The Role of microRNAs in Pain

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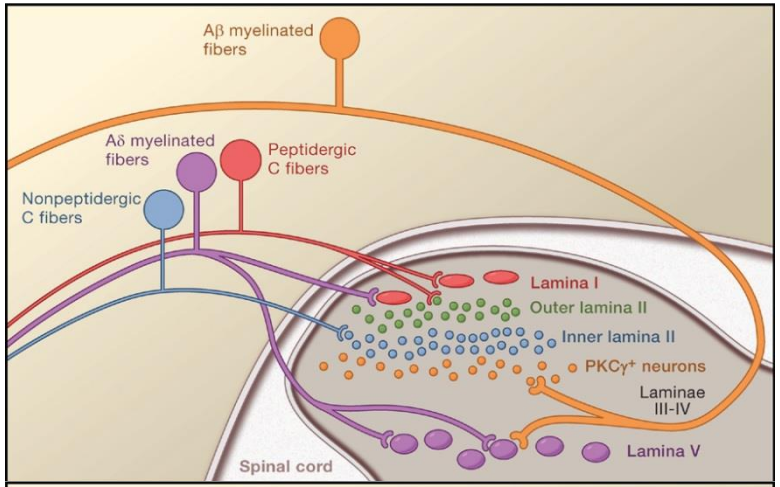
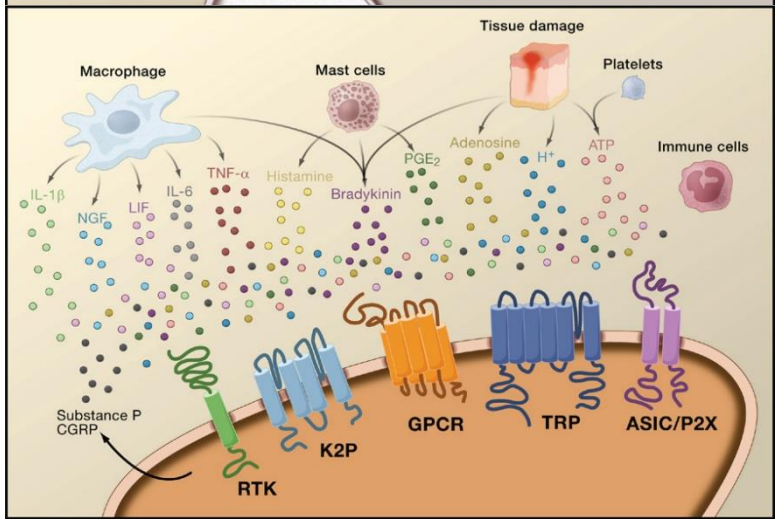
Motivation for molecular analysis of Pain

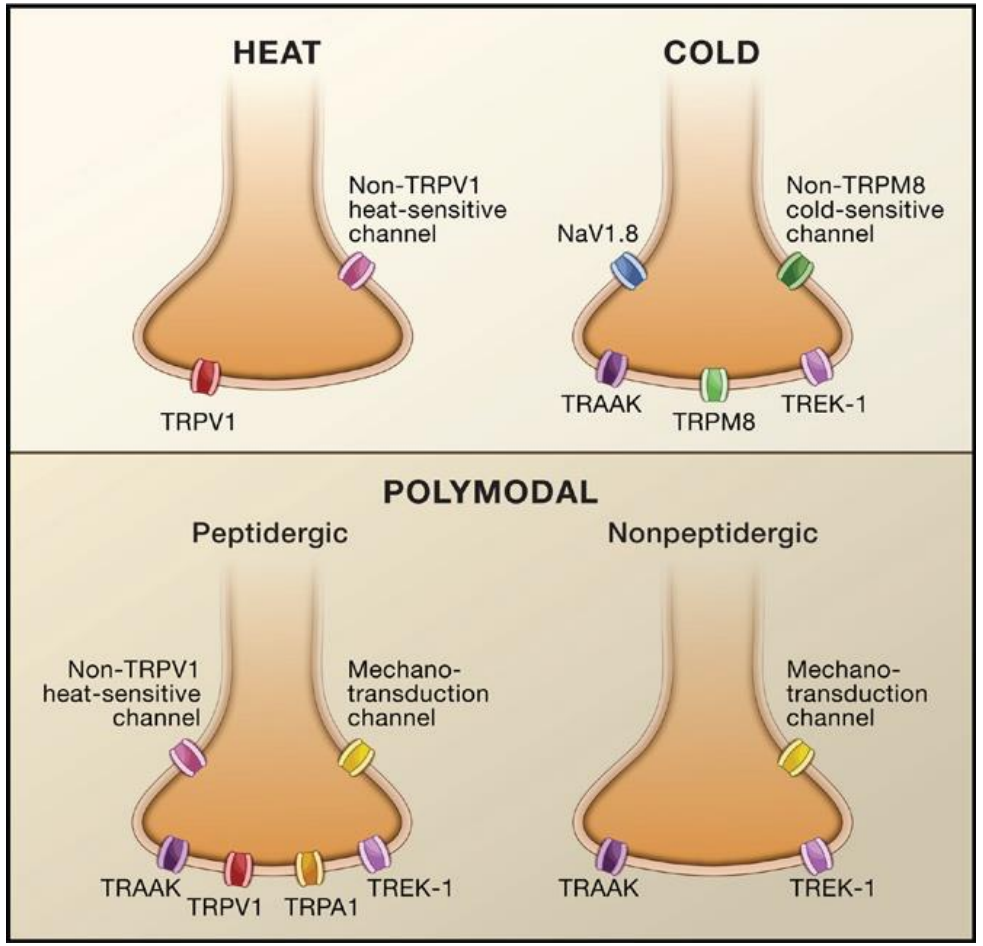
- Chronic pain one of the most common causes for seeking medical treatment
- Approximate cost to the US economy 635 billion dollars
- Current treatments effective only in 1/2 of patients.
- Complex etiology
  - damage or disease affecting any part of the nervous system
  - spinal cord injury, diabetes, alcoholism, chemotherapy, chronic viral infection, multiple sclerosis, and strokes
- Subjective assessment
- Need Biomarkers

Wong-Baker Faces Pain Rating Scale

Pain Biology







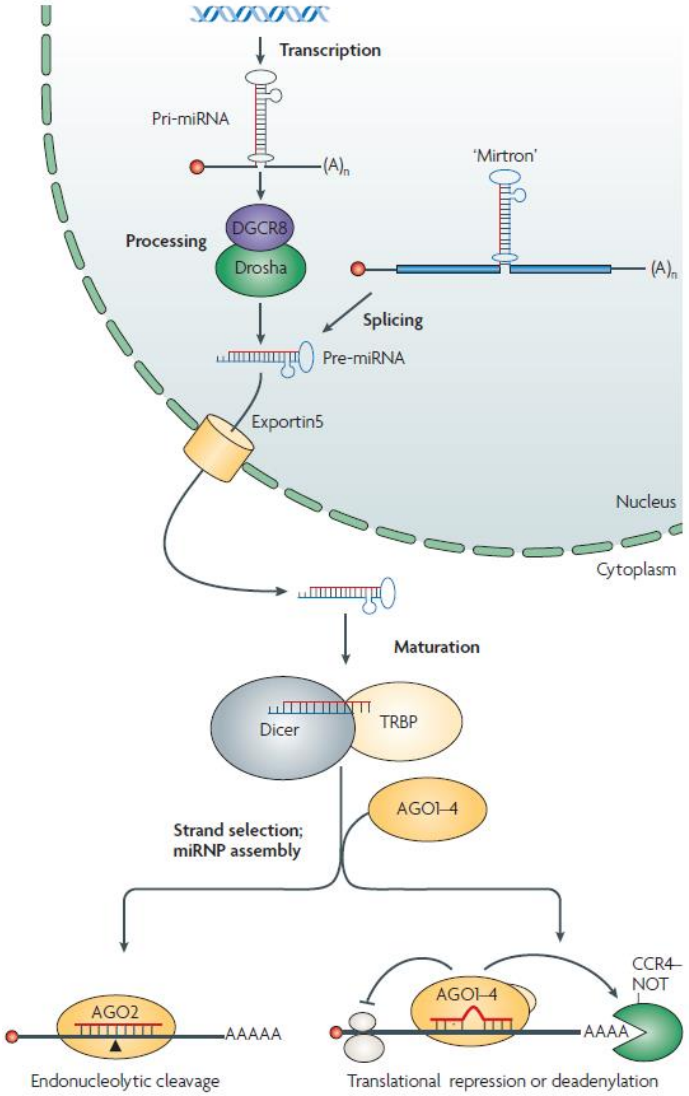
Basbaum AI, Bautista DM, Scherrer G, Julius D: **Cellular and Molecular Mechanisms of Pain**. *Cell* 2009, **139**(2):267-284.

