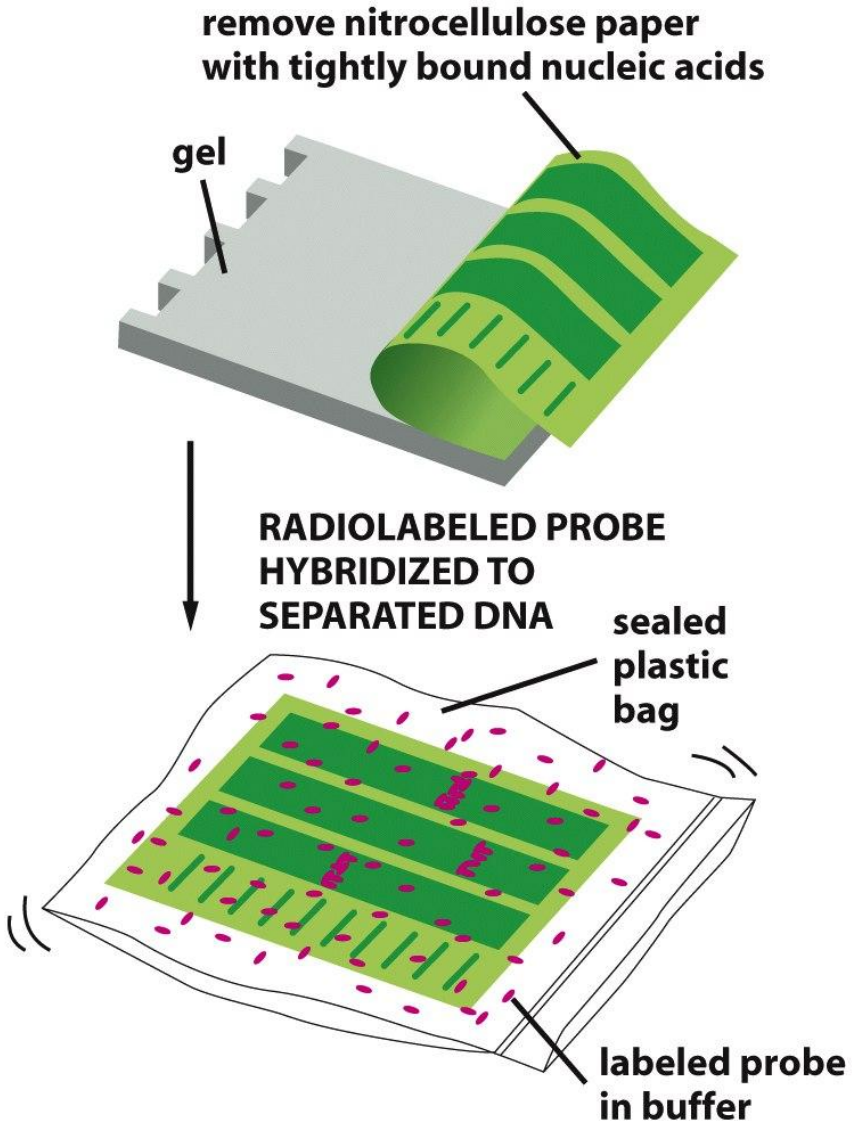


Figure 8-38 (part 3 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

Detection of specific RNA or DNA molecules by gel transfer hybridization (Northern (RNA) and Southern DNA blots)