microRNA

- Short RNA sequences
  - 17-22 nucleotides
  - Lin-4 and let-7 identified in C. Elegans
  - Classified in 2000
- miRNA causes downregulation of target genes
  - Degradation of mRNA transcript (more common in plants)
  - Translational repression is more common in mammals
- Involved in many diseases including cancer

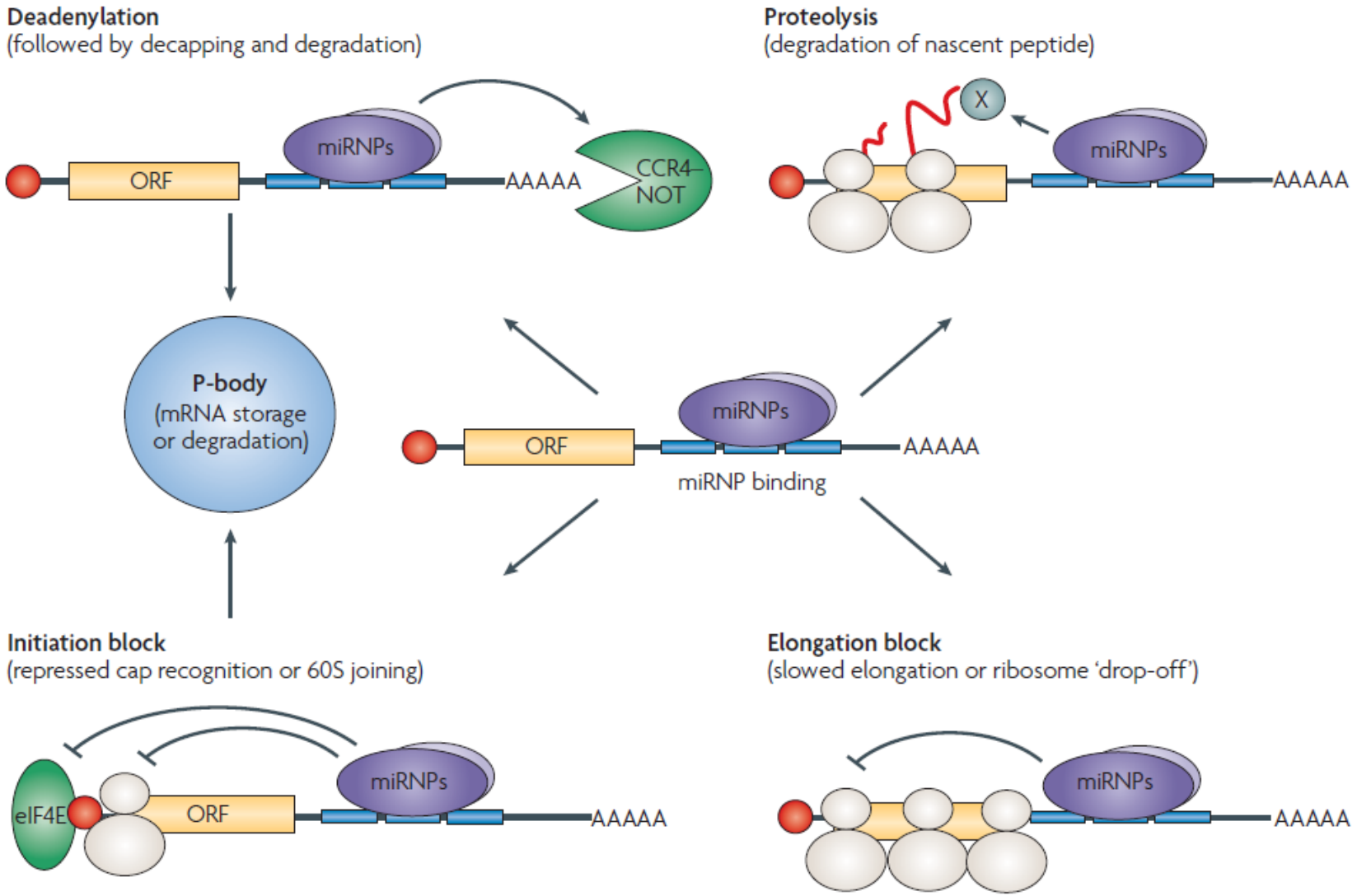
Biogenesis of miRNAs

- miRNA transcribed by RNA Pol II into pri-miRNA
- Pri-miRNA cleaved into pre-miRNA by Drosha and DGCR8
- Transported into cytoplasm by exportin-5
- Cleaved into miRNA by Dicer



Filipowicz et al. 2008 *Nature Reviews*

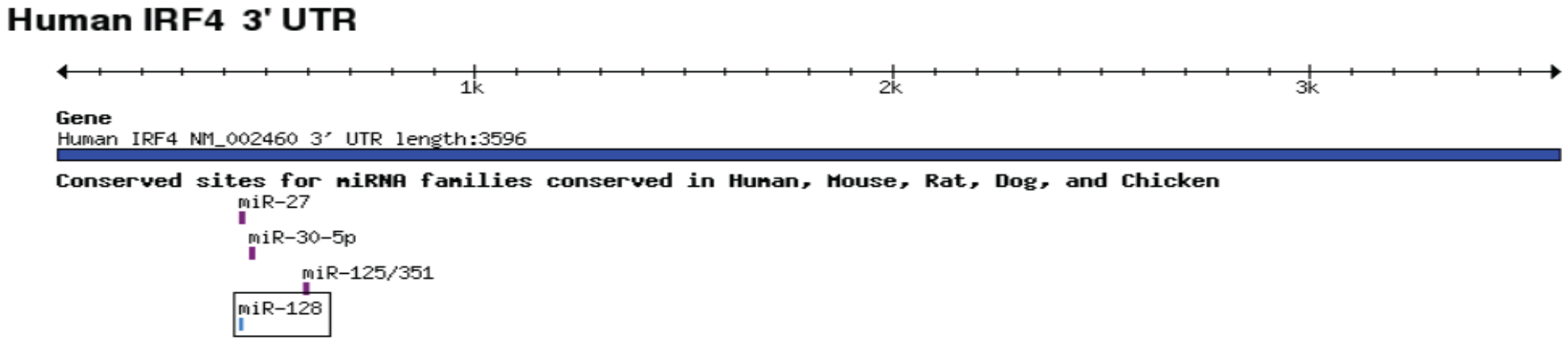
Mechanisms of Post-Transcriptional Regulation



Filipowicz et al. 2008 *Nature Reviews*

Computational Target Prediction

- Step 1: Identify miRNA binding sites in mRNA sequence
- Step 2: Assess prediction confidence by examining cross-species conservation of binding site