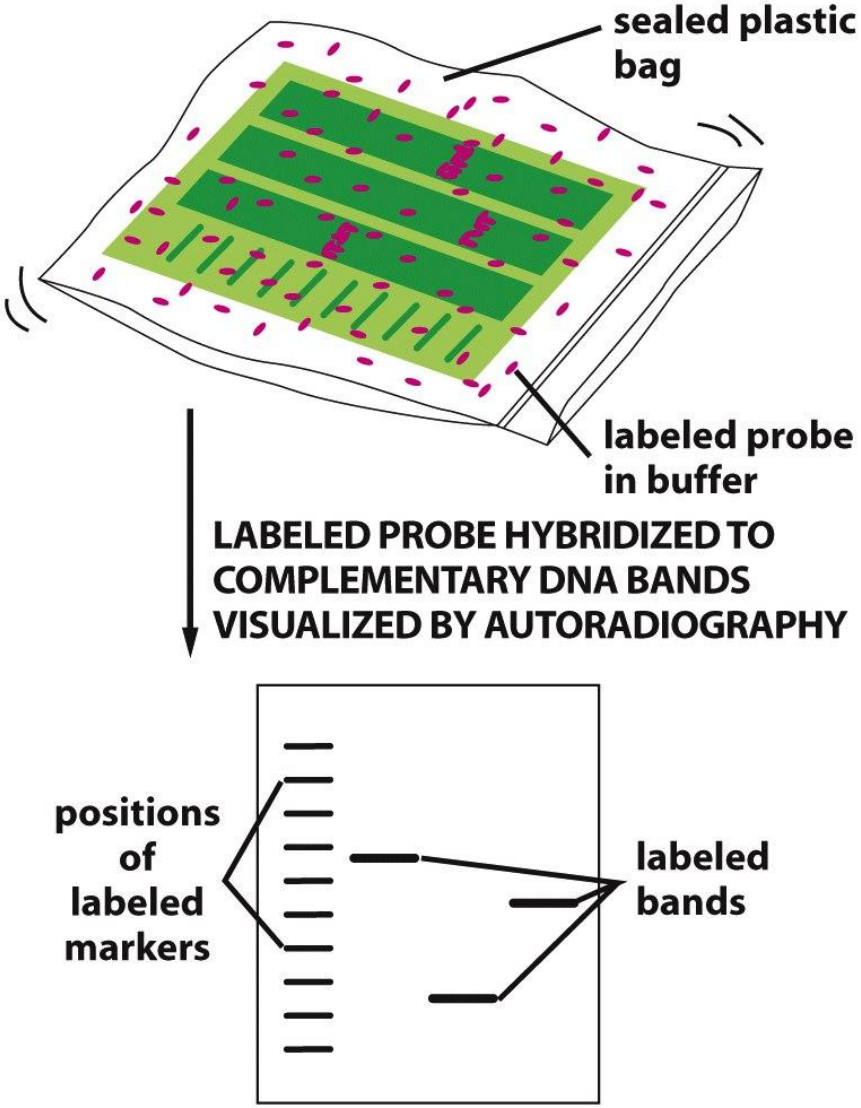
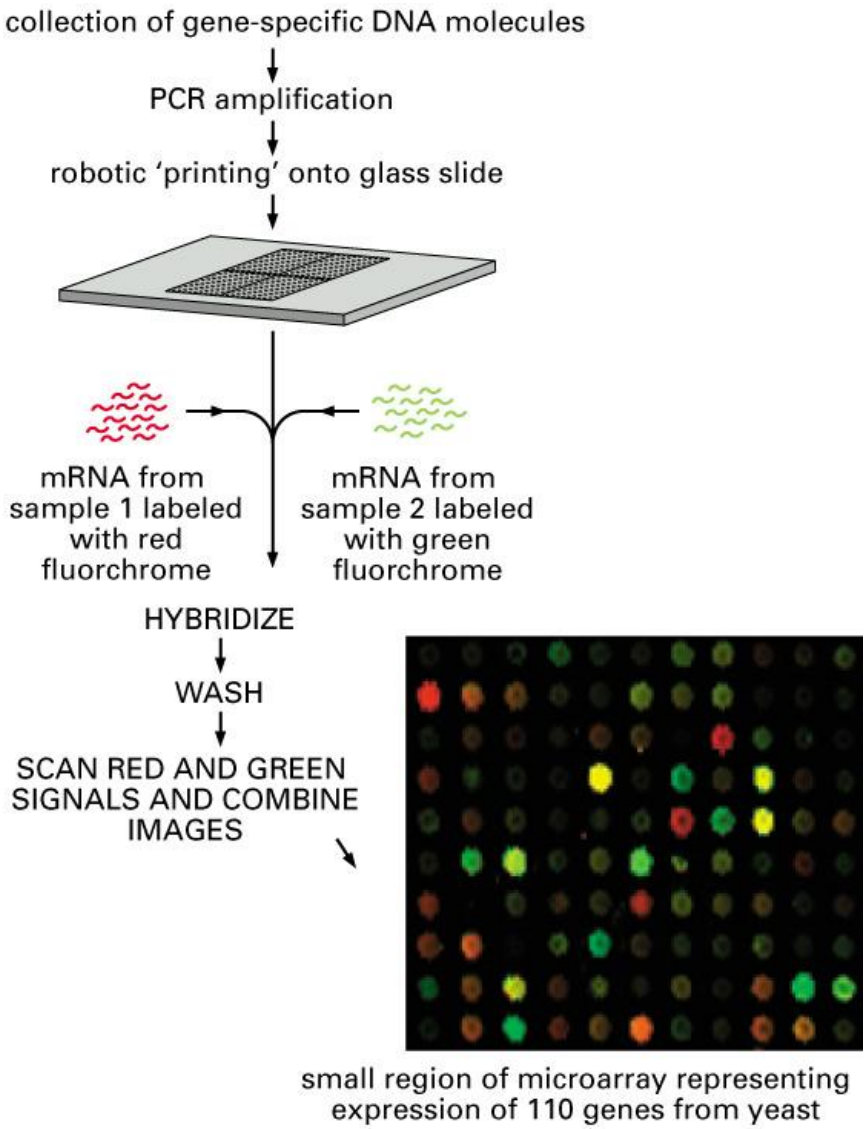
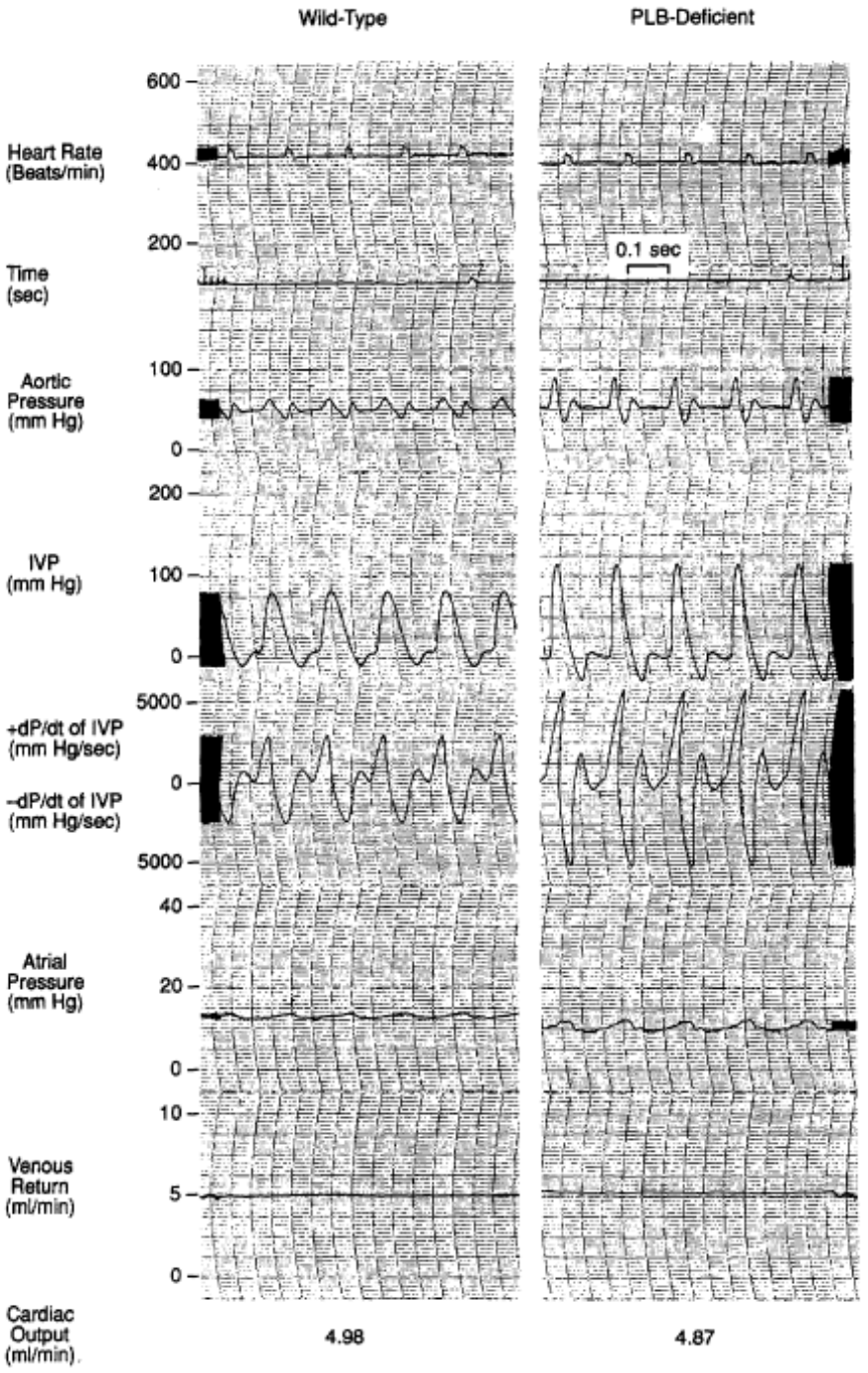


Figure 8-38 (part 4 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

Micro-Array 1



Analysis of phospholamban$^{-/-}$



Cardiovascular, Contractile, and Relaxation Parameters in Isolated Work-Performing Mouse Heart Preparations of Phospholamban-Deficient and Wild-Type Mice

	Wild-Type Mice	Phospholamban-Deficient Mice	*P* Value*
n	14	8	
Body wt, g	26.5±1.3	27.4±1.3	>.05
Heart wt (both ventricles), mg	195.0±12.6	200.3±12.2	>.05
Heart wt/body wt, mg/g	7.3±0.2	7.3±0.3	>.05
HR, bpm	379.0±7.3	385.0±6.1	>.05
MAP (afterload), mm Hg	50.8±0.3	50.6±0.3	>.05
Left IVP, mm Hg			
Systolic	85.8±3.2	108.0±4.0	.0001
Diastolic	−4.7±0.8	−17.4±1.8	.0001
End-diastolic	+11.7±1.1	+5.9±0.6	.0001
Cardiac output (venous return), mL/min	4.85±0.08	5.1±0.1	>.05
Aortic flow	1.9±0.23	1.3±0.3	>.05
Coronary flow	2.96±0.25	3.7±0.3	>.05
Stroke volume, μL	12.9±0.3	13.2±0.2	>.05
Cardiac power (left ventricle), mm Hg · mL · min^{-1}	249.0±7.3	256.0±2.4	>.05
Contraction properties			
+dP/dt, mm Hg/s	+3135±94	+5275±236	.0001
TPP/mm Hg, ms/mm Hg	0.546±0.01	0.317±0.02	.0001
Relaxation properties			
−dP/dt, mm Hg/s	−2637±116	−5209±246	.0001
RT½/mm Hg, ms/mm Hg	0.552±0.02	0.284±0.01	.0001