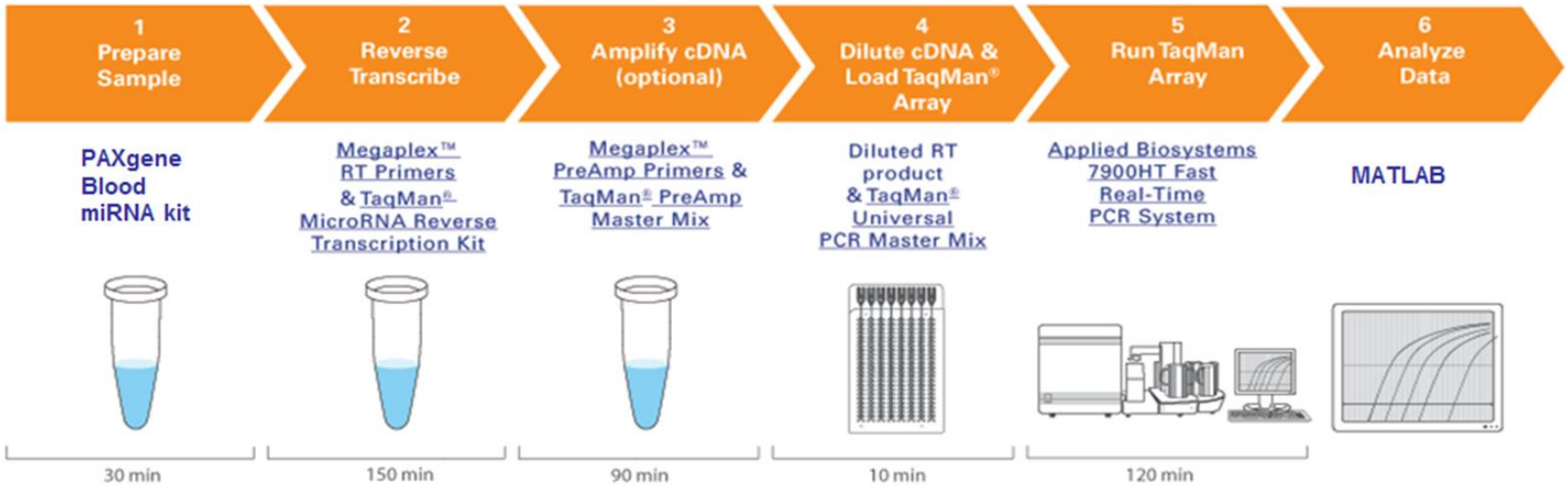
Circulating miRNAs

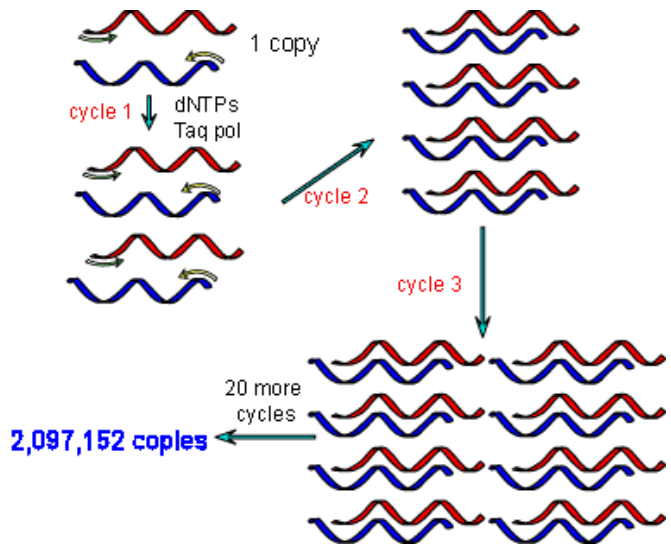
- miRNAs stable in extracellular fluid
  - Blood, saliva, CSF, urine
- In blood miRNAs may be packaged in exosomes or bound to Ago proteins
- miRNAs released into circulation
  - Macrophage derived exosomes
  - Dead or dying cells
  - In response to tissue injuries
- Circulating miRNAs are a source of biomarkers.

Goals of this study

- Identify circulating miRNAs as potential pain biomarkers
  - miRNA profiles to predict patient responsiveness to treatment
  - Enrich pre-clinical drug discovery
- Explore mechanisms of regulation of pain by circulating miRNAs