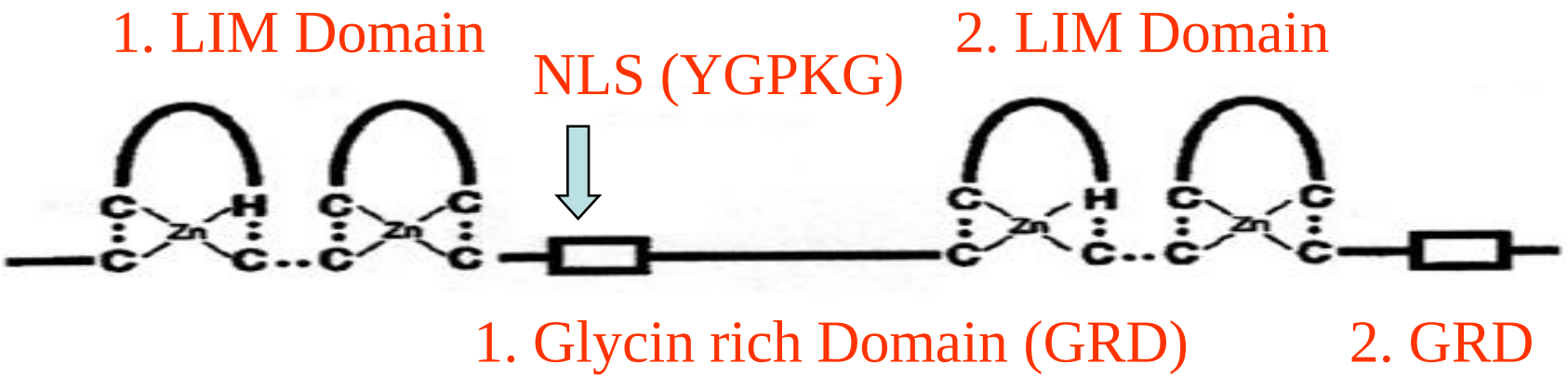
n indicates number of mice; HR, heart rate; bpm, beats per minute; MAP, mean aortic pressure; IVP, intraventricular pressure; TPP, time to peak pressure; and RT½, half-relaxation time. Values are mean±SEM.

*Assessed by *t* test for paired comparisons between control and mutant mice. Values were also tested by ANOVA using SUPER ANOVA software from Abacus.

Phospholamban$^{-/-}$

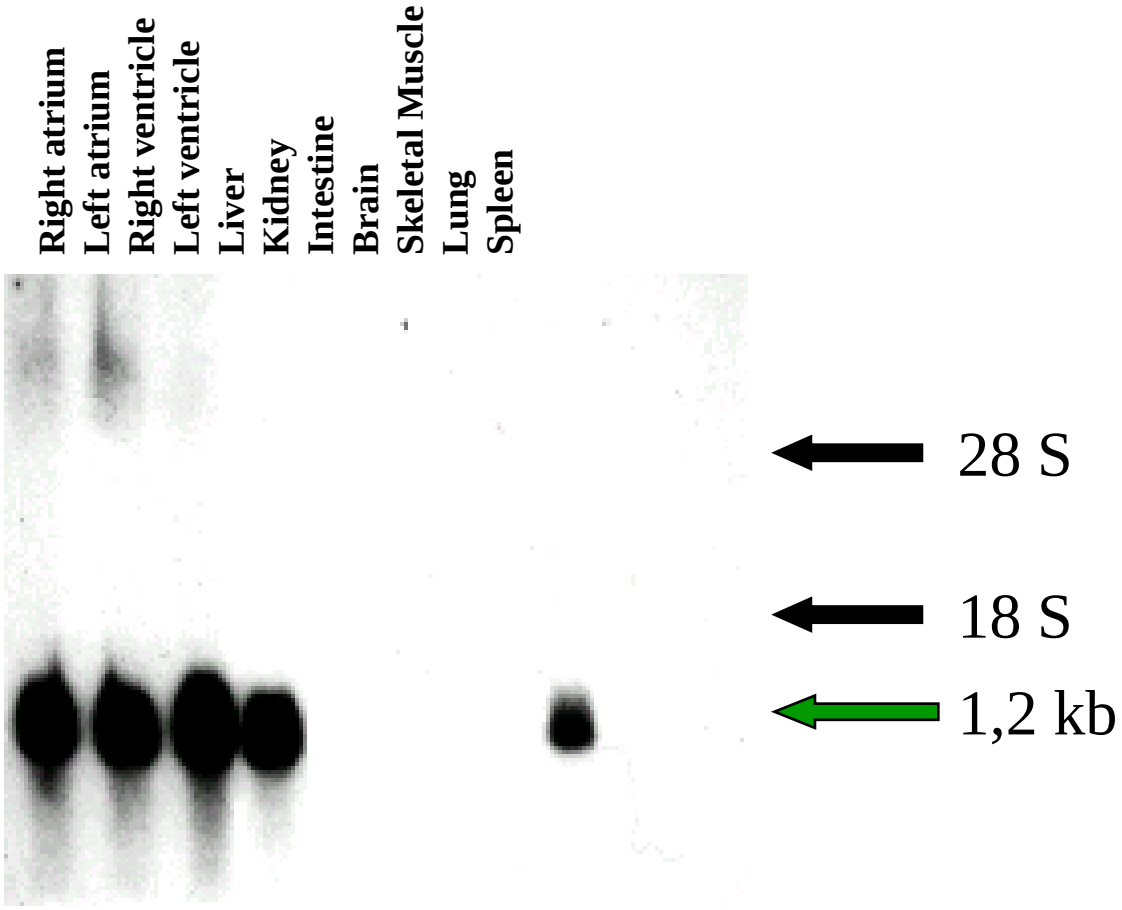
- Generation and analysis of phospholamban deficient animals helped significantly to define the function of this important gene.
- Today calcium cycling is an important target for gene therapy.

Muscle LIM Protein (MLP)



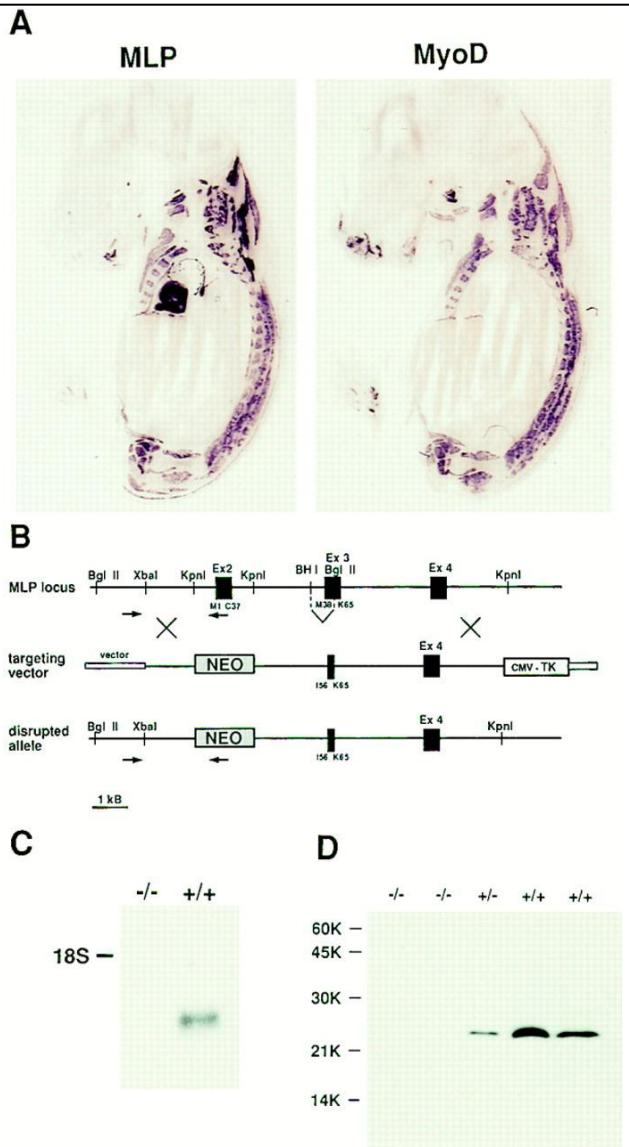
- 194 amino acid LIM (Lin11, Islet 1, Mec 3) LIM only protein (also known as: CSRP3, CRP3)
- Muscle specific expressed

Analysis of MLP gene expression via Northern Blot

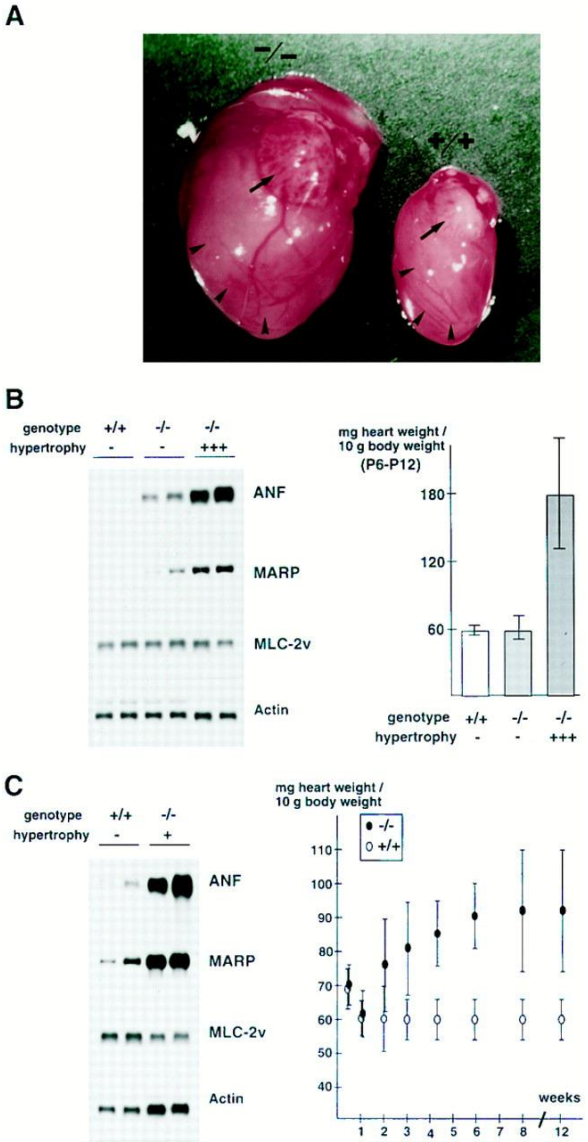


MLP is expressed in both atria and ventricles as well as in skeletal muscle

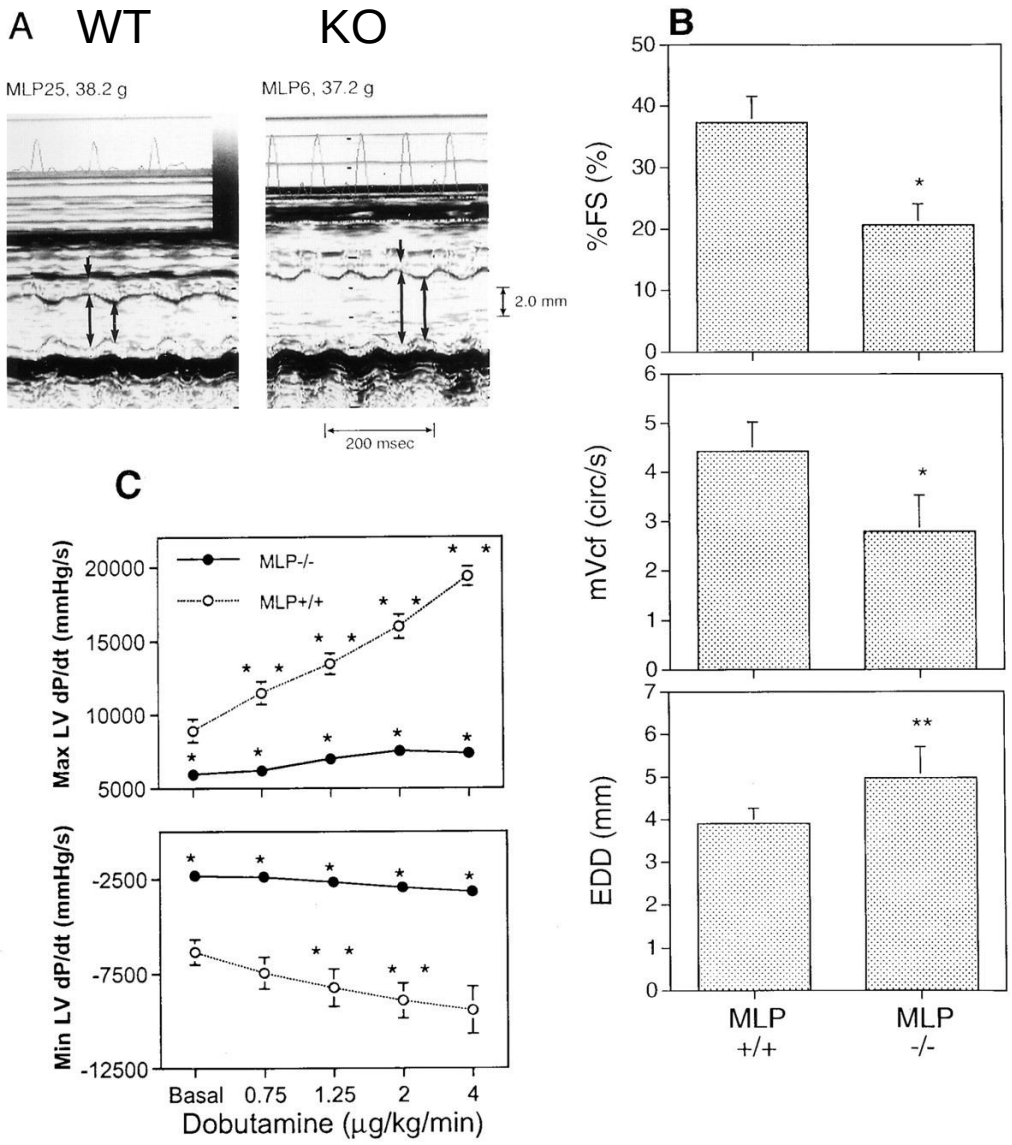
Strategy: MLP$^{-/-}$



Phenotype: MLP$^{-/-}$



Phenotype: MLP$^{-/-}$



Phenotype: $MLP^{-/-}$

- MLP knockout animals develop a dilated cardiomyopathy heart failure phenotype.
- MLP deficient animals were the first genetically altered mouse model to develop this phenotype.

Gene Targeting

- ...But what if deletion of a gene is embryonically lethal, and you don’t get viable offspring???

How a conservative site-specific recombination enyzme from bacteria can be used to delete specific genes from particular mouse tissues

- This approach requires the insertion of two specially engineered DNA molecules into the animals germ line. The first contains the gene for a recombinase (in this case the Cre recombinase from the bacteriophage P1) under the control of a tissue-specific promoter, which ensures that the recombinase is expressed only in that tissue. The second DNA molecule contains the gene of interest flanked by recognition sites (in this case lox P site) for the recombinase.