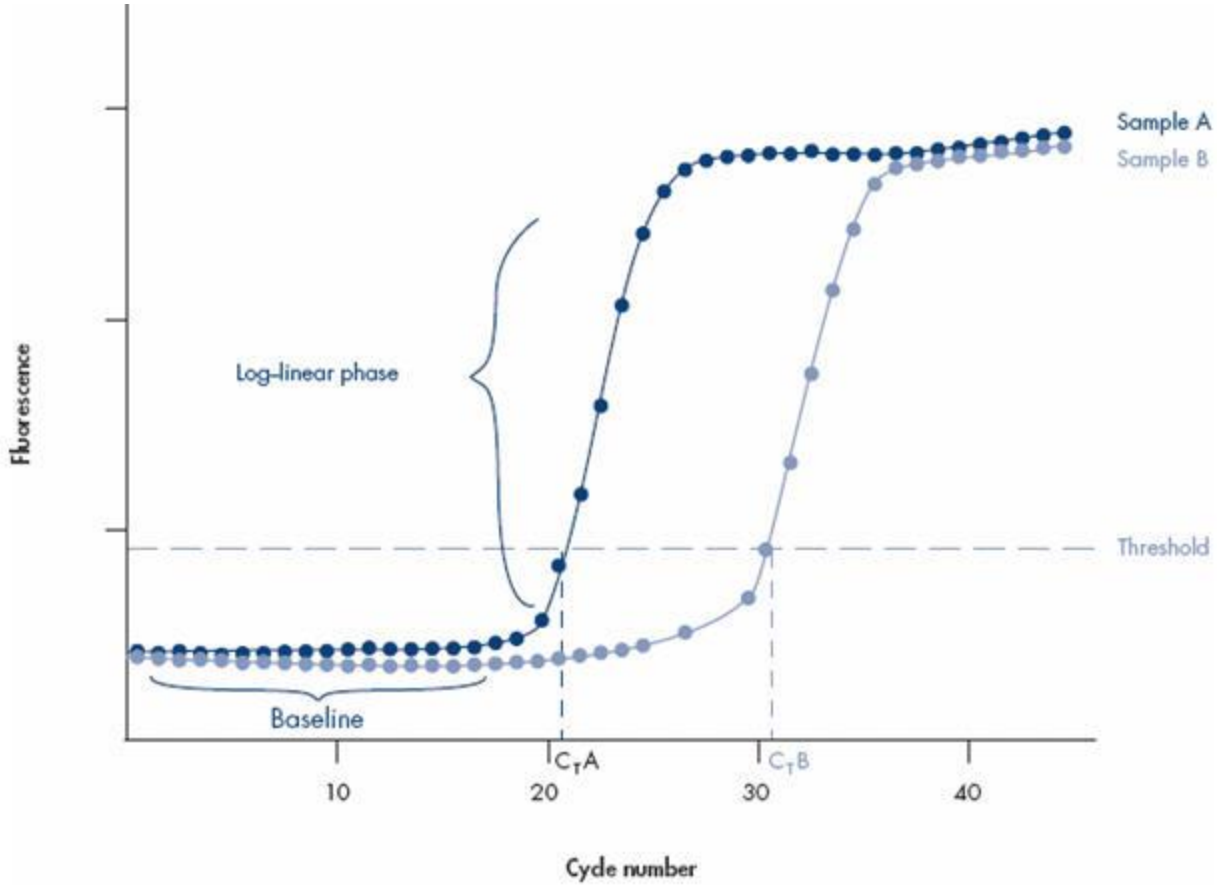
RT-PCR





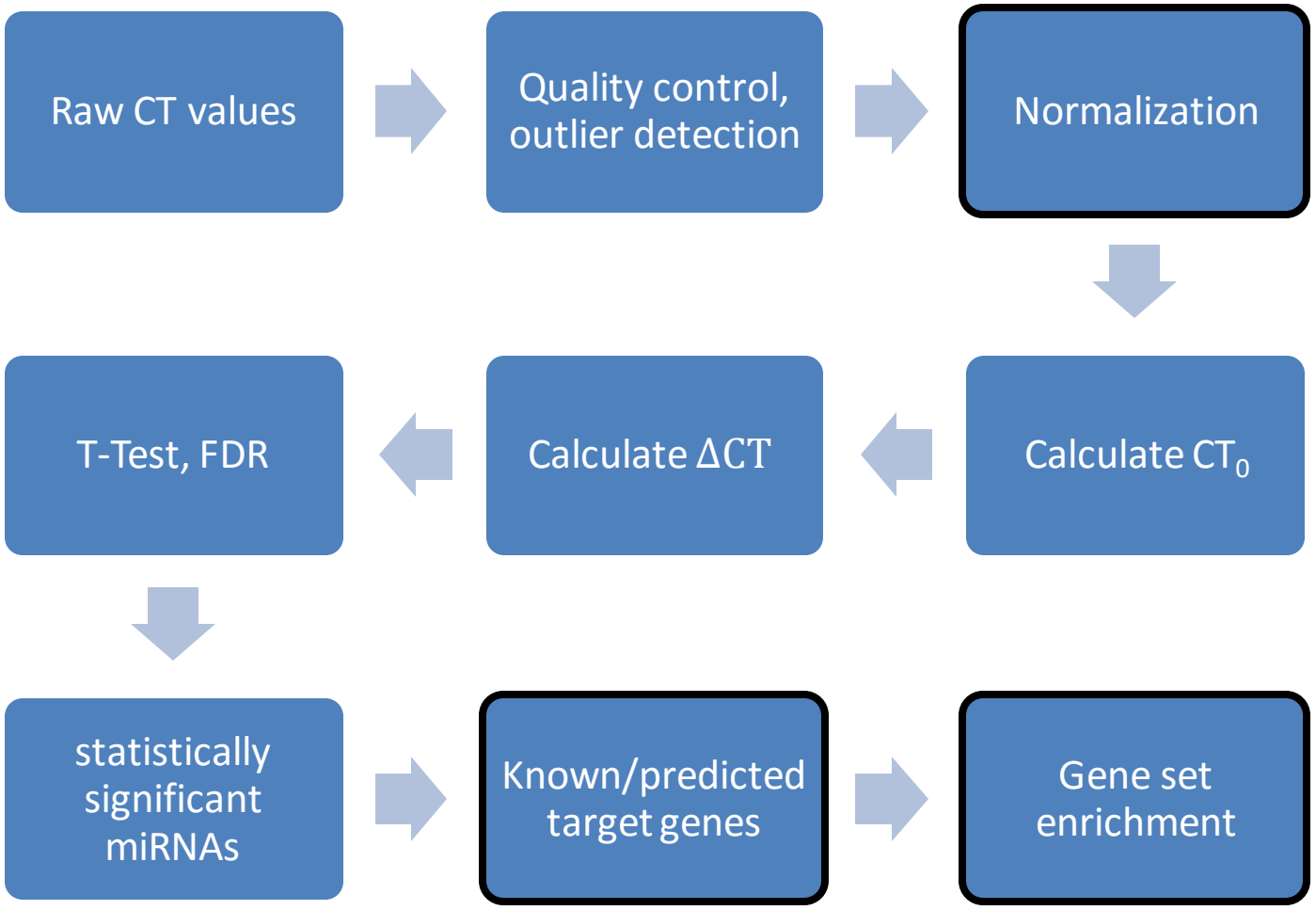
http://www.scq.ubc.ca/polymerase-chain-reaction/

Cycle Threshold



http://www.qiagen.com/resources/info/guidelines_rtpcr/dataanalysis_sybr.aspx

Data Analysis Pipeline



Normalization and Calculation of Fold Change

$$\Delta CT = CT - CT_0 \quad \textbf{(1)}$$
$$\Delta\Delta CT = \Delta CT - \Delta CT_{ctrl} \quad \textbf{(2)}$$
$$FC = 2^{-\Delta\Delta CT} \quad \textbf{(3)}$$

- Select endogenous control (CT_0) and use it to adjust CT values of all samples
  - Endogenous controls have high expression and low variance across multiple samples
  - Small nuclear RNAs (snRNAs) often used

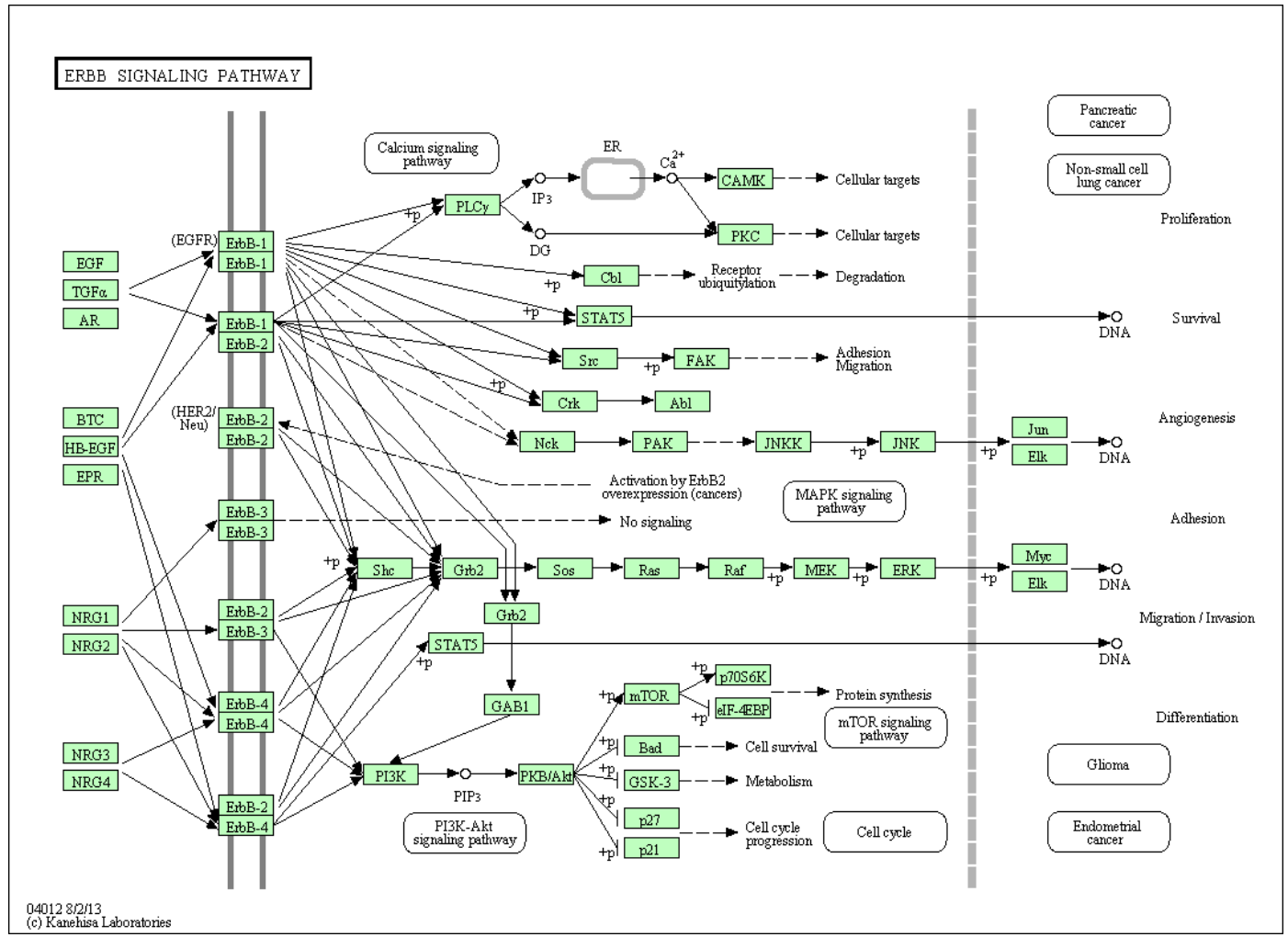
Normalization Methods

- Endogenous Controls such as RNU44 and RNU48
  - Caution: Detected to be significantly different
  - Many endogenous controls are differentially expressed in cancer
- Mean normalization
- Z-normalization
- Quantile normalization