How a conservative site-specific recombination enyzme from bacteria can be used to delete specific genes from particular mouse tissues

- The mouse is engineered so that this is the only copy of this gene. Therefore, if the recombinase is expressed only in the liver, the gene of interest will be deleted there, and only there!
- Many tissue specific promoters are known and available. Moreover many of these promoters are active only at specific times in development. Thus it is possible to study the effect of deleting specific genes at many different times during the development of each tissue.

Cre mediated recombination

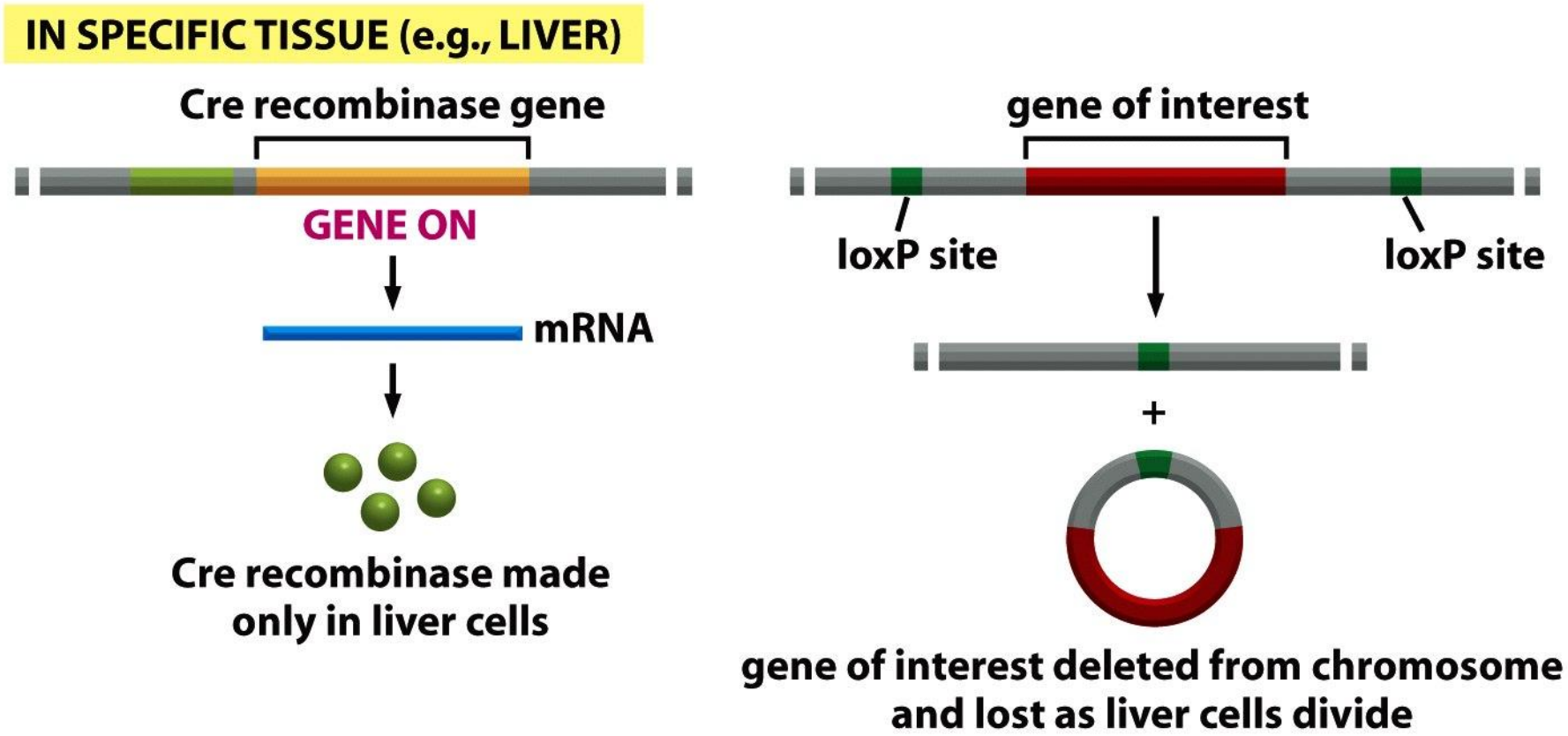


Figure 5-79a *Molecular Biology of the Cell* (© Garland Science 2008)

Cre mediated recombination

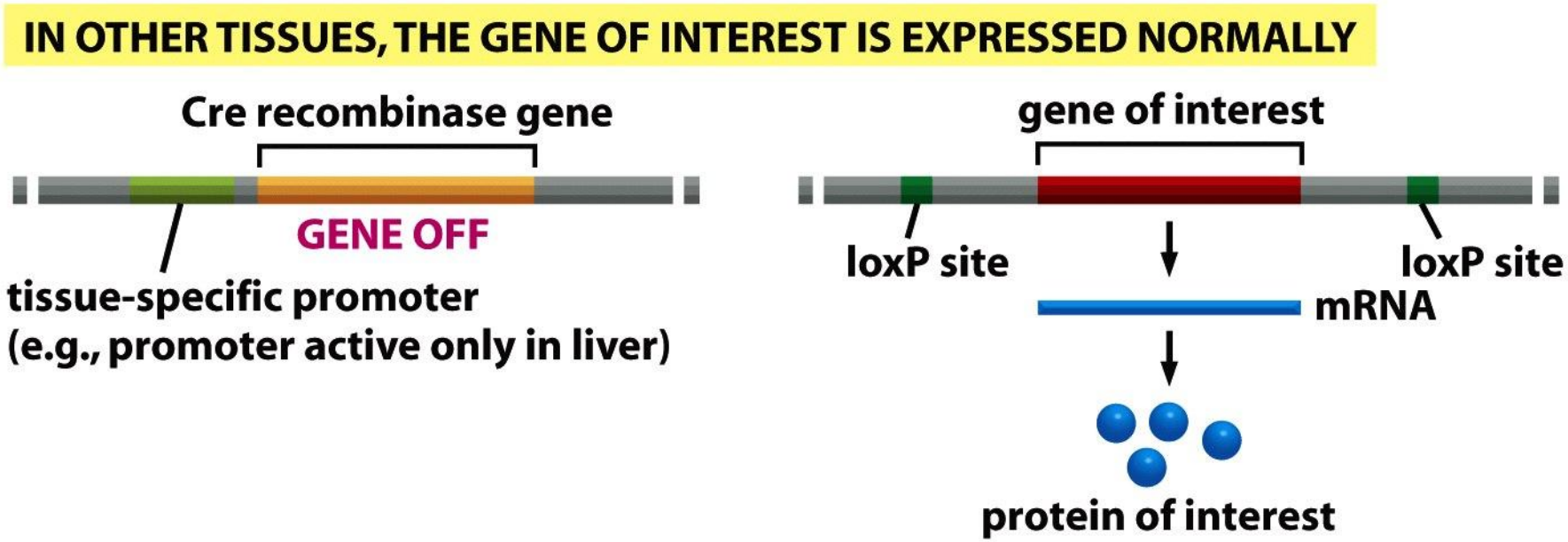
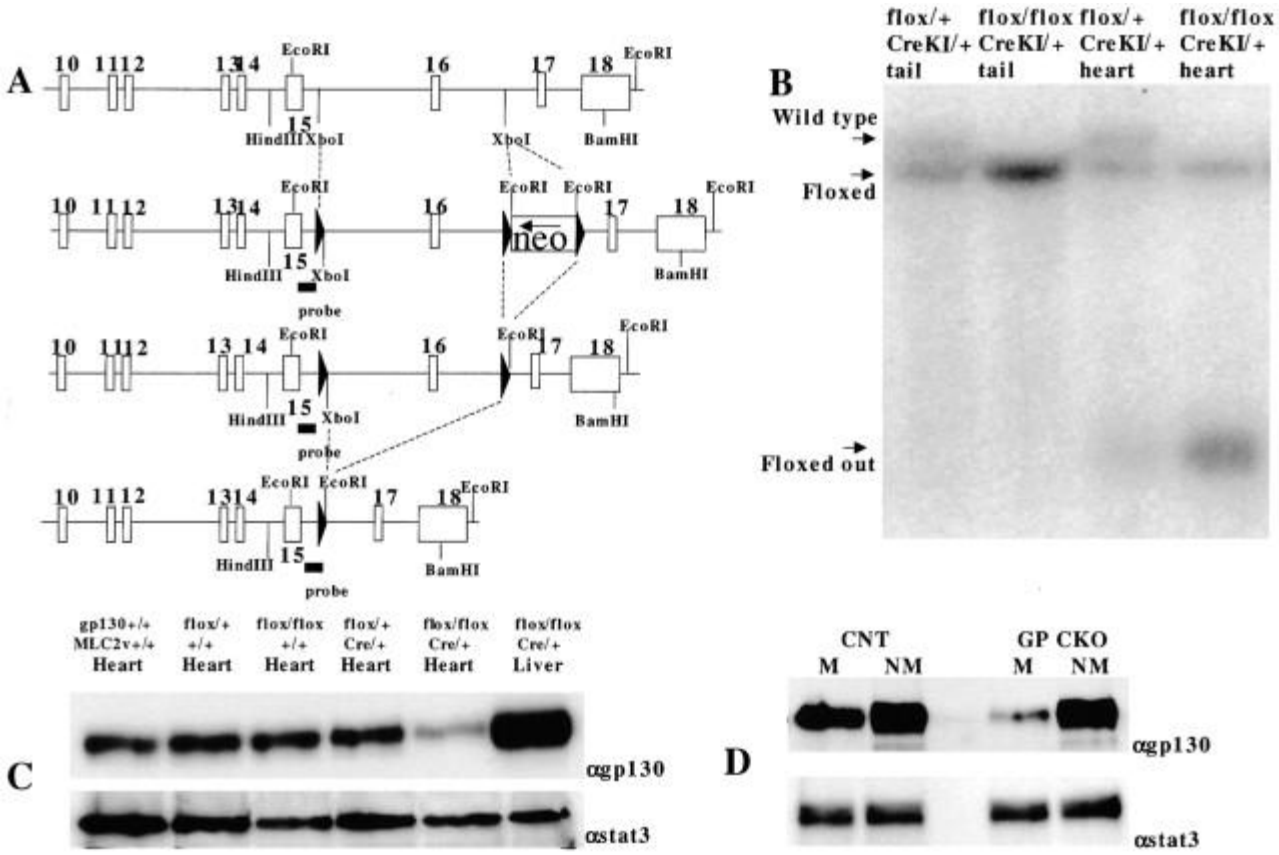


Figure 5-79b *Molecular Biology of the Cell* (© Garland Science 2008)

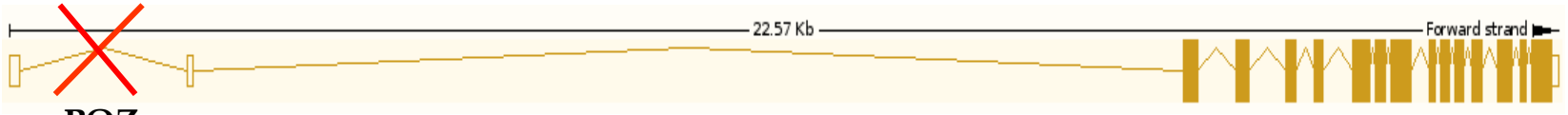
gp130

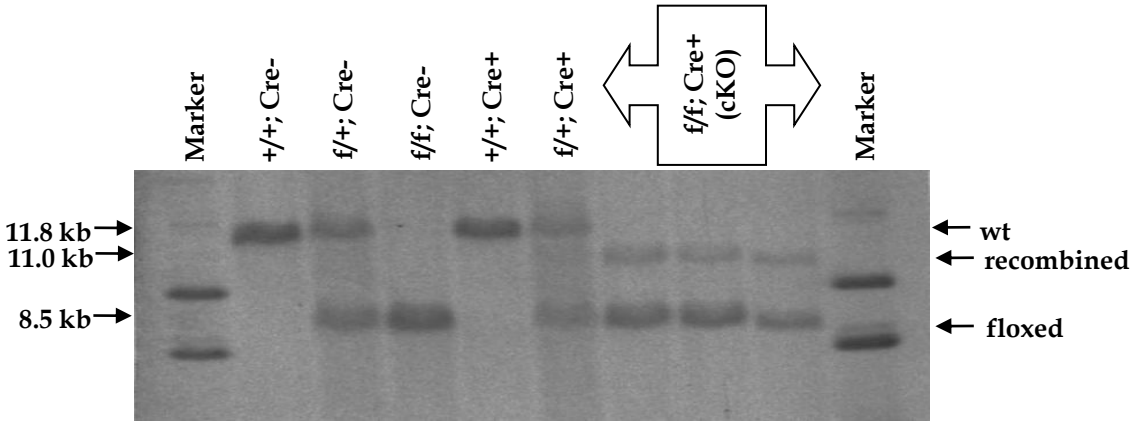
- Gp130 is a cytokine receptor expressed in a variety of different cells
- Conventional gp130 knockout results in embryonic lethality, thus no viable gp130 animals are born and the heart can’t be analyzed.