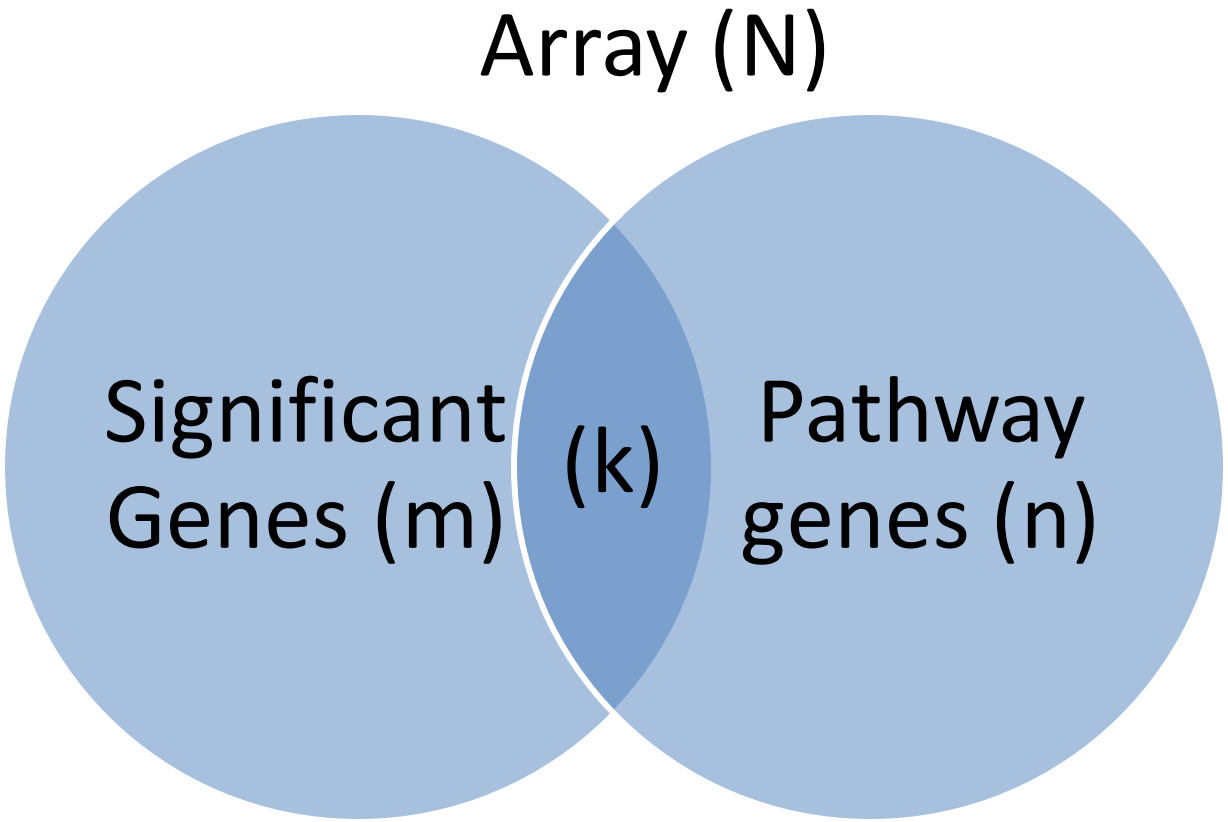
Gene Set Enrichment

- Optimize pathway enrichment methodologies in order to characterize the gene regulatory networks and molecular interaction pathways affected by differential miRNA regulation in pain

An Example Pathway

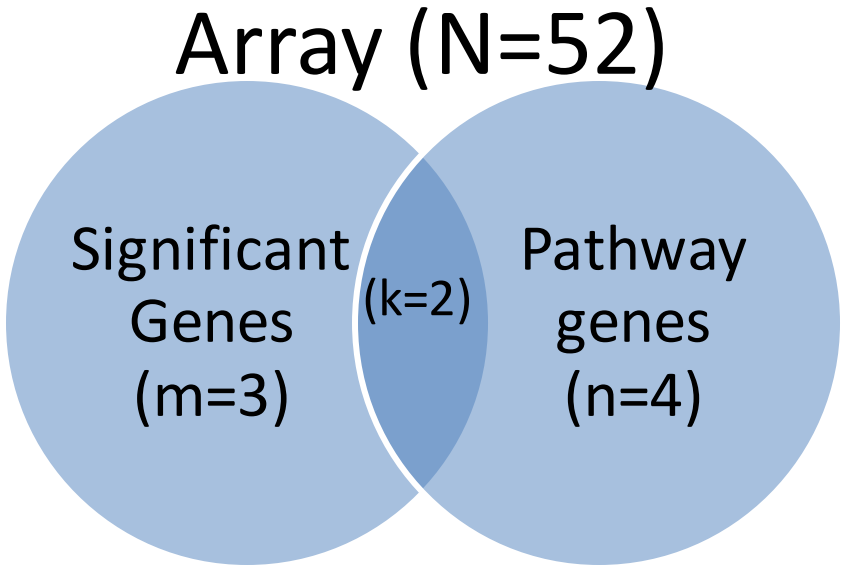


Probability that *k* significant genes are also in the Pathway



Exercise

- From a deck of cards (N=52), m=3 cards are randomly selected.
- What is the probability of exactly k=2 cards being Aces? (Number of Aces in deck: n=4)



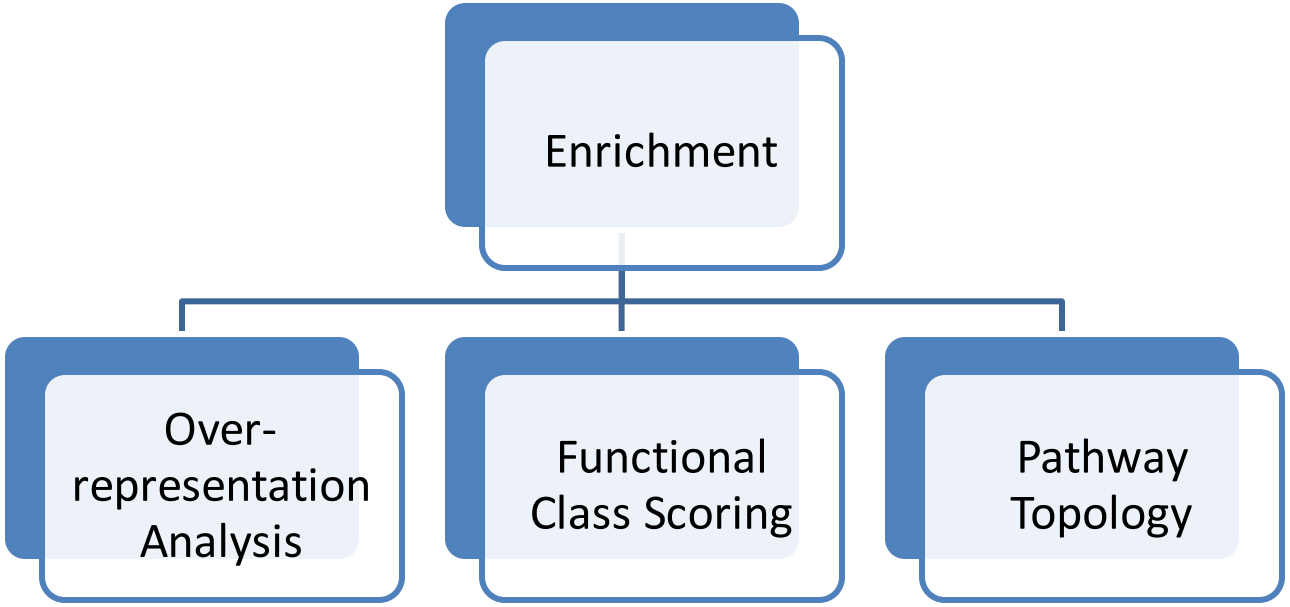
Probability at least *k* significant genes are present in the pathway

- Hypergeometric Probability (exact):
  - $P(X > k) = 1 - \sum_{r=0}^{k} \frac{\binom{m}{r} \times \binom{N-m}{n-r}}{\binom{N}{n}}$
- X^2 (approximation):
  - $\chi^2 = \frac{N(|n_{11}n_{22} - n_{12}n_{21}|)^2}{N_{1r}N_{2r}N_{1c}N_{2c}}$

$$df = (r-1)(c-1)$$

	Genes on Array	Significant Genes	
In Pathway	n_{11}	n_{12}	$N_{1r}=n_{11} + n_{12}$
Not in Pathway	n_{21}	n_{22}	$N_{2r}=n_{21} + n_{22}$
	$N_{1c}=n_{11}+n_{21}$	$N_{2c}=n_{12}+n_{22}$	$N=n_{11}+n_{12}+n_{21}+n_{22}$

Enrichment



- Hypergeometric
- Chi-squared
- Binomial

- GSEA
- PAGE
- SAM-GS

- Pathway-Express
- SPIA
- ScorePage

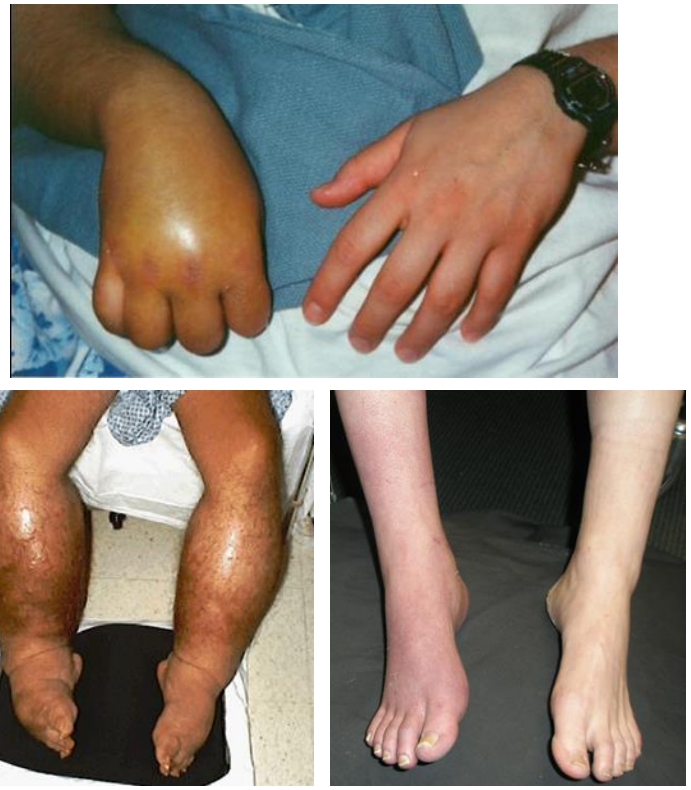
CRPS

- Characterize the miRNA expression in the blood of Complex Regional Pain Syndrome (CRPS) patients

Complex Regional Pain Syndrome (CRPS)

- CRPS is a chronic, painful and progressive neurological disease that affects skin, muscles, joints and bones
- CRPS is characterized by various degrees of burning pain, excessive sweating, swelling and sensitivity to touch
- Compare expression of miRNAs in blood from patients with CRPS and healthy age and sex matched controls

Complex Regional Pain Syndrome (CRPS)



Scores recorded for different types of pain

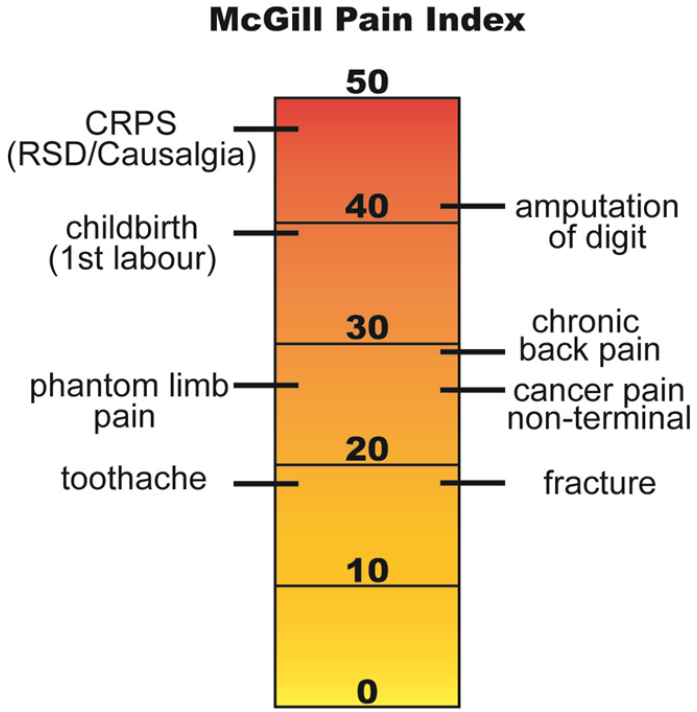


Figure adapted from Katz and Melzack (1999)

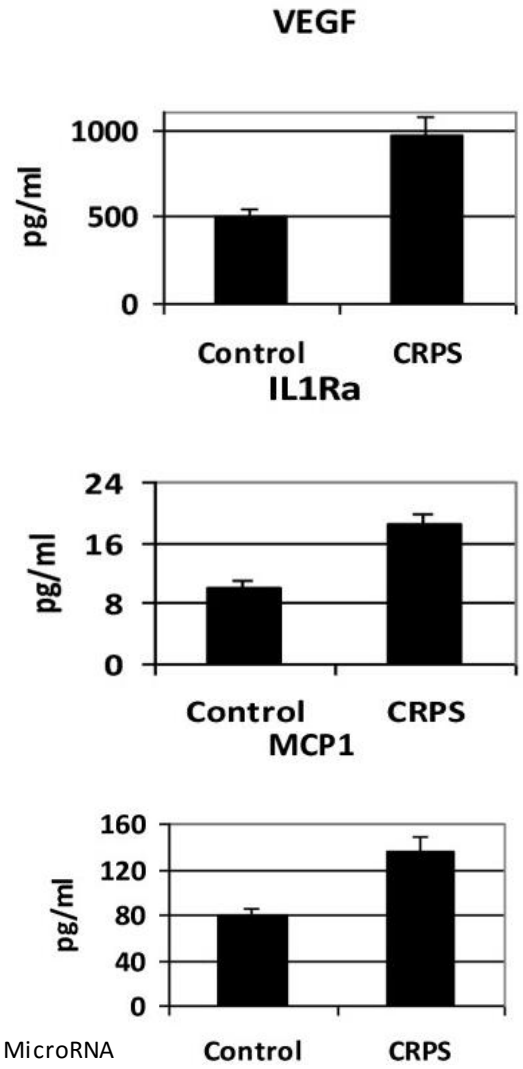
CRPS Study Design

- Profiled serum microRNAs CRPS patients and controls
  - Identify potential miRNA biomarkers
- 20 Controls
- 41 CRPS Patients
- Analyzed blood miRNA levels by qPCR
- Correlated miRNA expression with other data
  - Cytokine levels
  - Co-morbidities
  - Pain level

CRPS Circulation

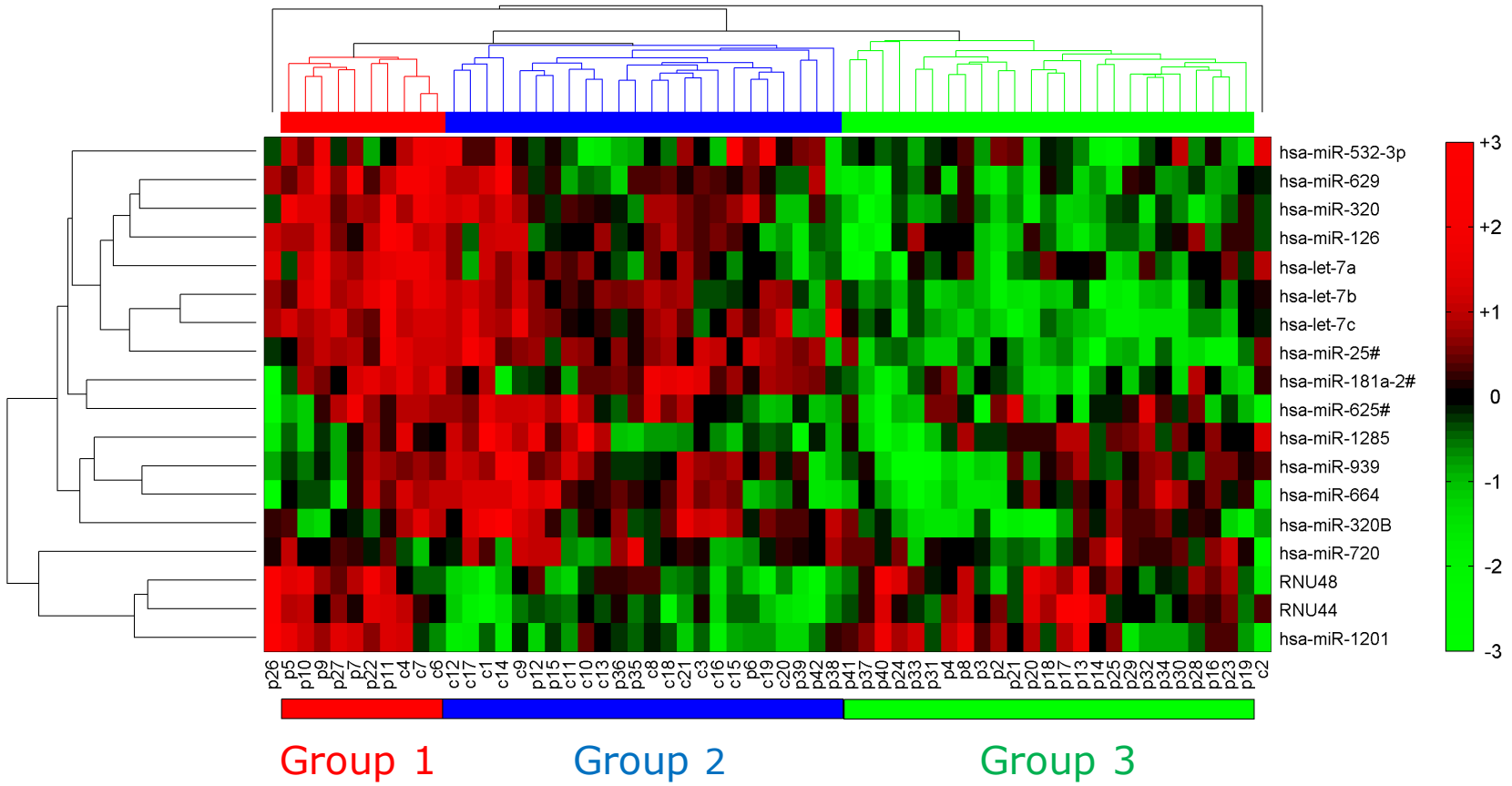
miRNA	Fold change	p value
hsa-miR-939	-4.59358	5.55E-06
hsa-miR-25#	-3.92328	1.09E-06
hsa-let-7c	-2.53502	2.07E-05
hsa-let-7a	-2.45923	0.002308
hsa-let-7b	-2.40344	5.49E-05
hsa-miR-320B	-2.0527	6.91E-06
hsa-miR-126	-2.02362	0.002469
hsa-miR-629.A	-1.71231	0.000653
hsa-miR-664	-1.54881	0.001487
hsa-miR-320	-1.44273	7.28E-05
hsa-miR-1285	-1.41594	0.003077
hsa-miR-625#	-1.33174	0.003542
hsa-miR-532-3p	-1.27226	0.001226
hsa-miR-181a-2#	-1.25927	0.000229
RNU48	1.348125	0.000391
hsa-miR-720	1.476853	0.003243
RNU44	1.854213	0.000904
hsa-miR-1201	2.14584	3.17E-05

Fold changes and p values of significantly altered miRNAs (minus sign indicates down-regulation; data sorted based on fold change). The statistical significance was calculated using 2-tailed t-tests on the miRNA expressions in CRPS patients versus control samples.



Orlova I*, Alexander G*, **Qureshi R***, Sacan A, Graziano A, Barrett J, Schwartzman R, Ajit S: MicroRNA modulation in complex regional pain syndrome. *Journal of Translational Medicine* 2011, 9(1):195

CRPS Clustering



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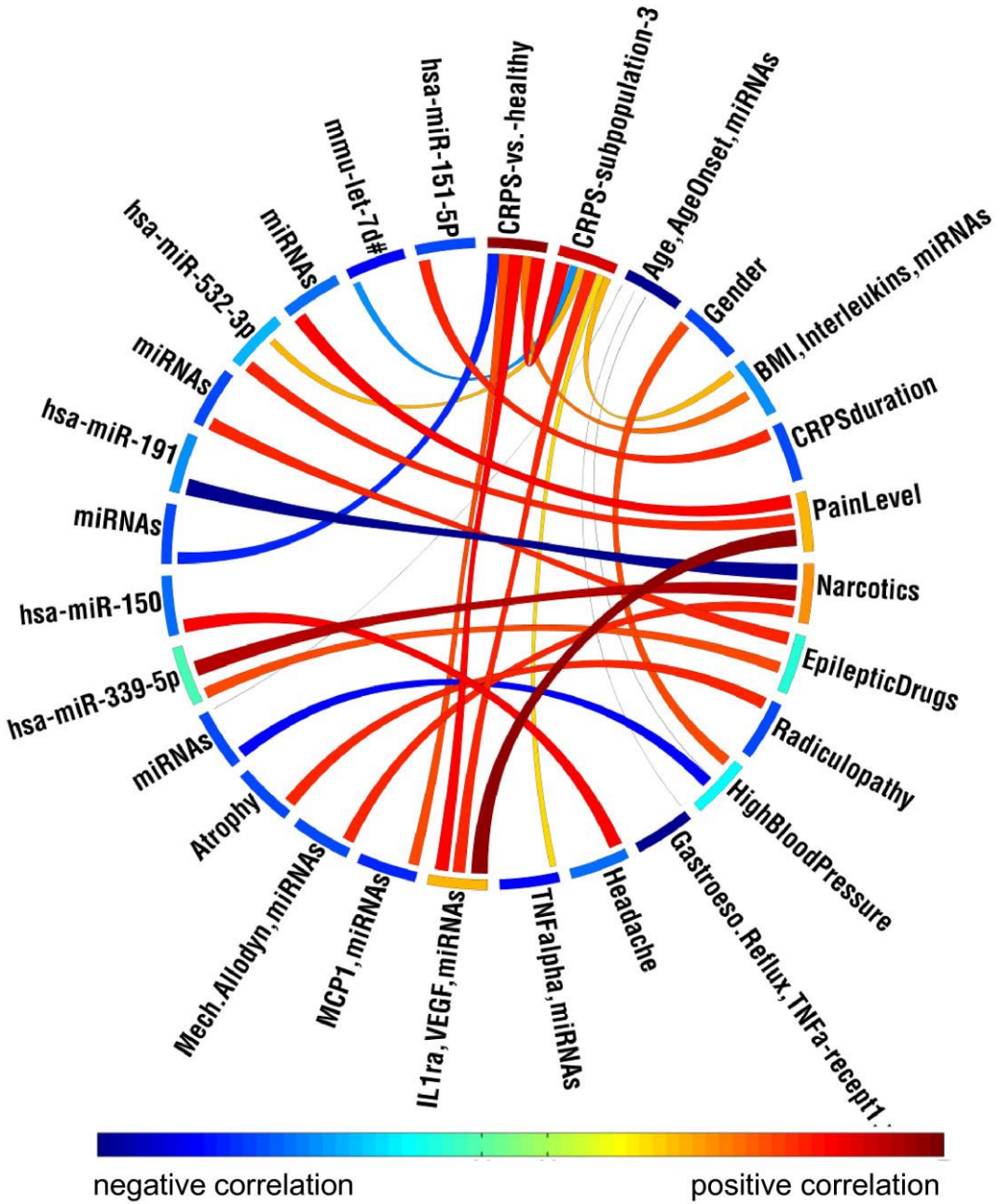
Correlation Analysis



miRNA correlations

- mir-532-3p → CRPS type
- mir-151-5p → CRPS duration
- mir-296-5p, 361-3p, 532-3p, 30d → Pain Level
- mir-92a, 484 → hypertension
- 219-1-3p, 204 → high cholesterol
- 150 → headache
- 339, 30c, 192, 140-3p, 152, 145 → narcotics
- 296-5p, 1290, 423-5p, 25, 1270 → BMI

Orlova I*, Alexander G*, **Qureshi R***, Sacan A, Graziano A, Barrett J, Schwartzman R, Ajit S: MicroRNA modulation in complex regional pain syndrome. *Journal of Translational Medicine* 2011, 9(1):195

miRNA meta-analysis

- Evaluate changes in miRNA expression in the blood of several rodent models of pain and characterize miRNA response to therapeutic intervention

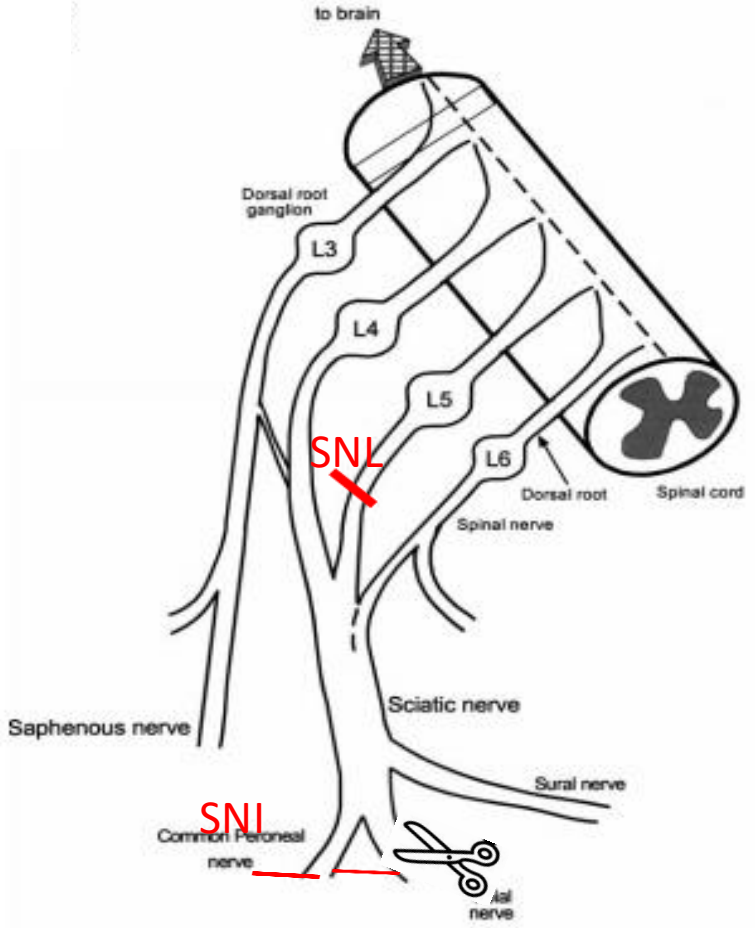
Animal Models of Pain

- Inflammatory Pain
  - Mice injected with Complete Freund's Adjuvant (CFA).
  - Relieved by COX-inhibitor celecoxib
- Chemotherapy Induced Pain
  - Mice treated with JNJ-26481585, an HDAC inhibitor currently in Phase II clinical trials for cancer
- Neuropathic Pain
  - Spinal Nerve Ligation (SNL) in rats
  - Spared Nerve Injury (SNI) in rats and mice
- Pain behavior confirmed by:
  - Mechanical sensitivity (von Frey test)
  - Thermal nociception (Hargreaves' test)
- Blood samples collected, RNA isolated. miRNA measured by rodent TLDA cards on ABI rt-PCR.

Circulating microRNA Signatures in Rodent Models of Pain
Qureshi et.al., Mol Neurobiol, 2016.

Rodent Neuropathic Pain Models

- Spinal Nerve Ligation
  - Peripheral neuropathic pain model
  - L5 spinal nerve or L5 and L6 spinal nerves bound with silk
  - 5 sham operated rats, and 4 SNL rats
- Spared Nerve Injury
  - Peripheral nerve injury model
  - Targets common peroneal nerve and tibial nerve
  - Nerves bound with silk and cut distal to ligation site
  - 5 Sham and 5 SNI rats
  - 5 Sham and 5 SNI mice



Adapted from Decosterd and Woolf, Pain, 2000

Commonly Enriched KEGG Pathways

Enriched KEGG Pathways

- The ERK/MAPK pathway, as a target for the treatment of neuropathic pain. Ma & Quirion. *Expert Opin Ther Targets*, 2005.
- MAP kinase and pain. Ji et al. *Brain Res Rev*, 2009.
- p38 MAPK, microglial signaling, and neuropathic pain. Ji & Suter. *Molecular Pain*, 2007.

- Regulation of Wnt signaling by nociceptive input in animal models. Shi et al. *Molecular Pain*, 2012.
- A new player in neuropathic pain pathogenesis. Harrison. Nature *Reviews Drug Discovery*, 2013.
- WNT signaling underlies the pathogenesis of neuropathic pain in rodents. *The Journal of Clinical Investigation*, 2013.

Enriched KEGG pathways (continued)

- Axon guidance pathway
- Neurotrophin Signaling Pathway
  - Siniscalco et al. Role of Neurotrophins in Neuropathic Pain. Curr Neuropharmacol. 2011
- ErbB signaling Pathway
  - Calvo et al. Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. J. Neurosci. 2010
- TGF-Beta Signaling Pathway
  - Utreras et al. TGF-β1 sensitizes TRPV1 through Cdk5 signaling in odontoblast-like cells. Molecular Pain 2013
- mTor Signaling Pathway
  - Lyu et al. The mTOR signaling pathway regulates pain-related synaptic plasticity in rat entorhinal-hippocampal pathways. Molecular Pain
- GnRH Signaling