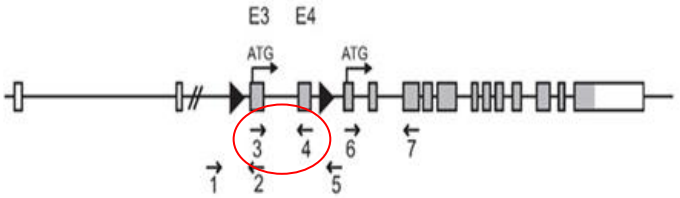
gp130 myocardium specific$^{-/-}$

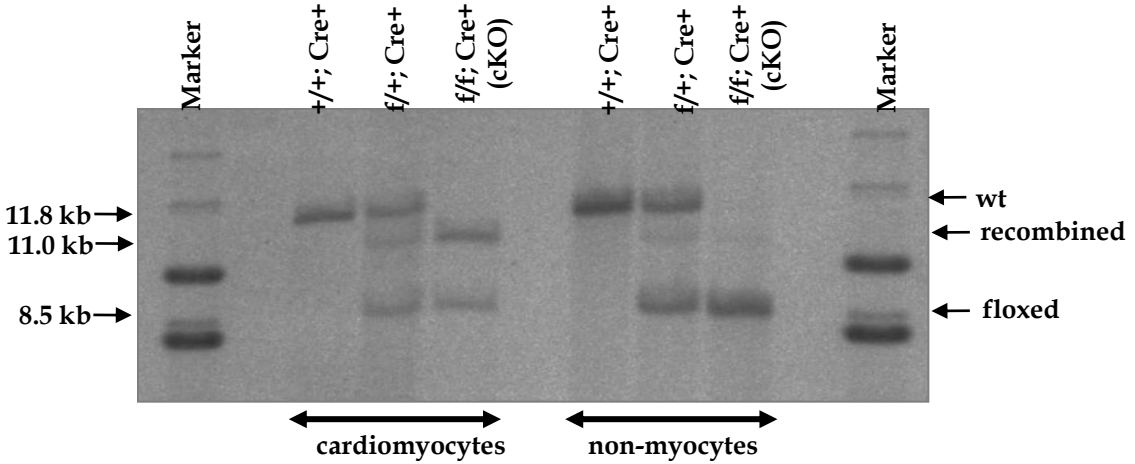


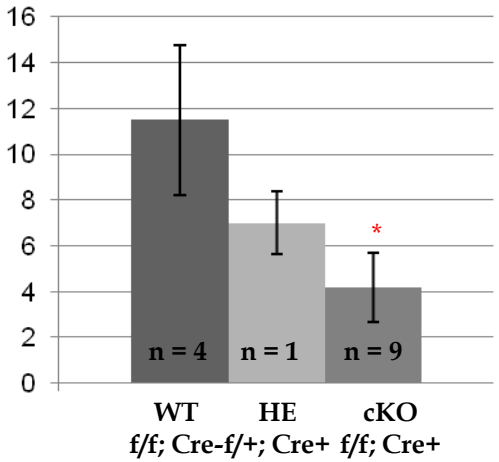
MiP1 conditional knockout mouse









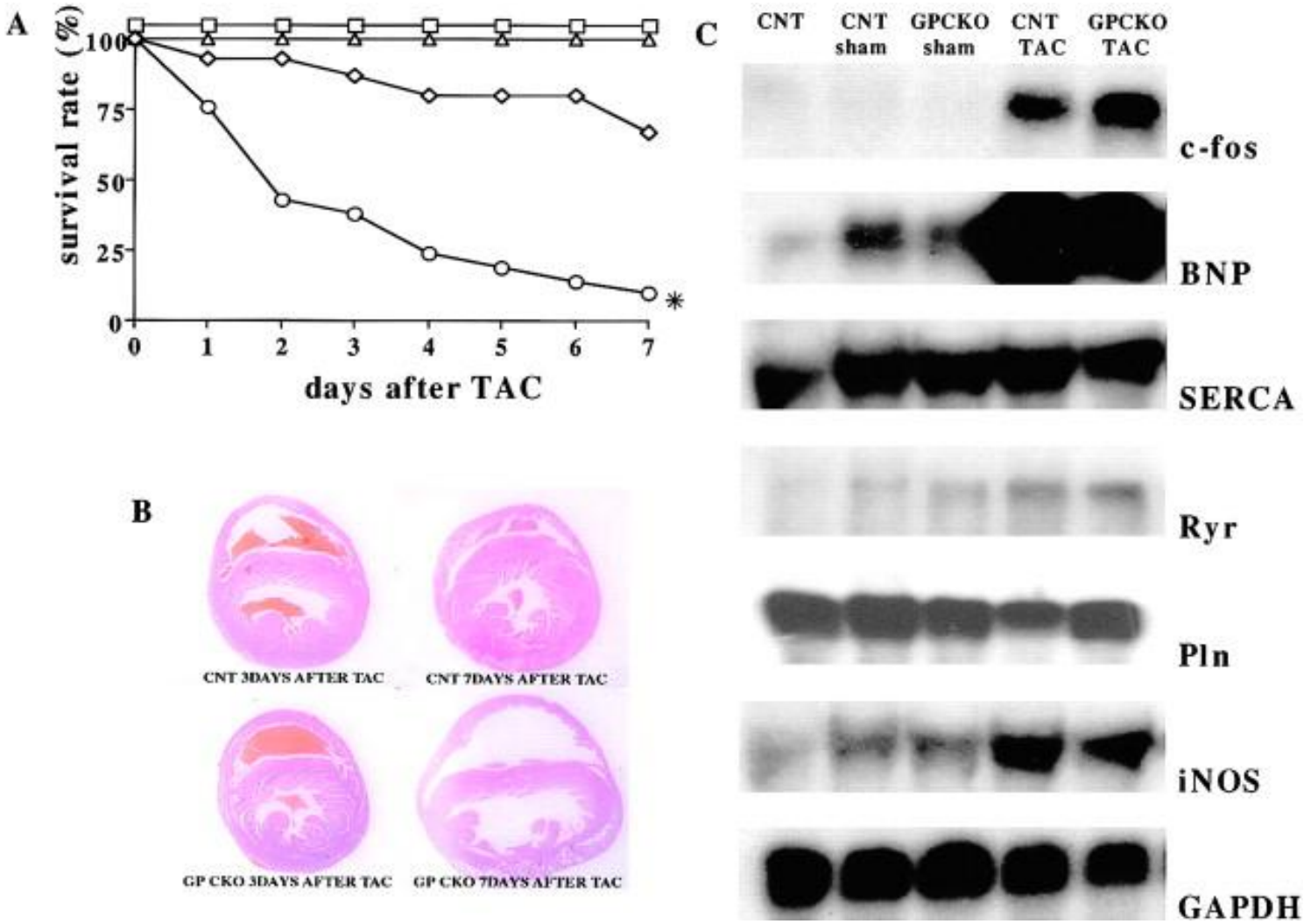


cKO/WT = 0,36

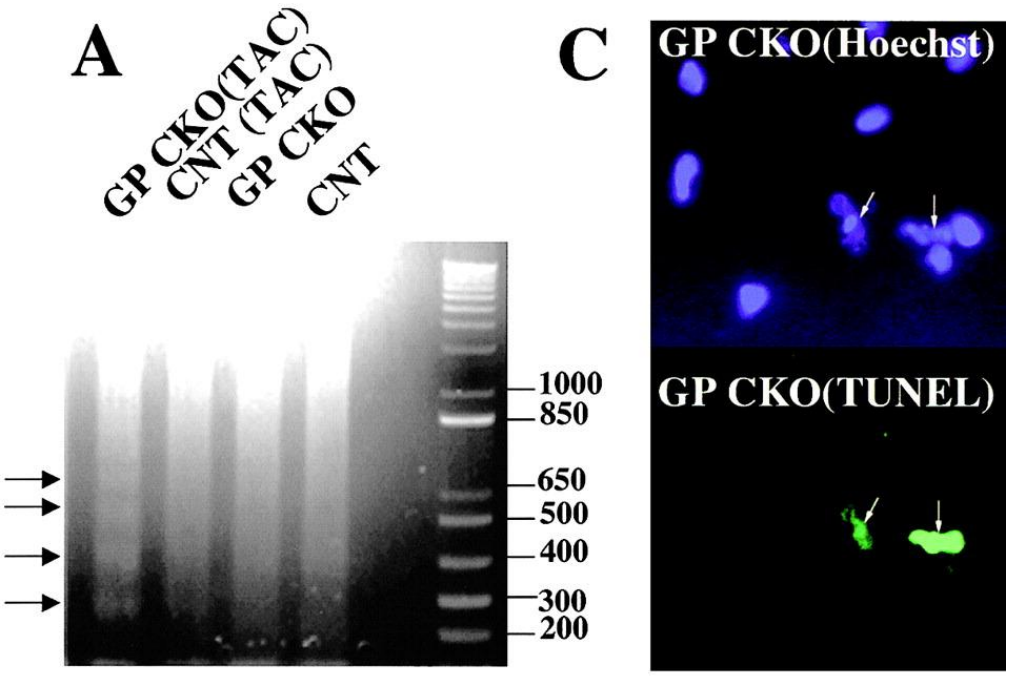
* P = 3,778E-08

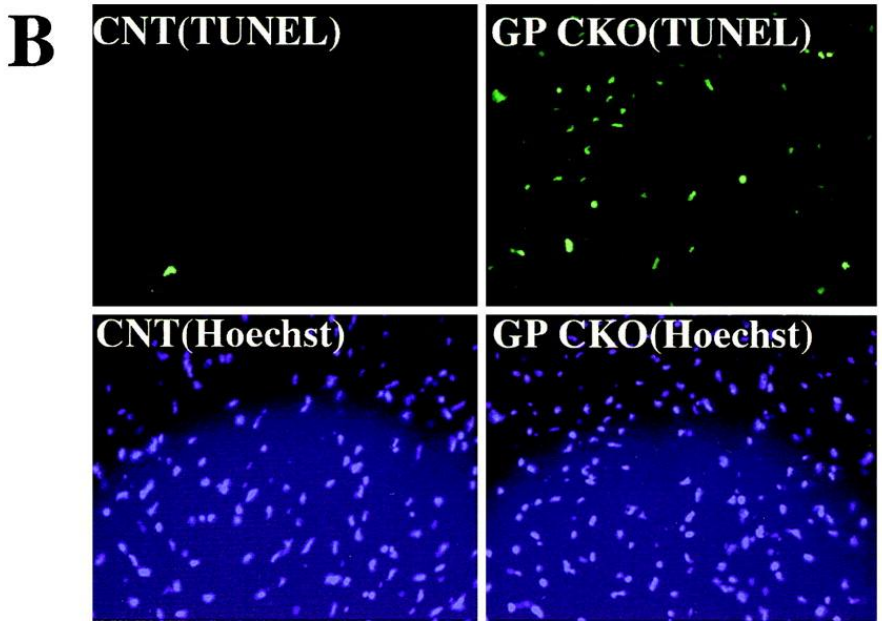
HE/WT = 0,61

Phenotypic Effects of Biomechanical Stress on GP130 Conditional KO Mice

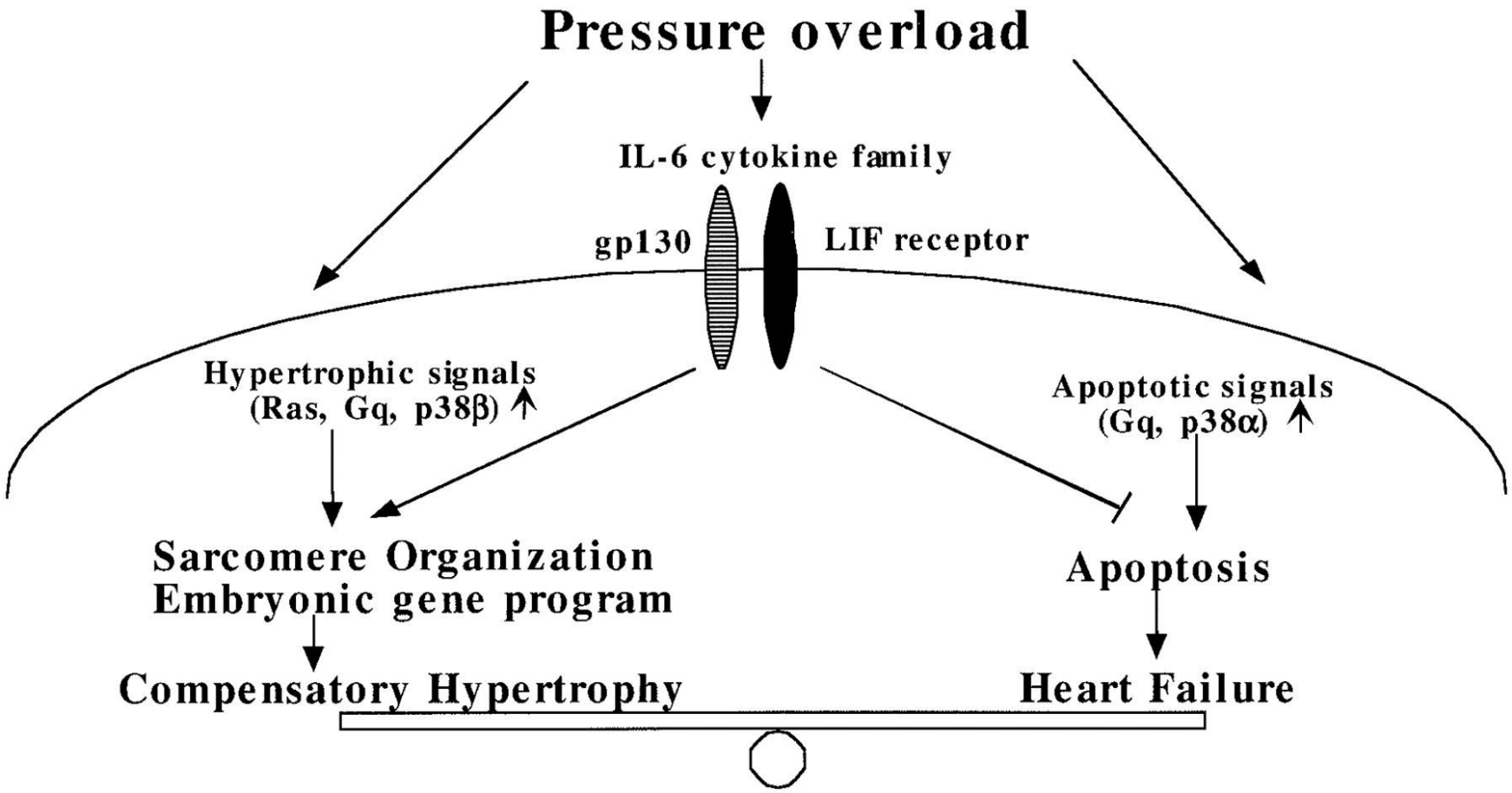


Analysis of gp130$^{-/-}$: apoptosis





Summary: gp130$^{-/-}$



Summary: gp130$^{-/-}$

- Gp130 myocardium specific knockout animals were the first conditional knockout animals in the cardiovascular system.

Other types of recombinases: FLP-FRT recombinase

- It is analogous to Cre-Lox recombination. It involves the recombination of sequences between short Flippase Recognition Target (FRT) sites by the Flippase recombination enzyme (FLP or Flp) derived from the 2µm plasmid of the baker's yeast *Saccharomyces cerevisiae*.

Tissue specific promoters

- Alpha myosin heavy chain promoter (αMHC)
- Myosin light chain 2 v (MLV2v)

...and what we don't know

- Although large international consortia aim to knockout every single gene in mouse genome, so far only 65% of genes are knocked out
- (EU: "Conditional Mouse mutagenesis Programme"
- (USA: "The National Institutes of Health" "Texas Institute for Genomic Medicine" "North American Conditional Mouse mutagenesis Program"

...and what we don't know

- Another very important issue:
- The basic helix-loop-helix transcription factor MRF4 has been knocked out by three different groups:

  Arnold, Olson, and Wold laboratories

  with the striking result of three completely different phenotypes ranging from complete lethality to unaffected viability.

  Why?

...and what we don't know

- In this particular case, the cis-acting effects of the different alleles created on an adjacent gene locus *myf5* were responsible.

...and what we don’t know

- In addition, although important, just to inactivate a particular gene does not help much....sometimes you may wish to test the effects of certain “point mutations” or the function of different domains.

RNA interference

- **RNA interference** (**RNAi**) is a system within living cells that helps to control which genes are active and how active they are. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference.