Regulation of DNA methylation by RNA

Daniel G. Tenen

Cancer Science Institute of Singapore
Harvard Stem Cell Institute

Fifth International Symposium on
Secondary Leukemia and Leukemogenesis
Rome, September 24, 2016

Genetics of this research in the Tenen lab

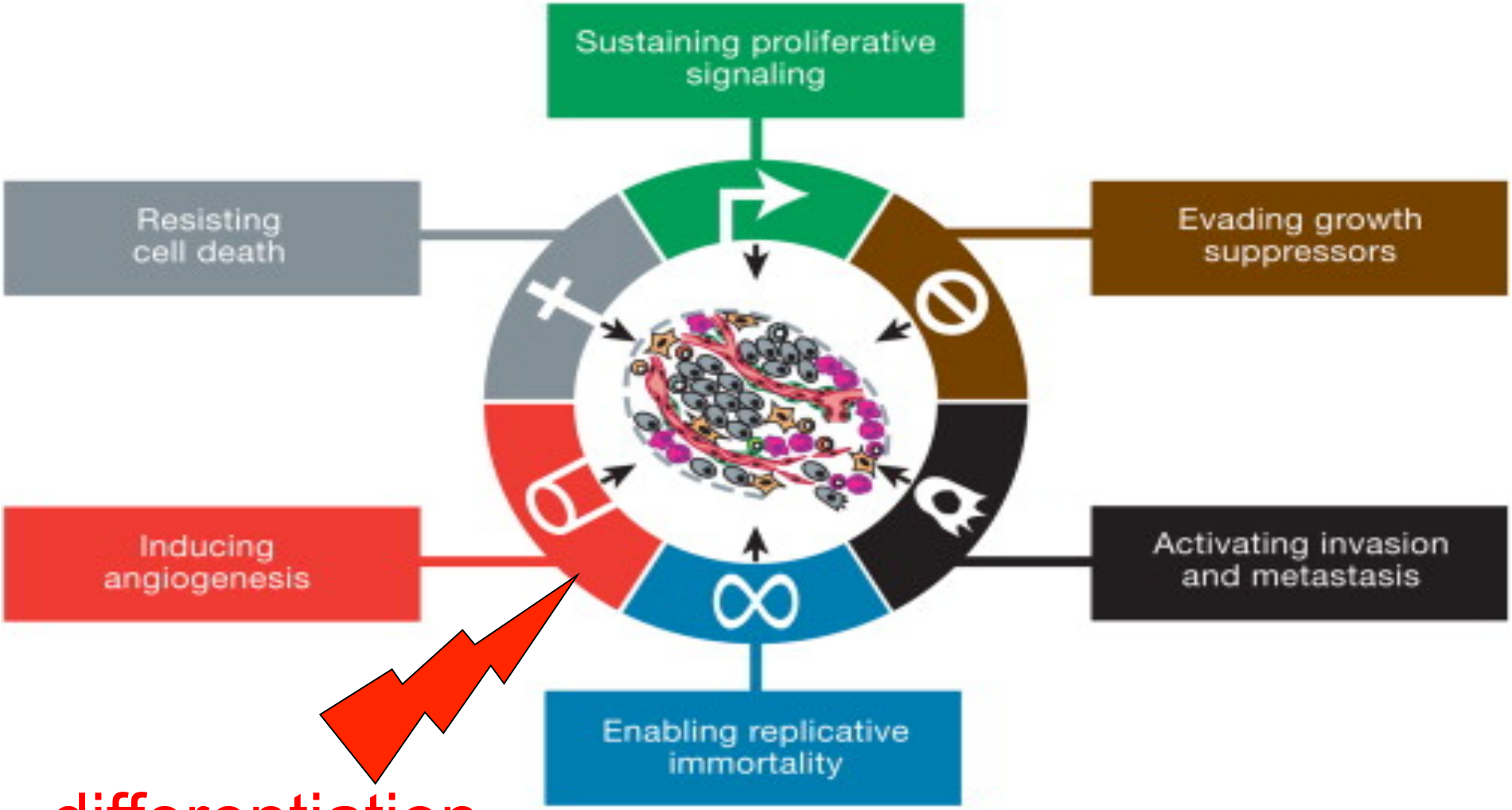
Maria Teresa Voso
PNAS, 1994

Stefan Hohaus
MCB, 1995

Annalisa Di Ruscio
Nature 2013,
Nature Comm 2015

- The BEST result the Tenen lab ever produced!
- TODAY is their wedding anniversary! (20+ years!)
- Can I spend the next 20 minutes telling the story of how they met in my lab?

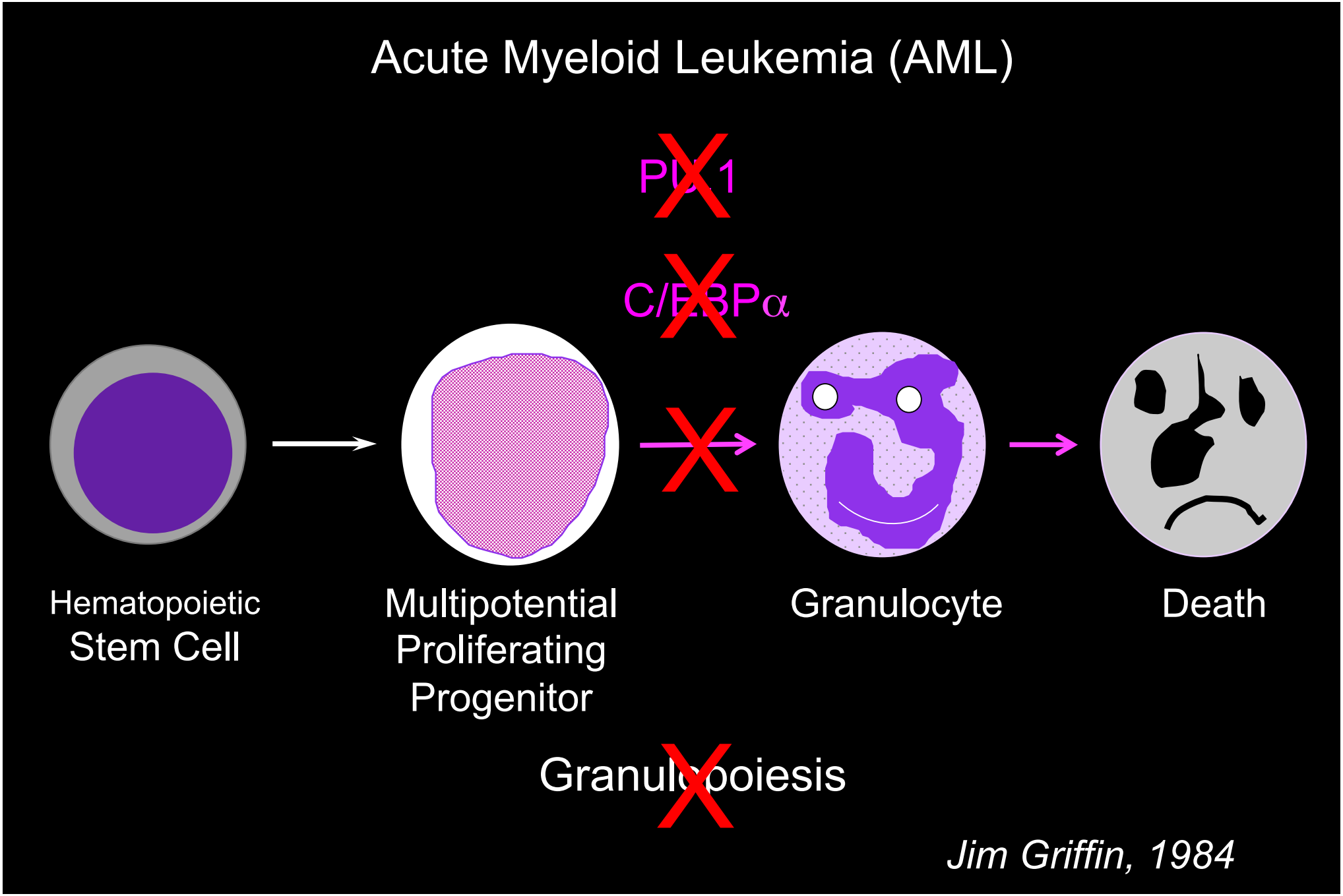
Hallmarks of Cancer: The Next Generation



differentiation block

Thanks to Doug Hanahan & Robert Weinberg
Cell 144:646, 2011

Part I: Myelopoiesis and AML made simple

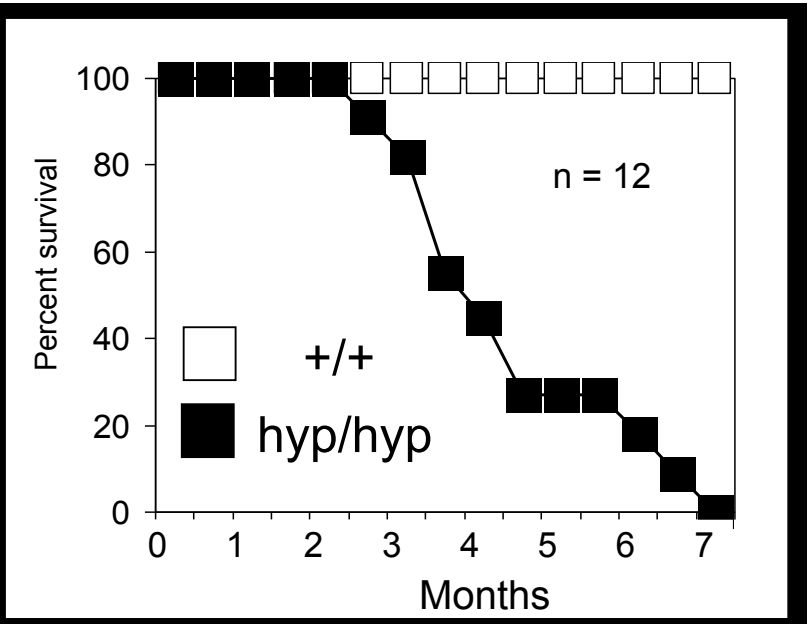

Acute Myeloid Leukemia (AML)
PU.1
C/EBPα
Hematopoietic
Stem Cell
Multipotential
Proliferating
Progenitor
Granulocyte
Death
Granulopoiesis
Jim Griffin, 1984

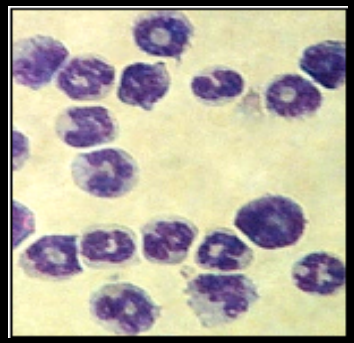
Graded reduction in PU.1 results in AML

PU.1 heterozygous KO mice are normal

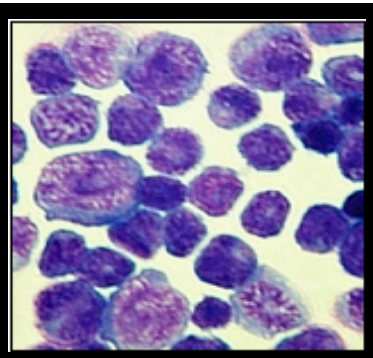
KO of the upstream regulatory element (URE)
Results in 20% of wild type PU.1 => AML

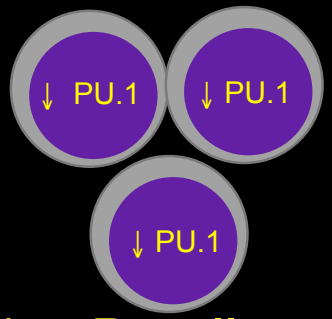
Rosenbauer, Nat Genet 2004



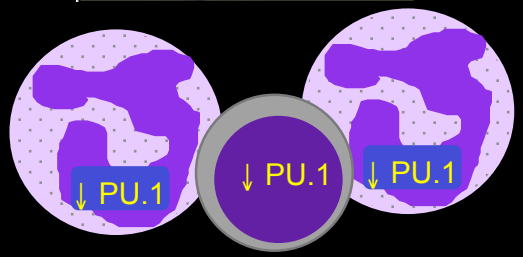


spleen

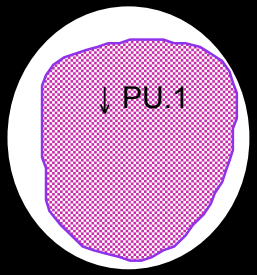




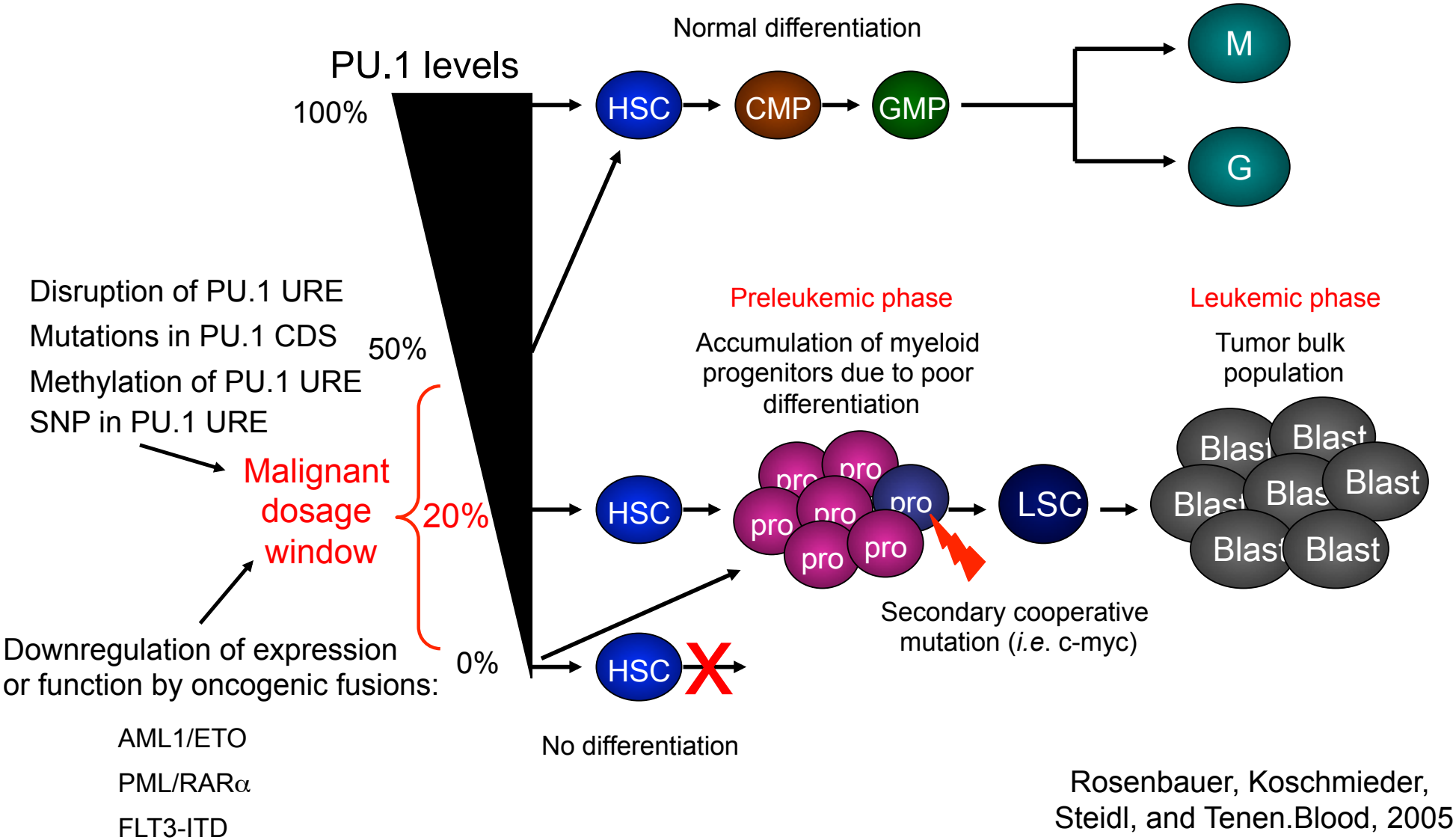
excess
G-CSF
effect?

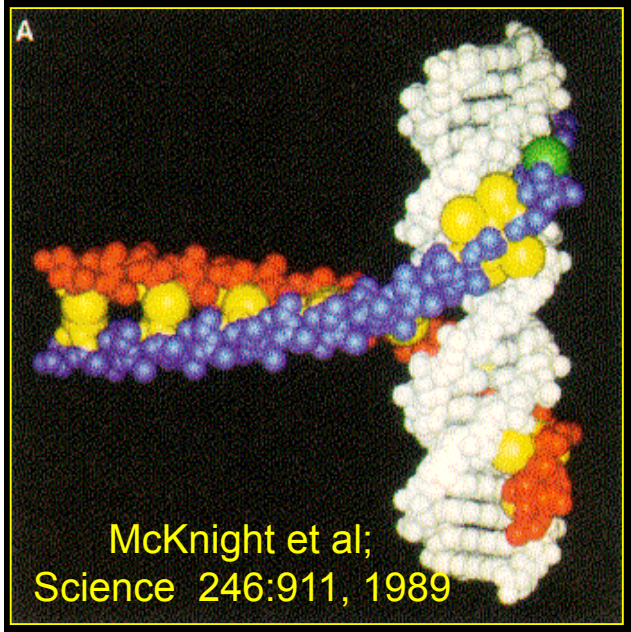


?

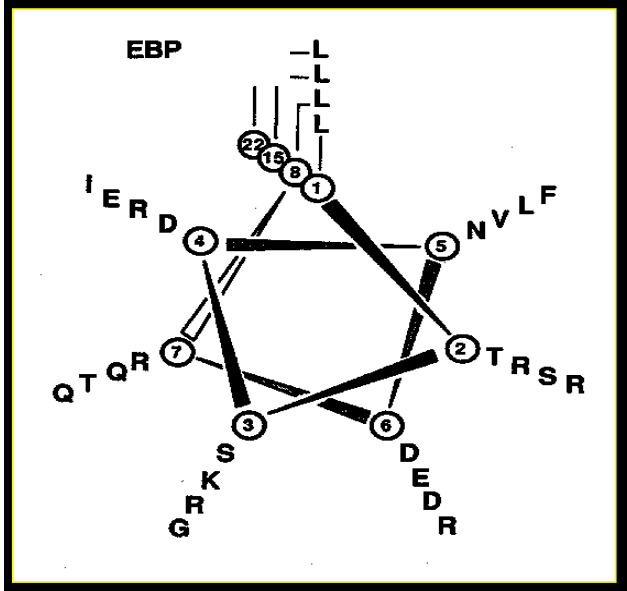


1.↓ B cells, macrophages 2. Pre-leukemic 3. AML


Normal differentiation
PU.1 levels
100%
HSC
CMP
GMP
M
G
Disruption of PU.1 URE
Mutations in PU.1 CDS
Methylation of PU.1 URE
SNP in PU.1 URE
50%
Preleukemic phase
Accumulation of myeloid progenitors due to poor differentiation
Leukemic phase
Tumor bulk population
Malignant dosage window
20%
HSC
pro
LSC
Blast
Secondary cooperative mutation (i.e. c-myc)
Downregulation of expression or function by oncogenic fusions:
0%
HSC
No differentiation
AML1/ETO
PML/RARα
FLT3-ITD
Rosenbauer, Koschmieder, Steidl, and Tenen.Blood, 2005



McKnight et al;
Science 246:911, 1989

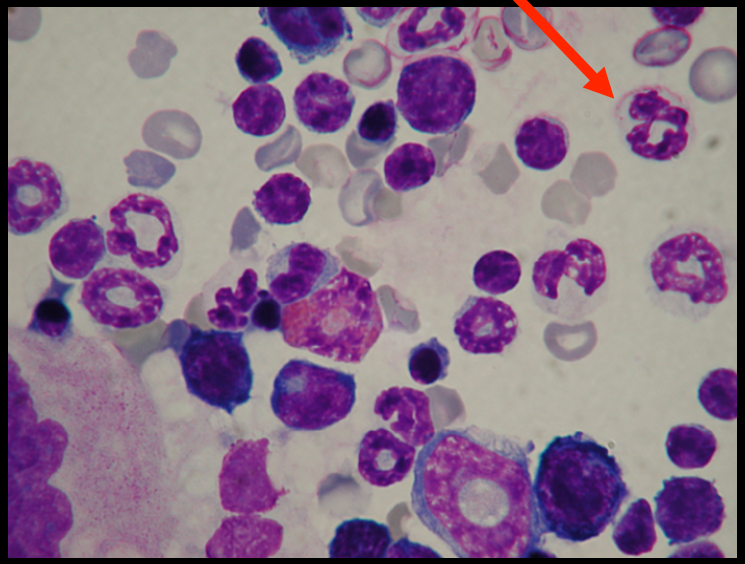


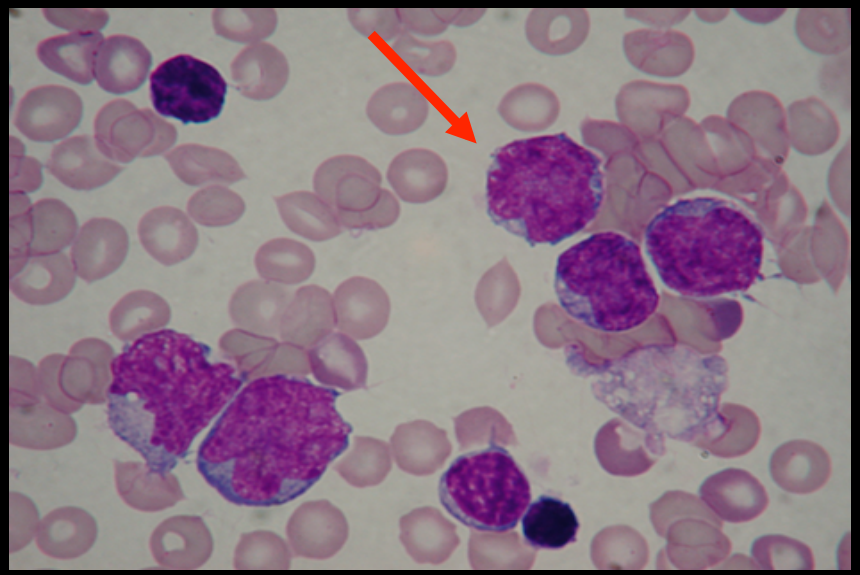
CCAAT Enhancer Binding Protein alpha C/EBPα:

- "original" basic leucine zipper transcription factor
- C/EBPα knockout mice: block in differentiation
  - In blood, CMP to GMP→ AML
  - In lung, Type II to Type I alveolar cells → lung Ca
- activates differentiation genes (G-CSFr, C/EBPe), and represses self-renewal genes (Bmi-1, c-myc, N-myc, Sox4)
- An iPS reprogramming factor
- induces cell cycle arrest and inhibits proliferation by
  - p21, CDK inhibition, calpain cleavage of cyclin A
  - interaction with and inhibition of E2F
    downregulation of c-myc
- a differentiation factor and tumor suppressor in acute myeloid leukemia, lung cancer, & liver

C/EBPα knockouts mimic human acute myeloid leukemia

WT

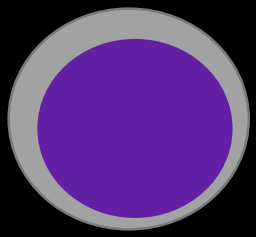


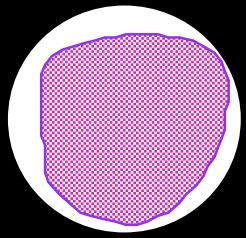


KO

Hematopoietic Stem Cell; self renewing

Multipotential Proliferating Progenitor (GMP)

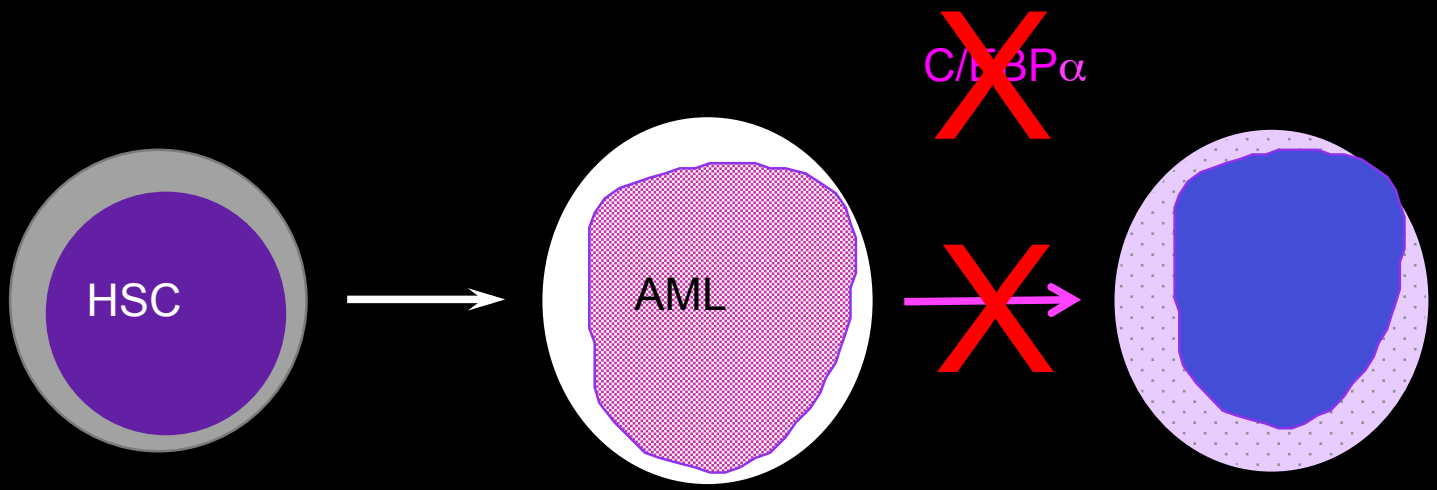




Pu Zhang

Zhang et al, PNAS 1997; Immunity, 2004
Ye et al, Nature Cell Biology 2013

Acute Myeloid Leukemia (AML)

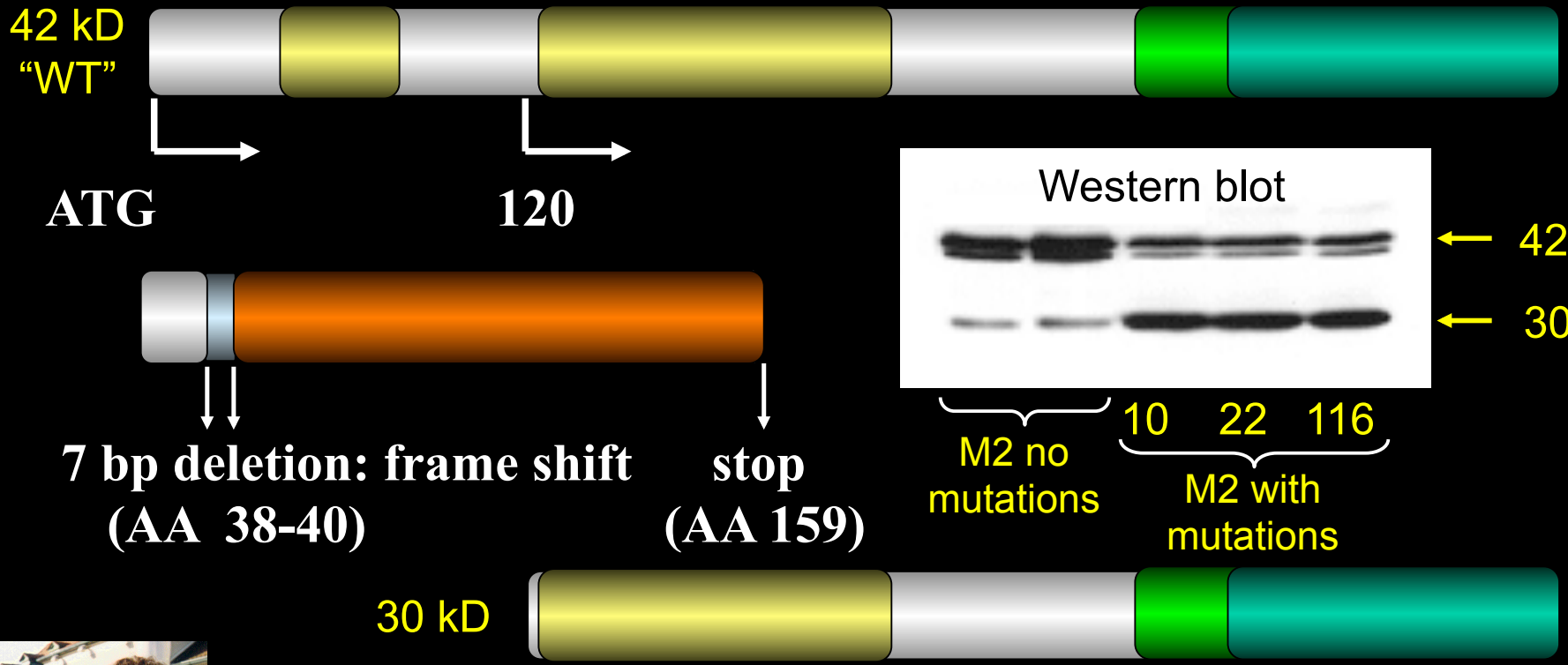


C/EBPα disruption does a LOT more than just block differentiation!

- Loss of control of proliferation

- Enhanced self-renewal by lack of repression of important targets:
  - Myc (Lisa Johansen, 2001; Min Ye, 2013)
  - Bmi-1 (Pu Zhang, 2004; Levantini, Sci Trans. Med, 2016)
  - Sox4 (Hong Zhang, 2013)

C/EBPα dominant negative mutations in AML ***



Dominant-negative mutations of *CEBPA*, encoding CCAAT/enhancer binding protein-α (C/EBPα), in acute myeloid leukemia

Thomas Pabst[1], Beatrice U. Mueller[1], Pu Zhang[1], Hanna S. Radomska[1], Sailaja Narravula[1], Susanne Schnittger[2], Gerhard Behre[2], Wolfgang Hiddemann[2] & Daniel G. Tenen[1]

*** *they do not exist anymore because whole genome sequencing misses them!*

Genetic inheritance of AML through CEBPA mutations

The NEW ENGLAND JOURNAL of MEDICINE

NEJM, 2008

BRIEF REPORT

Mutation of *CEBPA* in Familial Acute Myeloid Leukemia

Matthew L. Smith, M.B., B.S., Jamie D. Cavenagh, M.D., T. Andrew Lister, M.D., and Jude Fitzgibbon, Ph.D.

VOLUME 26 · NUMBER 31 · NOVEMBER 1 2008

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Pabst et al, JCO, 2008

Somatic *CEBPA* Mutations Are a Frequent Second Event in Families With Germline *CEBPA* Mutations and Familial Acute Myeloid Leukemia

Thomas Pabst, Marianne Eyholzer, Simon Haefliger, Julian Schardt, and Beatrice U. Mueller

Loss of C/EBPα function in AML

- I hate to break it to the genomicists, *but:*
- Many, many studies (including those done by hand) demonstrate 10% of AMLs have C/EBPα mutations
- In fact, part of the clinical workup of human AML (take that, you evil reviewers of our 2001 Nature Genetics paper!)
- So obviously the DNA sequencers are missing important things! (like calreticulin in P Vera)
- What about the remaining 90% of AML without mutations?

➢ Decreased expression and/or function by kinases or fusion genes (Pabst et al, Nature Med, 2001)

Zhang et al, PNAS 1997; Immunity, 2004
Ye et al, Nature Cell Biology 2013

Some myeloid differentiation is required for development of AML

HSC
self renewing

Multipotential Proliferating
Progenitor (GMP-like)

Granulocyte

Min Ye

with Scott Armstrong

LIC

Human studies:

Weissman, 2000
Jamieson, 2004
Majeti 2014
Dick 2014

Min Ye, Cell Stem Cell, 2015

CML myeloid blast crisis made simple

Transformation of CML chronic phase to blast crisis requires suppression of C/EBPα

BCR-ABL suppresses C/EBPα expression through inhibitory action of hnRNP E2

Danilo Perrotti et al, Nature Genetics, 2002

Again, emphasizes the importance of regulation of expression or function, not just mutations

Drugs used in humans with AML which target C/EBPs: Drugging the Undruggable!

- ATRA in APL -> increases C/EBPβ and then C/EBPε
  (Park et al, JCI 1999; Duprez, et al, EMBO J 2003)

- FLT3 inhibitors: restore C/EBPα expression and function: Ser21
  (Mizuki et al, Blood 2003; Zhang et al, Blood 2004; Radomska, 2006)

- Imatinib in CML myeloid blast crisis: restore C/EBPα
  by a post-transcriptional mechanism
  (Perrotti et al, Nature Genetics, 2001)

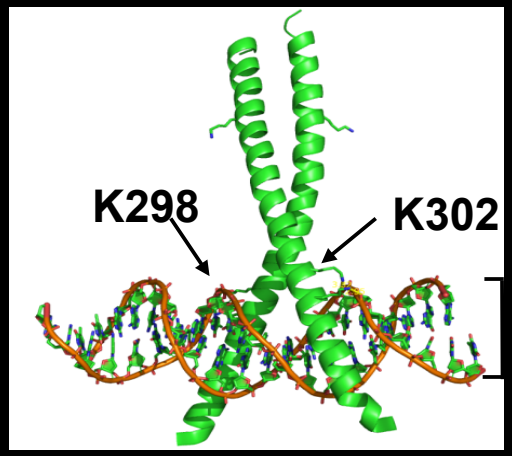
- CDDO (alter translation)
  (Koschmieder et al, Blood 2007)

- CDC2/CDK1 inhibitors
  (Radomska, Alberich-Jorda et al, JCI 2012)

- HDAC inhibitors
  (Liss, Alberich-Jorda et al, Haematologica 2014)

Targeting C/EBPα: new things coming

1) Acetylation by HATs (GCN5, a lysine acetyltransferase):



nature COMMUNICATIONS

Deepak Bararia and Hui si Kwok, 2016

ARTICLE

Received 9 Mar 2015 | Accepted 7 Feb 2016 | Published xx xxx 2016

DOI: 10.1038/ncomms10968 OPEN

Acetylation of C/EBPα inhibits its granulopoietic function

2) Targeting downstream targets of C/EBPα: Bmi-1 in lung cancer (Elena Levantini, Sci. Trans. Med, in press). Phase I trials in lung cancer, leukemia soon.

Elena Levantini
Institute of Biomedical Technologies, National Research Council, Pisa
Harvard Medical School

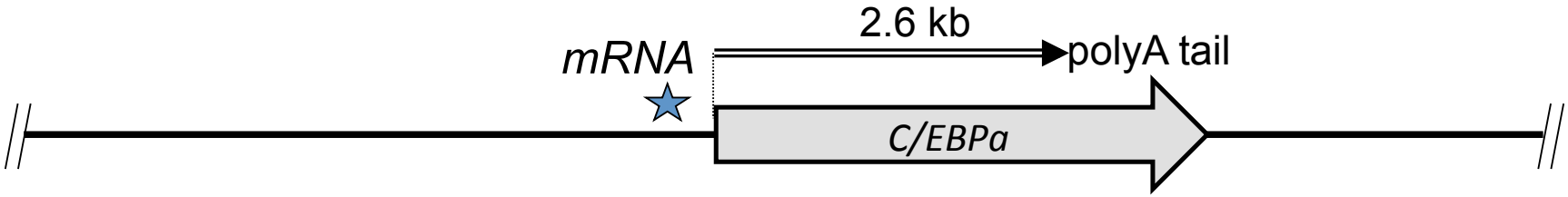
Part II: Regulation of DNA methylation by RNA
Can we demethylate selectively with RNA?

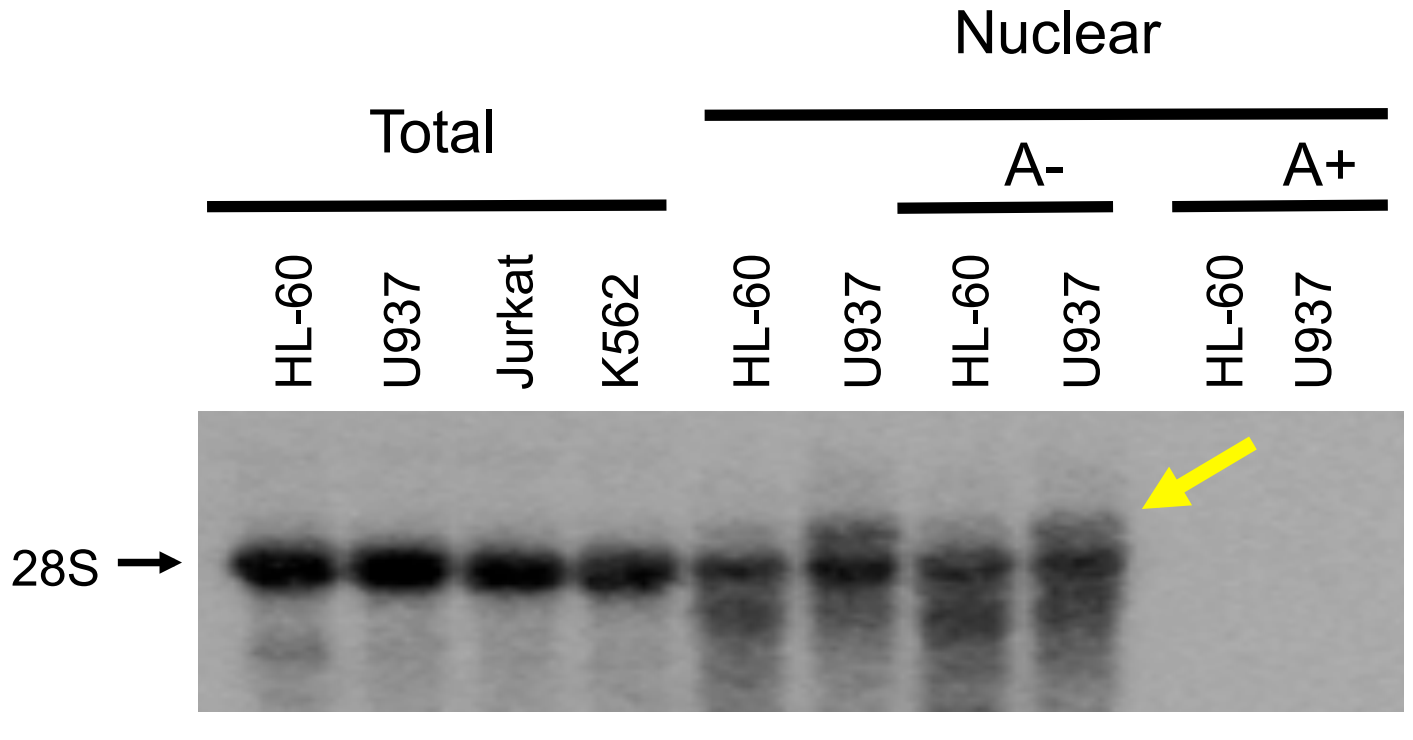
Annalisa Di Ruscio
Novara
Harvard Medical School

Alex Ebralidze
Tenen group, Boston

Di Ruscio et al, Nature, 2013

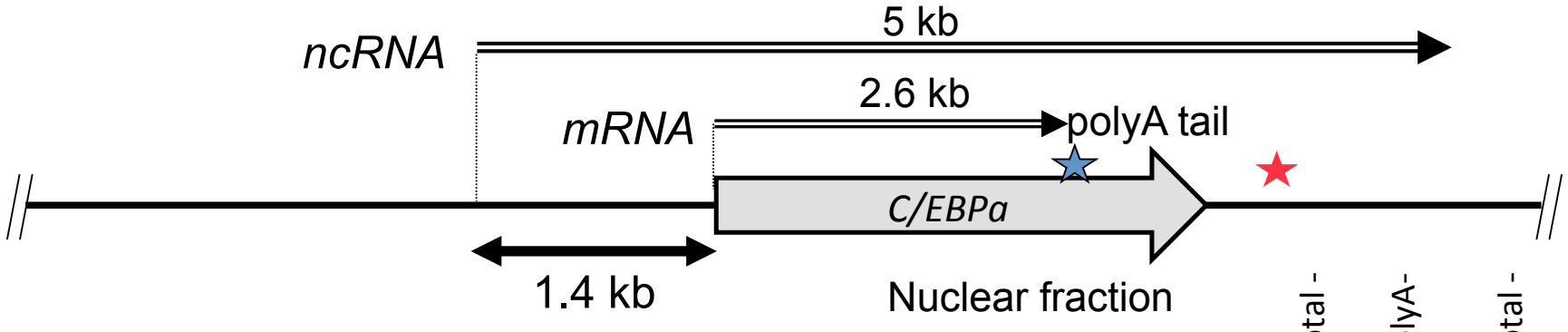
Using ancient technology (Northerns) to study C/EBPα transcripts…



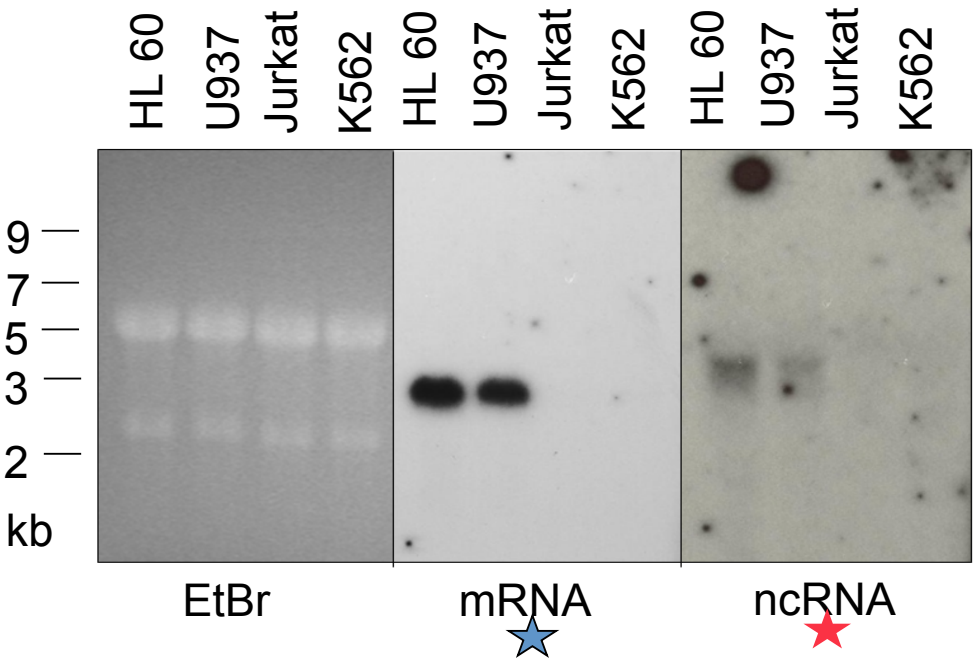


Alex Ebralidze

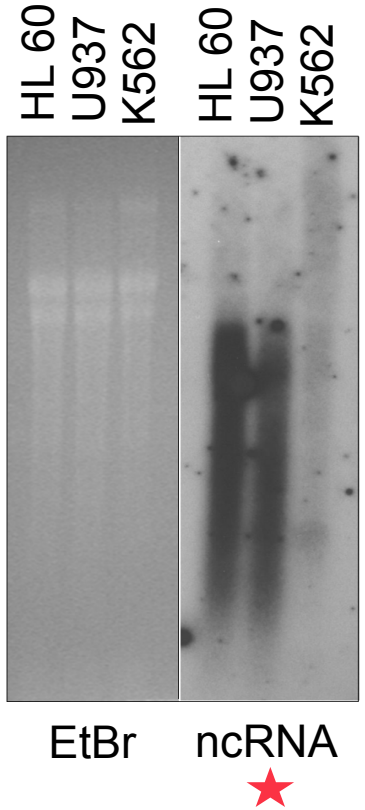
A nuclear polyA- “extracoding” RNA overlapping the C/EBPα gene locus

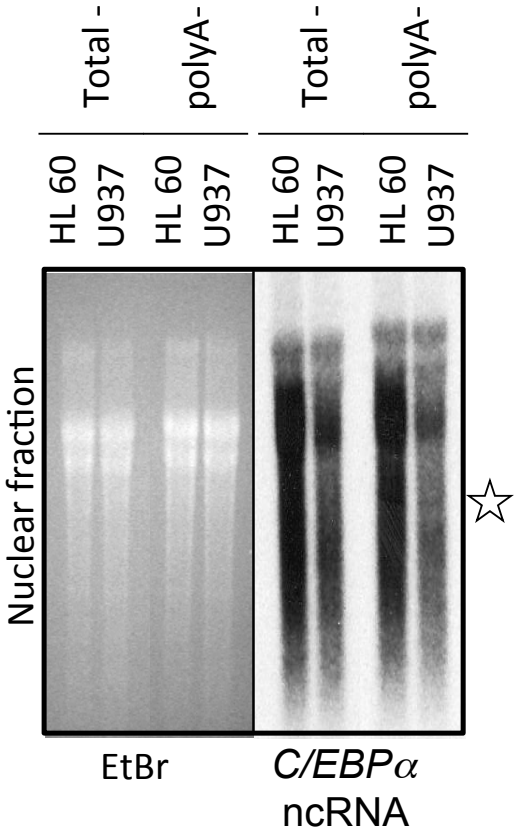


Total cellular fraction



Nuclear fraction





★ Coding probe

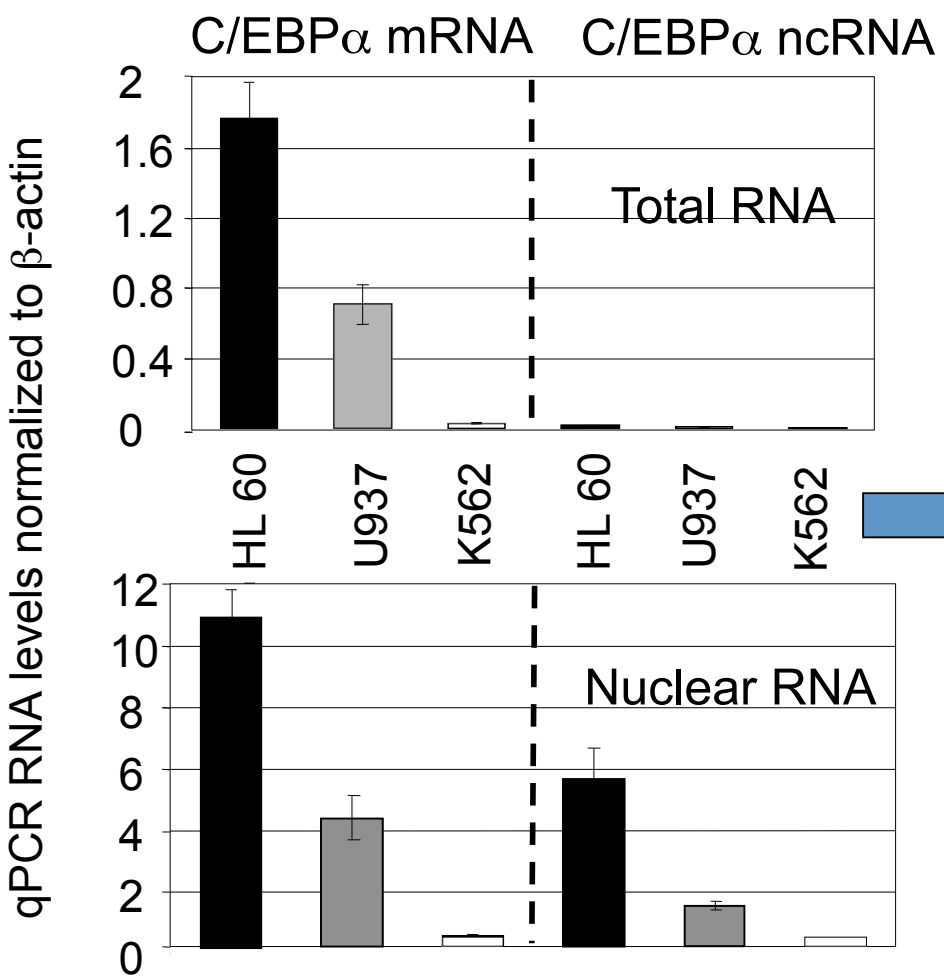
★ Non-coding probe

ecRNA is nuclear

ecRNA is polyA-

C/EBPα ncRNA levels correlate *with* C/EBPα mRNA in normal and AML cells

C/EBPα ncRNA levels are ~2% of mRNA levels in total RNA…
but are almost same as mRNA in the nucleus…



So RNA-seq studies using:

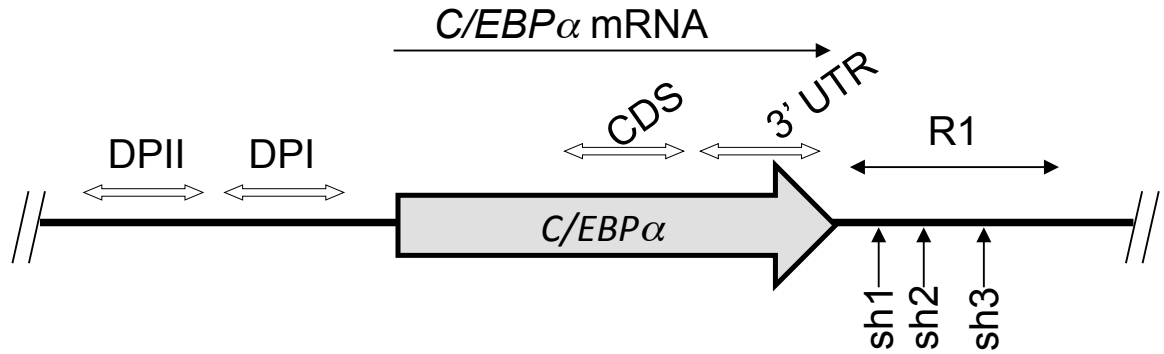
total RNA
polyA+ RNA
Ribo-depleted RNA

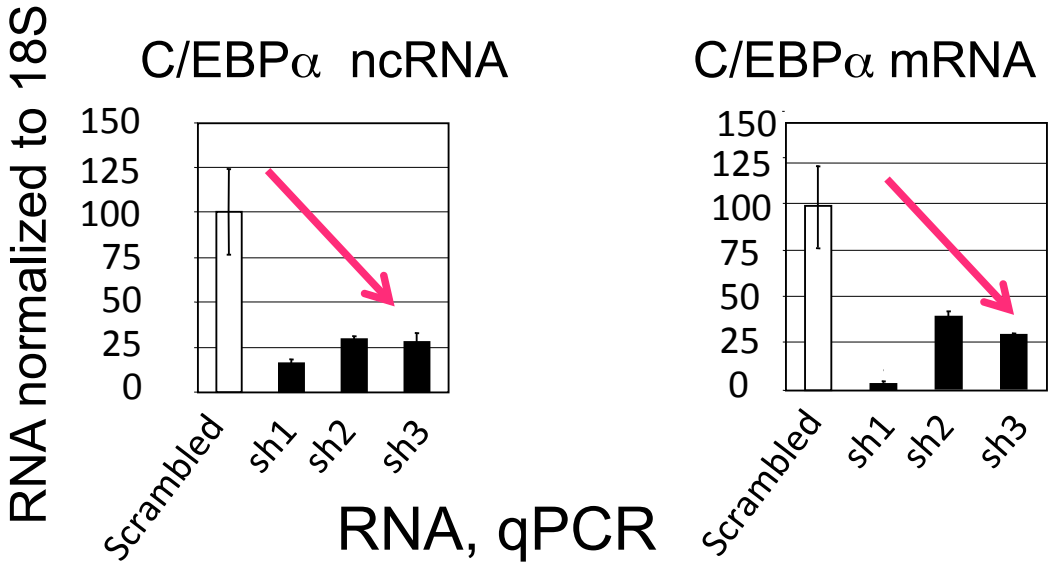
…are missing the important ones !!!

(5,000 in a myeloid cell)

ncRNA loss of function studies in HL-60 myeloid cells (C/EBPα gene is unmethylated and mRNA and ncRNA expressed)

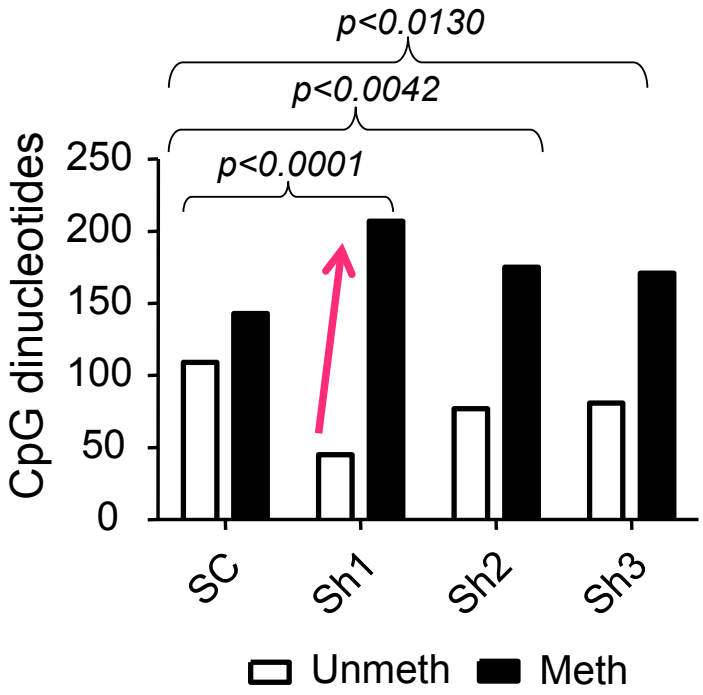
knockdown of C/EBPα ncRNA decreases C/EBPα mRNA…





RNA, qPCR

…and increases methylation

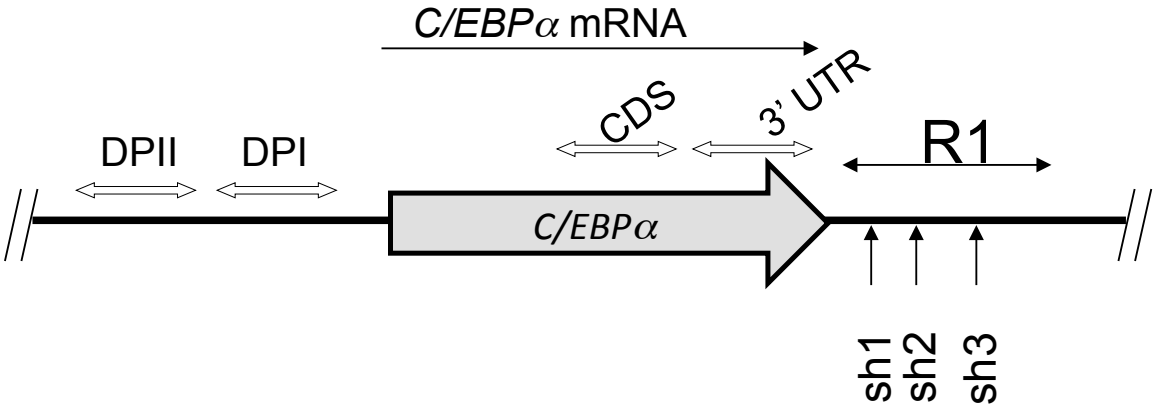


Methylation

DP1, bisulfite sequencing

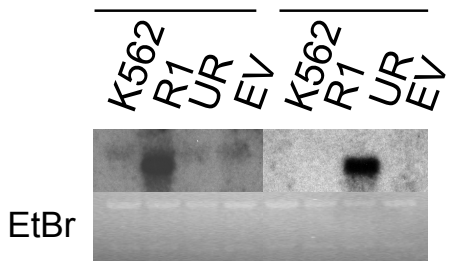
ncRNA gain of function in K562 cells
(C/EBPα methylated, mRNA and ncRNA are not expressed)

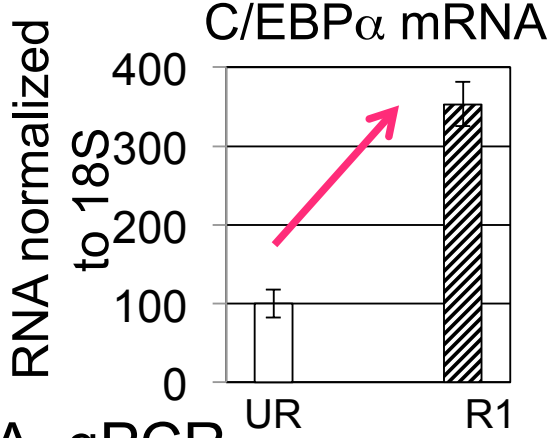
Expression of C/EBPα ncRNA increases C/EBPα mRNA…



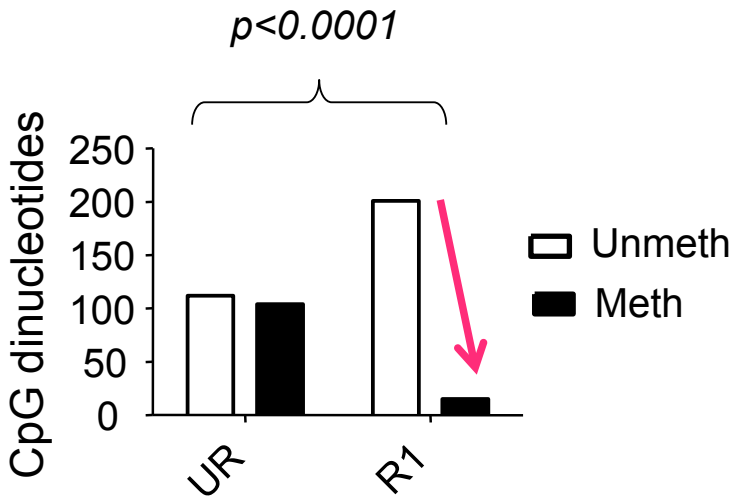
…and decreases methylation

ncRNA (Northern) in K562 cells





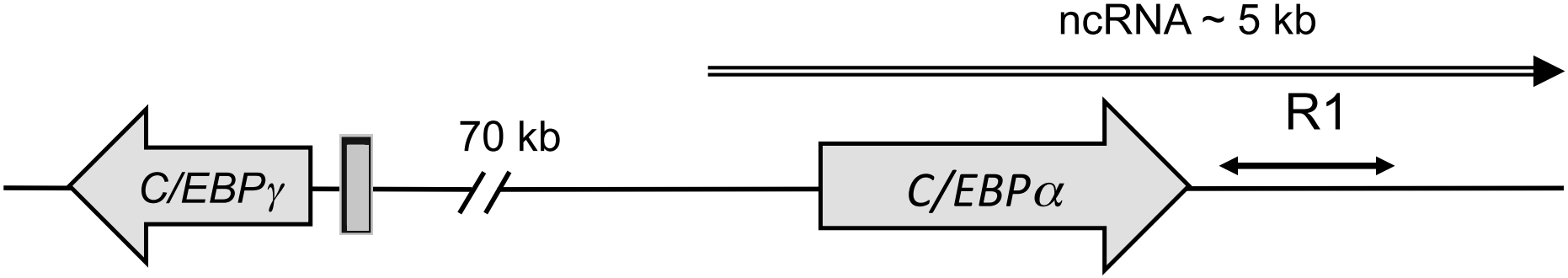
RNA, qPCR

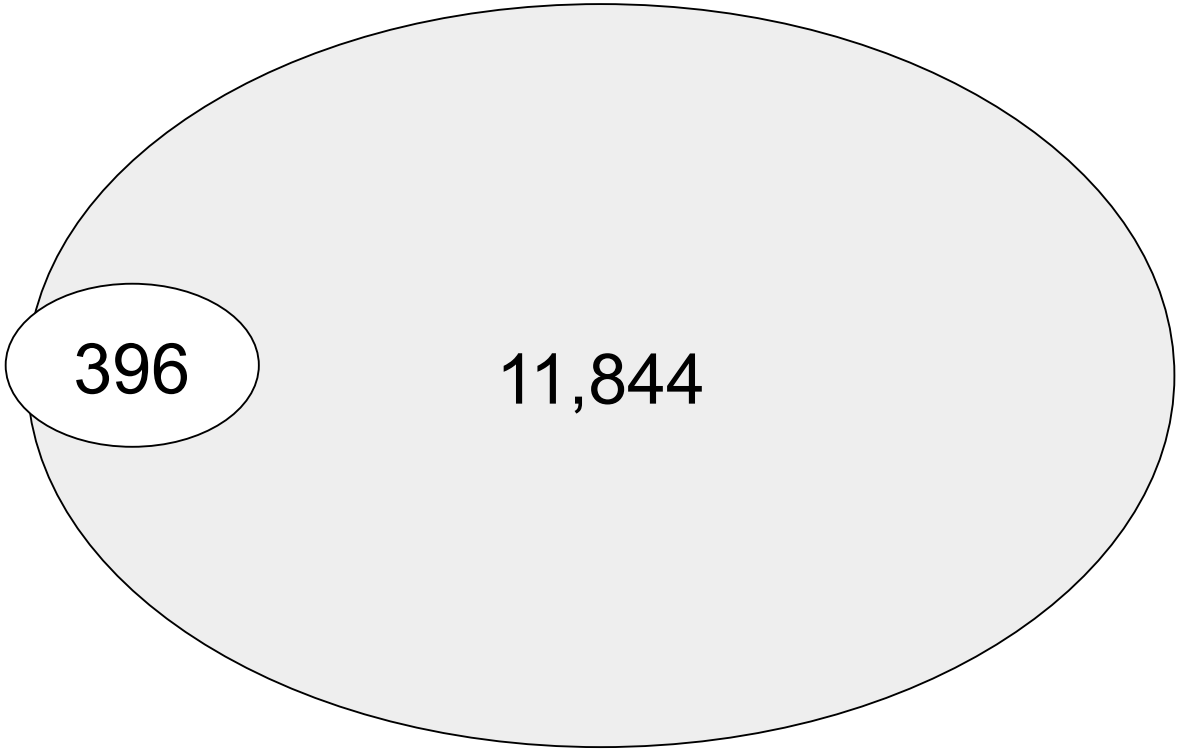


Methylation
coding region
bisulfite sequencing

ncRNA demethylation is localized and gene selective

Expressing the ncRNA R1 does not demethylate C/EBPg (70 kb away)



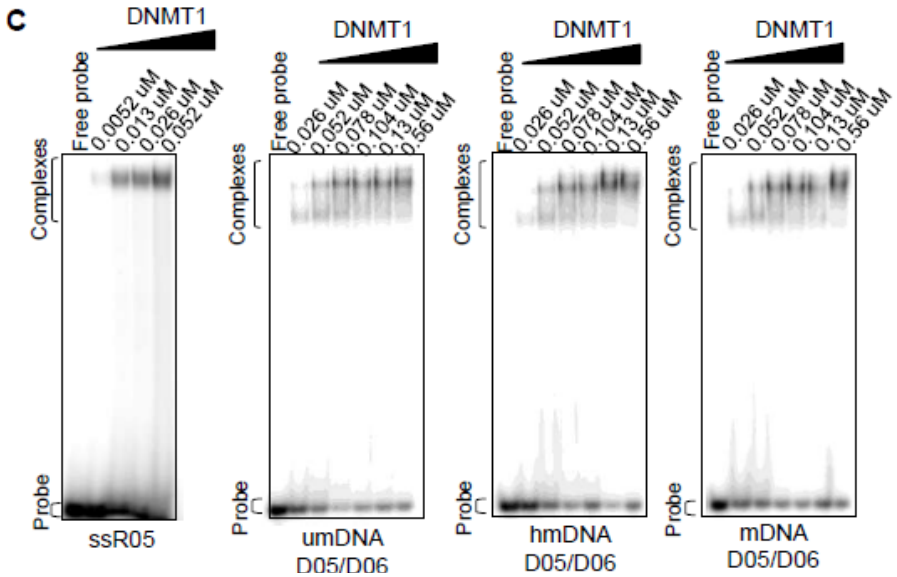


And does not induce genome wide demethylation

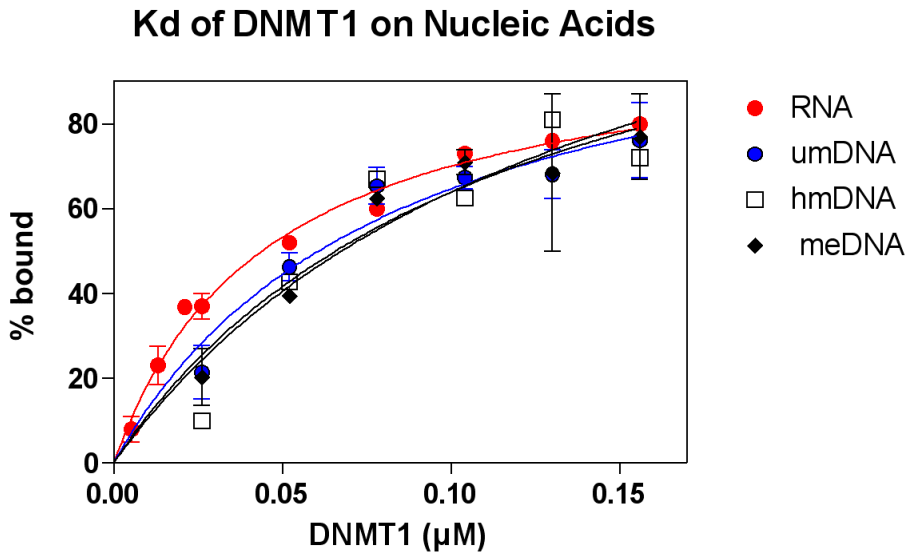
Of ~12,000 loci, only 400 were demethylated similar to C/EBPα (~ 3% of genome) by RRBS

Mechanism: DNMT1 binds RNA better than DNA

(and there are many more molecules of RNA than DNA in the nucleus)



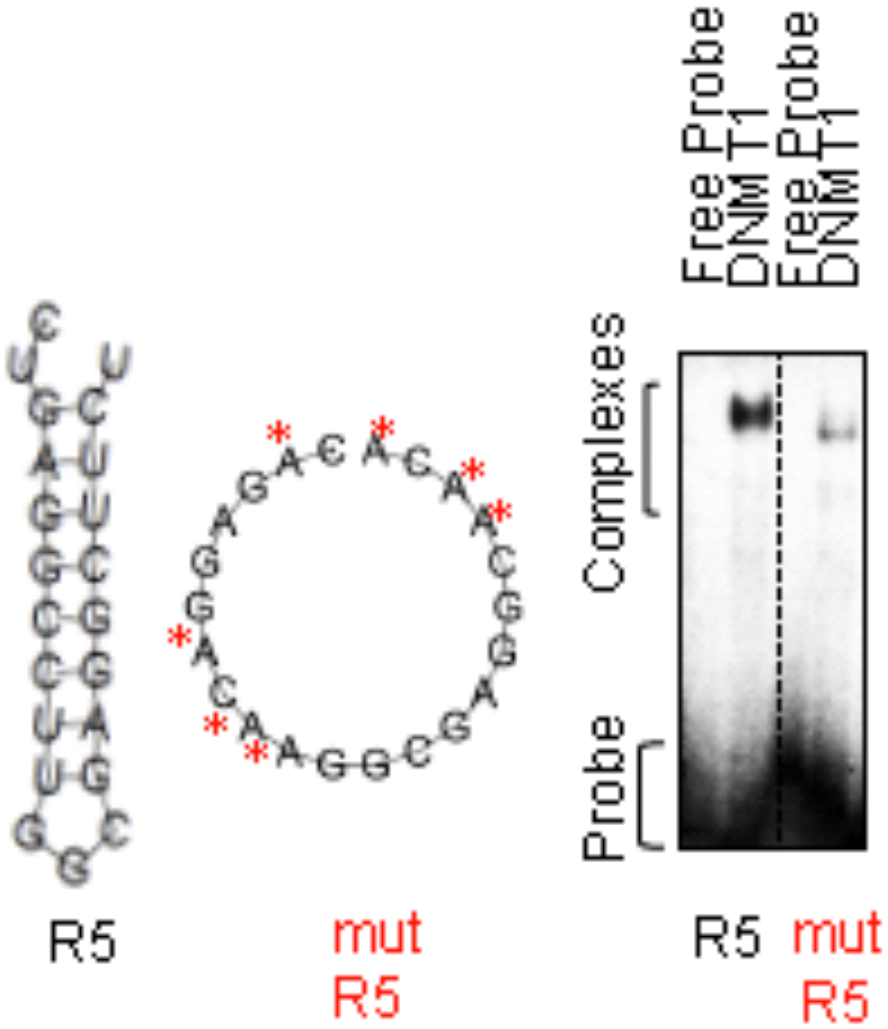
REMSA



Dissociation constant: K_d, mM
(smaller numbers means higher affinity)

RNA	0.045
unmethylated DNA	0.082
methylated DNA	0.11
hemimethylated DNA	0.14

DNMT1 recognizes RNA secondary structure (likely not sequence specific)



REMSA:

DNMT1 binds poorly to RNA which cannot form a stem loop (mutR5) compared to RNA which can form secondary structure (R5)

Do DNMT1-interacting RNAs (DiRs) only exist in the CEPBA gene?
Or is this a general mechanism?

To answer this question, we turned to:

epigeria = epigenetic hysteria
a.k.a.*epimania*:

a condition in which performing superficial genomic studies outstrip the science

Is this a general mechanism? Mapping the episcriptome

Touati
Benoukraf

1. DNMT1 RNA binding (Rip-Seq) (in HL-60 cells)
2. methylation analysis by RRBS
3. RNA expression by microarray and now RNAseq

DNMT1-interacting RNAs (DiRs):

- DNTM1 binds RNA structures, not sequences, with affinity > DNA
- DiRs inhibit DNMT1 activity in vitro and in cell culture models
- DiRs are mostly over genes, not distal regions
- Over 5,000 gene loci in myeloid cells have DiRs
- Almost all nuclear and polyA- (so not in the RNA databases)
- Genes with DiRs are highly expressed and unmethylated
- Genes without DiRs are low/undetectable and methylated
- DiRs can demethylate in a gene selective manner

Independent confirmation of our findings:

Hendrickson *et al. Genome Biology* (2016) 17:28
DOI 10.1186/s13059-016-0878-3

Genome Biology

RESEARCH Open Access

Widespread RNA binding by chromatin-associated proteins

David G Hendrickson[1,2†], David R. Kelley[1,2*†], Danielle Tenen[1,2], Bradley Bernstein[2] and John L. Rinn[1,2,3*]

Danielle E. Tenen

Rip-sequencer extraordinaire

nature COMMUNICATIONS

ARTICLE

Received 27 Jan 2016 | Accepted 28 May 2016 | Published 7 Jul 2016

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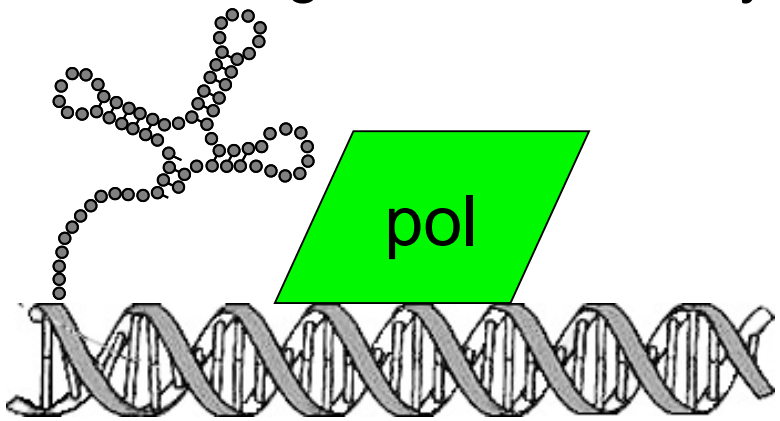
Extra-coding RNAs regulate neuronal DNA methylation dynamics

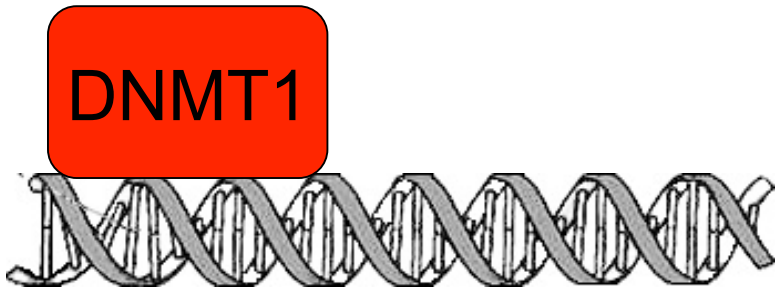
Katherine E. Savell[1], Nancy V.N. Gallus[1], Rhiana C. Simon[1], Jordan A. Brown[1], Jasmin S. Revanna[1], Mary Katherine Osborn[1], Esther Y. Song[1], John J. O'Malley[1], Christian T. Stackhouse[1], Allison Norvil[2], Humaira Gowher[2], J David Sweatt[1] & Jeremy J. Day[1]

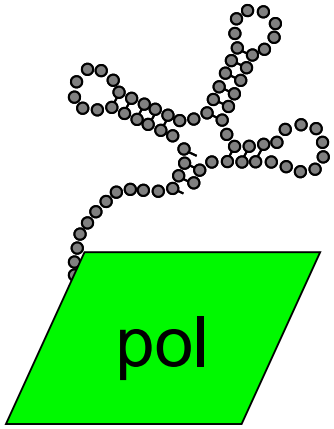
Are genes silenced by methylation? What is the evidence?



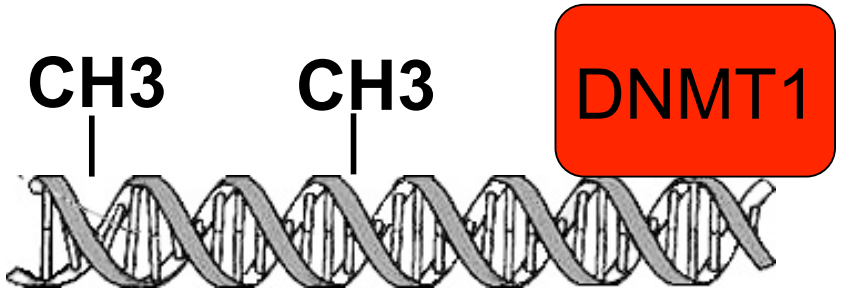
model:

unmethylated expressed
tumor suppressor



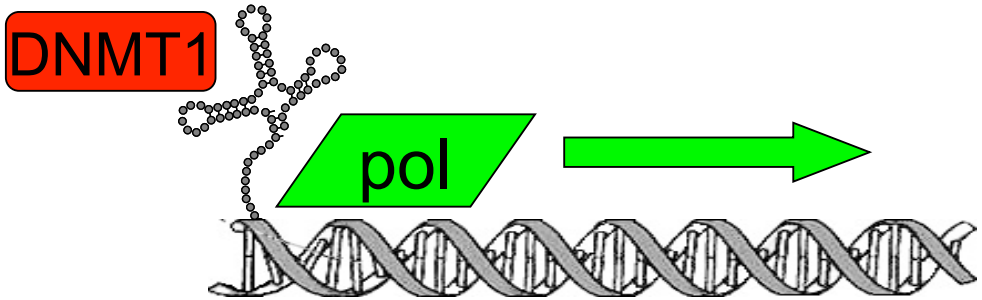


DNMT1
silences expression

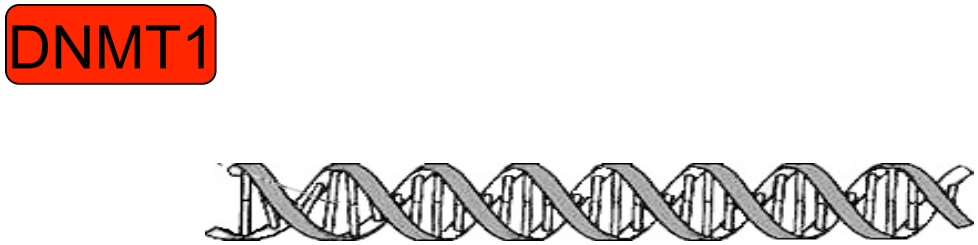


methylated and silenced

We hypothesize gene silencing precedes methylation

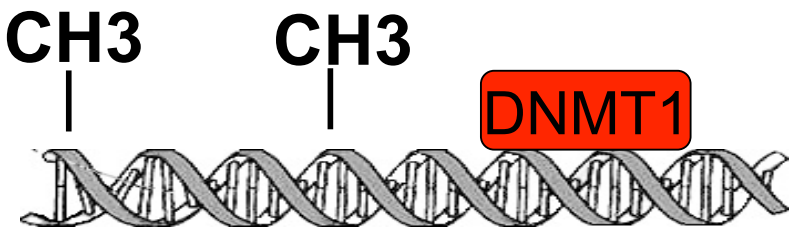


unmethylated expressed gene
protected from methylation by
RNA binding to DNMT1



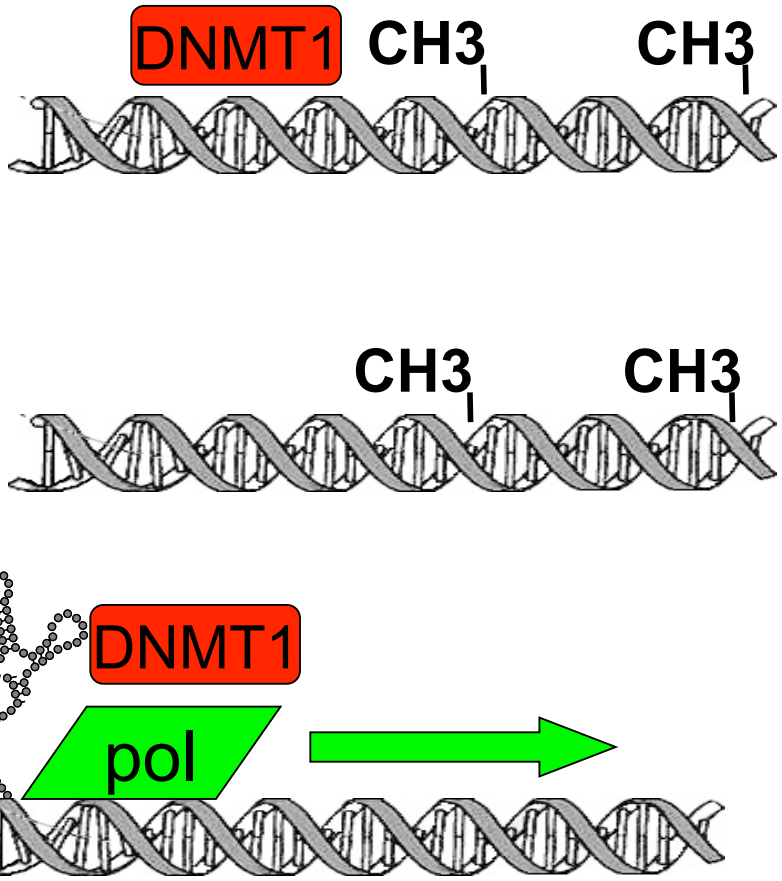
Gene shuts off RNA;
DNA is not protected from DNMT1

DNMT1 can bind DNA



silenced methylated gene

RNA can be used to remove methylation and activate tumor suppressors

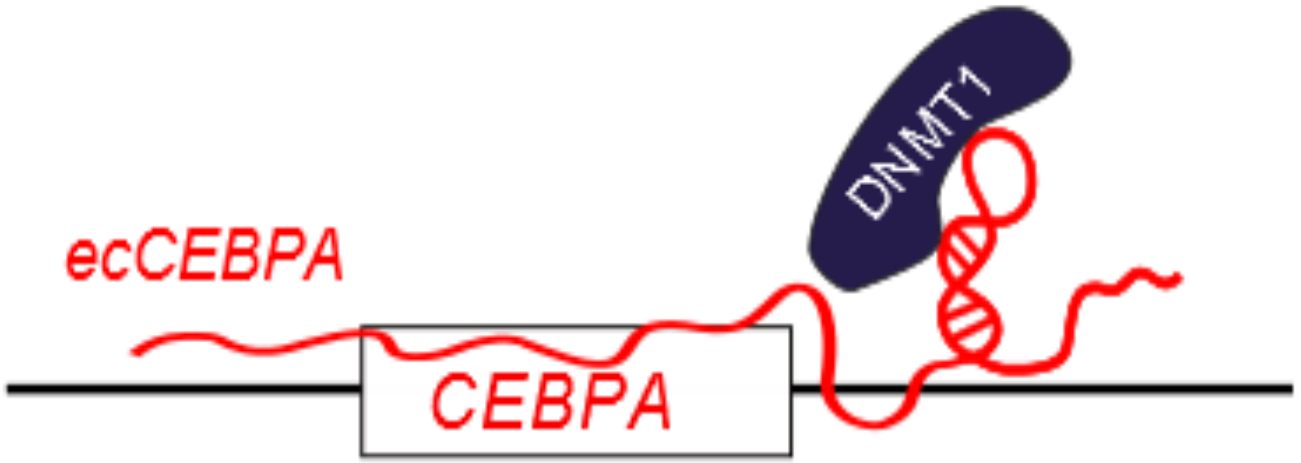


silenced methylated
tumor suppressor

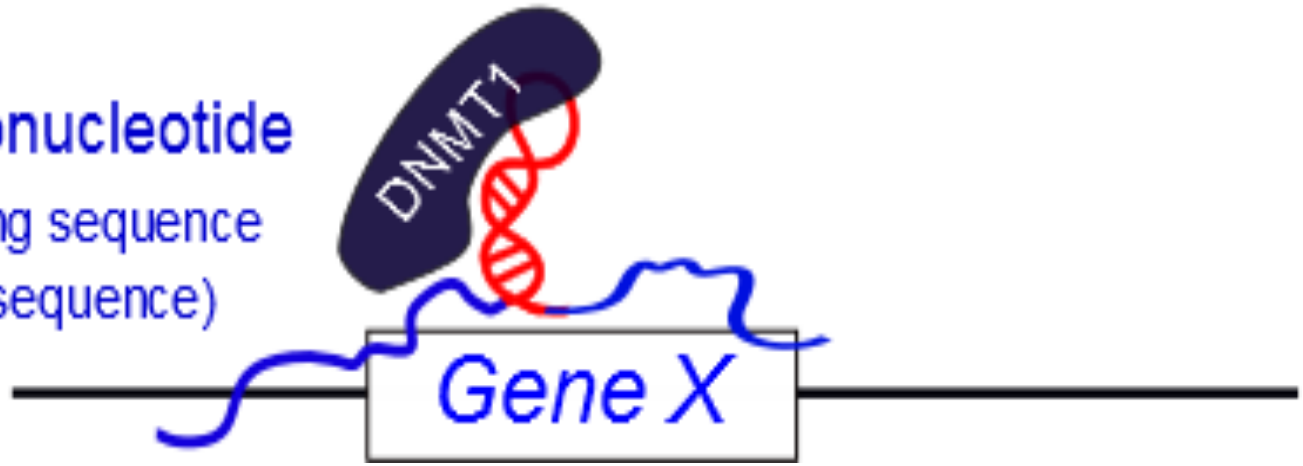
RNA binds to DNMT1
and inhibits methylation

gene is unmethylated and expressed
(demonstrated for C/EBPα)

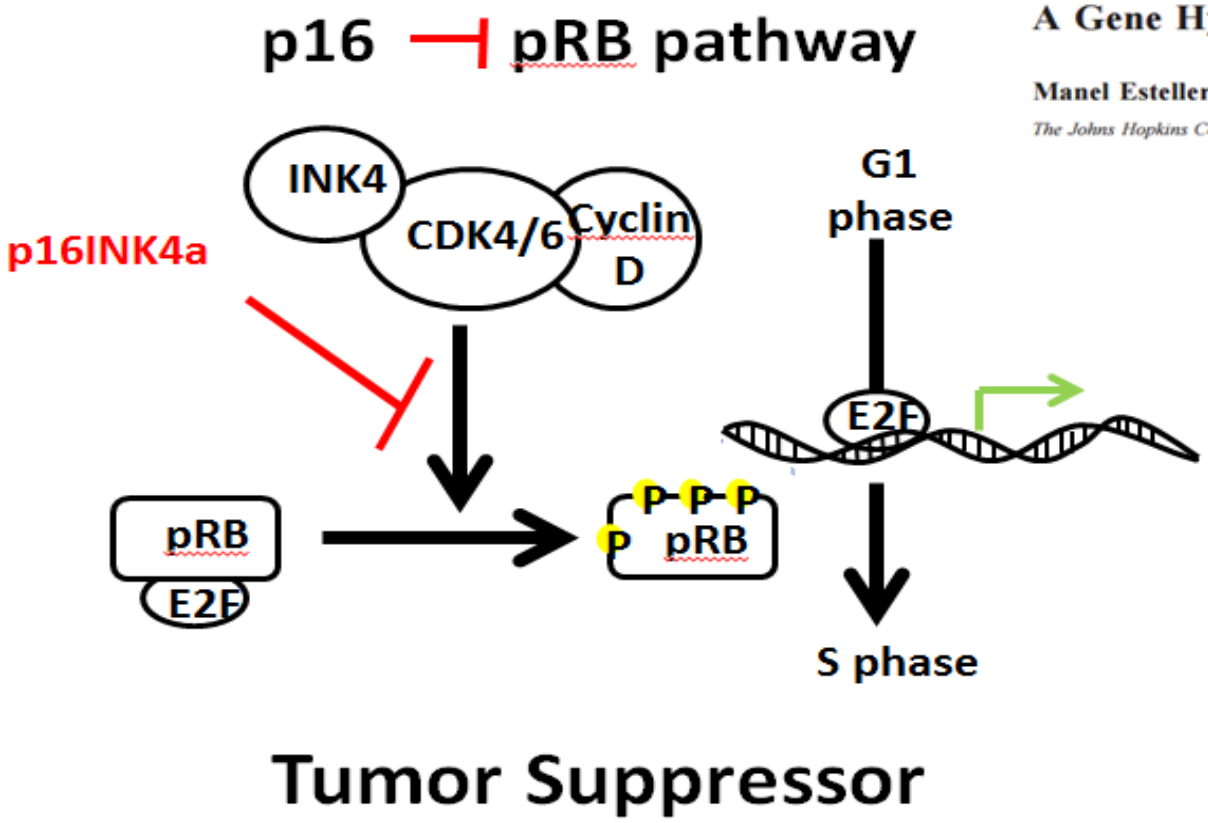
- Demethylation is local and selective
- Can be used to induce gene-selective demethylation
- Can activate tumor suppressors (i.e., C/EBPα)



Composite RNA oligonucleotide
(*ecCEBPA-DNMT1* binding sequence
+Gene X specific RNA sequence)



Gene X：CDKN2A (p16)



[CANCER RESEARCH 61, 3225–3229, April 15, 2001]

Perspectives in Cancer Research

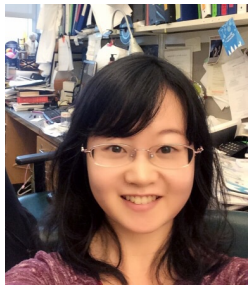
A Gene Hypermethylation Profile of Human Cancer[1]

Manel Esteller,[2, 3] Paul G. Corn,[2] Stephen B. Baylin, and James G. Herman[4]

The Johns Hopkins Comprehensive Cancer Center, Baltimore, Maryland 21231

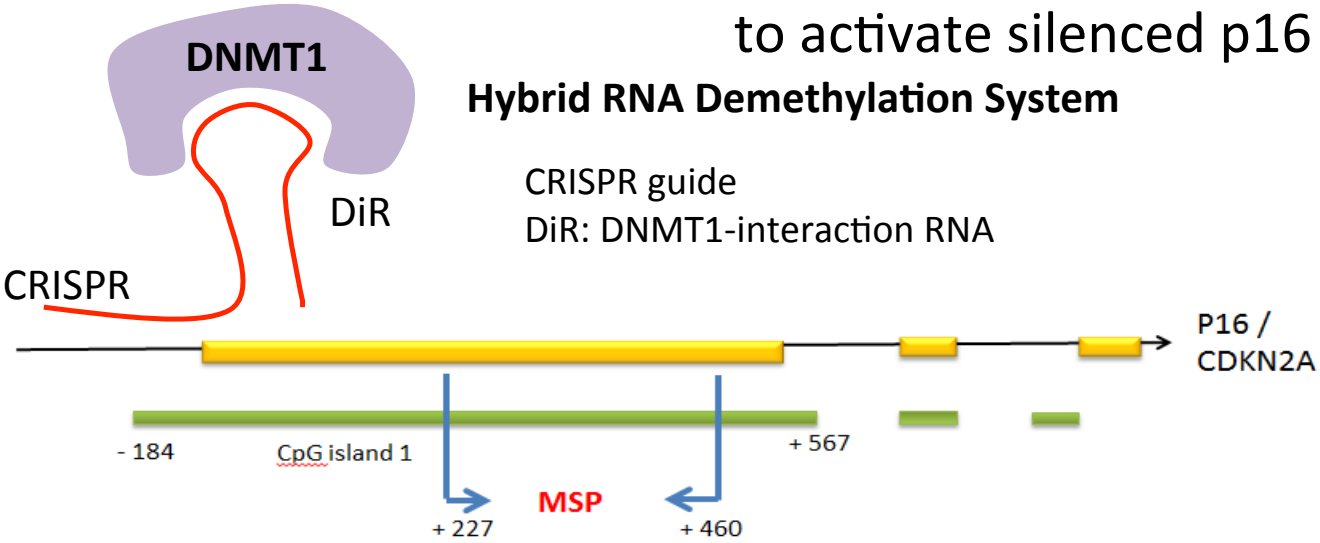
	p16 INK4a	*p14 ARF*	*p15 INK4b*
Colon	37%,41/110	28%,37/132	0%,0/19
Breast	17%,11/66	0%,0/20	0%,0/16
Ovary	18%,4/22	5%,1/20	N.D.
Uterus	20%,6/29	16%,4/25	N.D.
Lung	31%,28/89	6%,4/62	0%,0/21
Head-Neck	27%,26/95	4%,1/25	N.D.
Leukemia	1%,1/150	5%,1/20	62%,93/150
Lymphoma	48%,12/25	0%,0/22	24%,6/25
Brain	30%,3/10	9%,2/22	N.D.
Kidney	23%,6/25	13%,5/38	N.D.
Bladder	9%,1/11	5%,1/20	N.D.
Esophagus	33%,5/15	8%,3/37	N.D.
Stomach	36%,8/22	26%,31/118	N.D.
Pancreas	39%,7/18	0%,0/20	N.D.
Liver	15%,3/20	0%,0/20	N.D.

Using CRISPR-DiR hybrid RNAs to target methylation to activate silenced p16



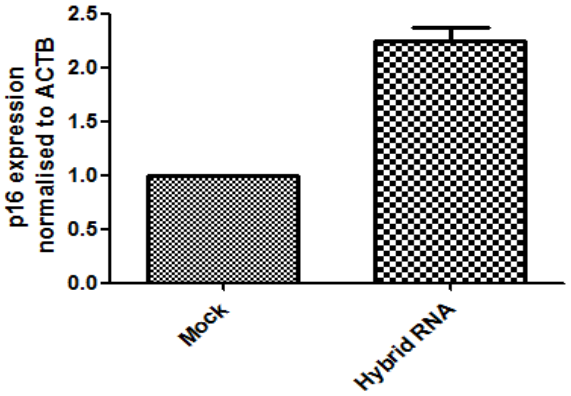
Yanjing Liu

Hybrid RNA Demethylation System

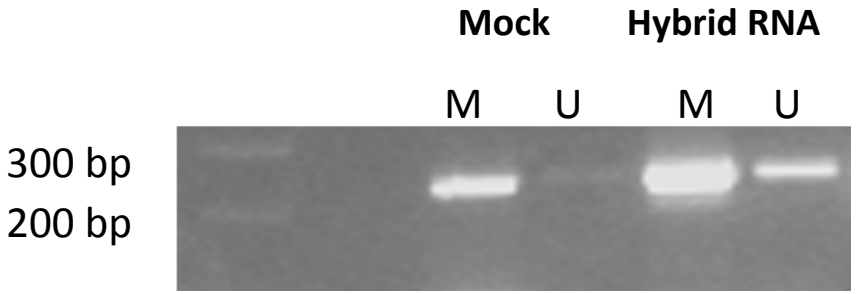


72 hours treatment in SNU398 liver cancer cells

p16 Expression



p16 Methylation



Targeting C/EBPα: new things coming

Dual action therapeutic approaches:
Inhibiting cancer cells while promoting normal cell function

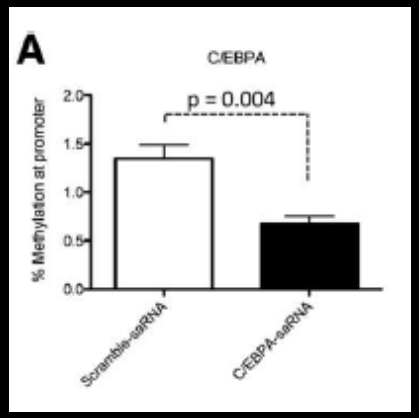
Short activating RNAs (saRNAs)
saRNA to CEBPA activates its expression in liver cancer cells

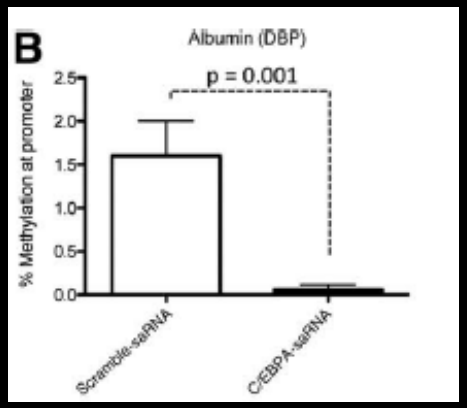
Phase I trial in liver cancer initiated, AML in the future

C/EBPα saRNAs improve liver function in hepatocarcinoma cells

Reebye et al, Hepatology 59:216, 2014

Decreased methylation

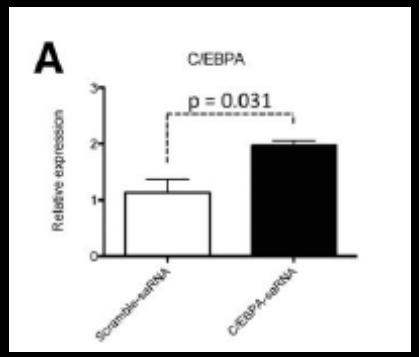


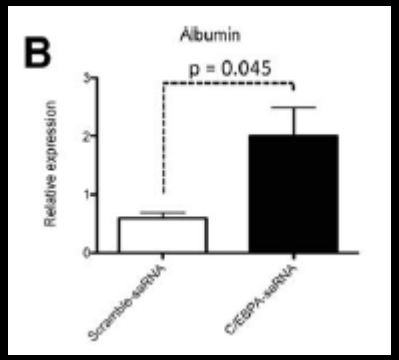


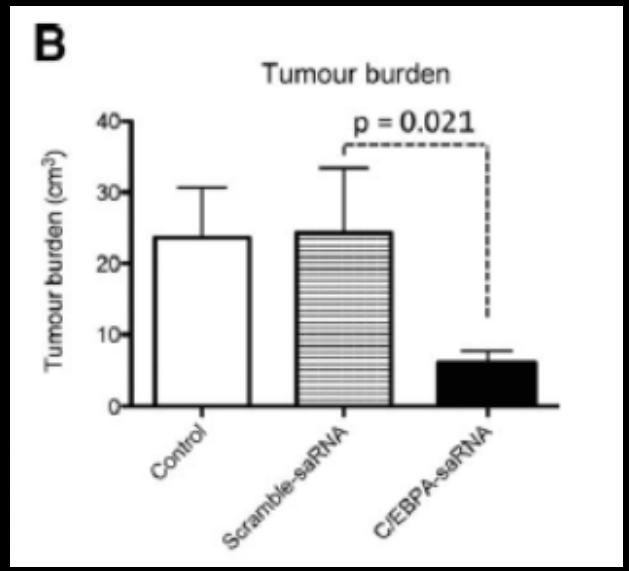
C/EBPα

albumin

Increased mRNA







Xenograph tumor inhibition

Do they act by inhibiting DNA methyltransferases?

Differentiation, transformation, reprogramming, leukemia

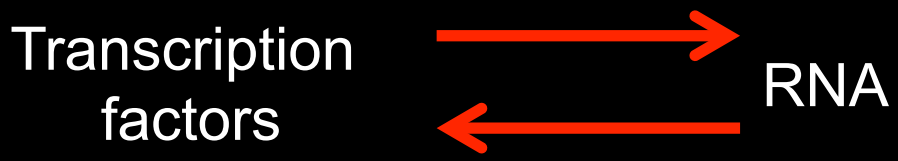
DRIVERS

PASSENGERS

Transcription factors

Differentiation, transformation, reprogramming, leukemia

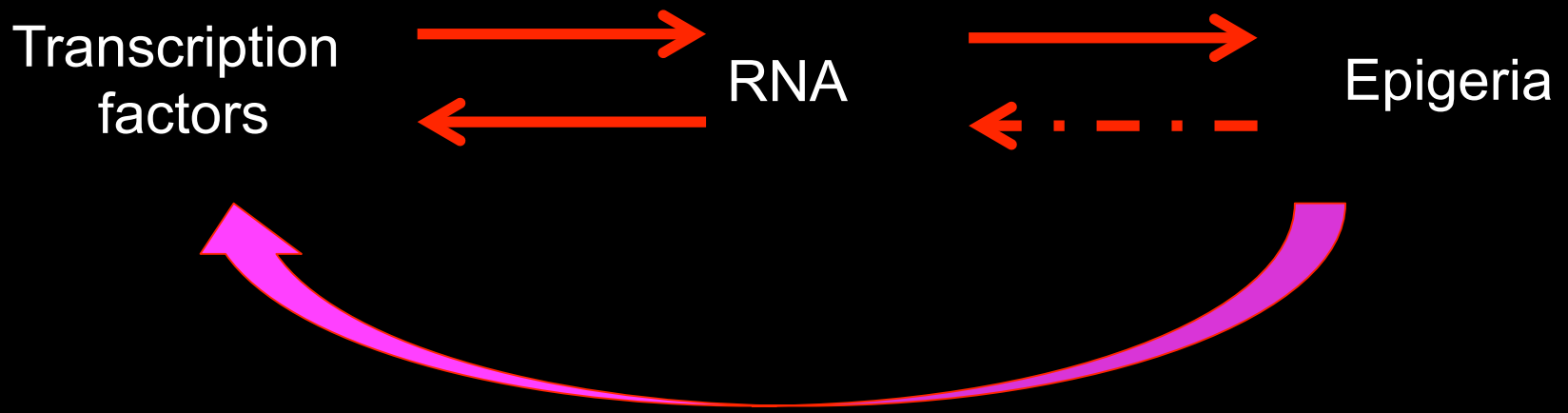
DRIVERS



Differentiation, transformation, reprogramming, leukemia

DRIVERS

ENFORCERS



Future Directions:

- CRISPR-like RNAs to induce demethylation of tumor suppressors use in myelodysplastic syndrome (MDS)

- ? binding of RNA to DNMT3a in MDS and AML?
  - The RNA binding region of DNMT1 and DNMT3a are conserved
  - What is the RNA binding of wild type and mutant DNMT3a?
  - Does this have any role in MDS/AML?

- Binding of RNA to Polycomb and other epigenetic modifying enzymes

- RNA binding by transcription factors ("DNA" binding proteins):
  - PU.1
  - C/EBPα
  - HoxA9
  - SALL4

Acknowledgments

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  - Annalisa DiRuscio
  - Alex Ebralidze
  - Min Ye
  - Philipp Staber
  - Akos Czibere

- Li Chai
  - Chai lab members

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- Sriharsa Pradhan (New England Biolabs)
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- Konstantin Ebralidse

CSI SINGAPORE
Cancer Science Institute of Singapore

Nicole C. Tenen

Danielle E. Tenen

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(Cancer Science Institute at the National University of Singapore)

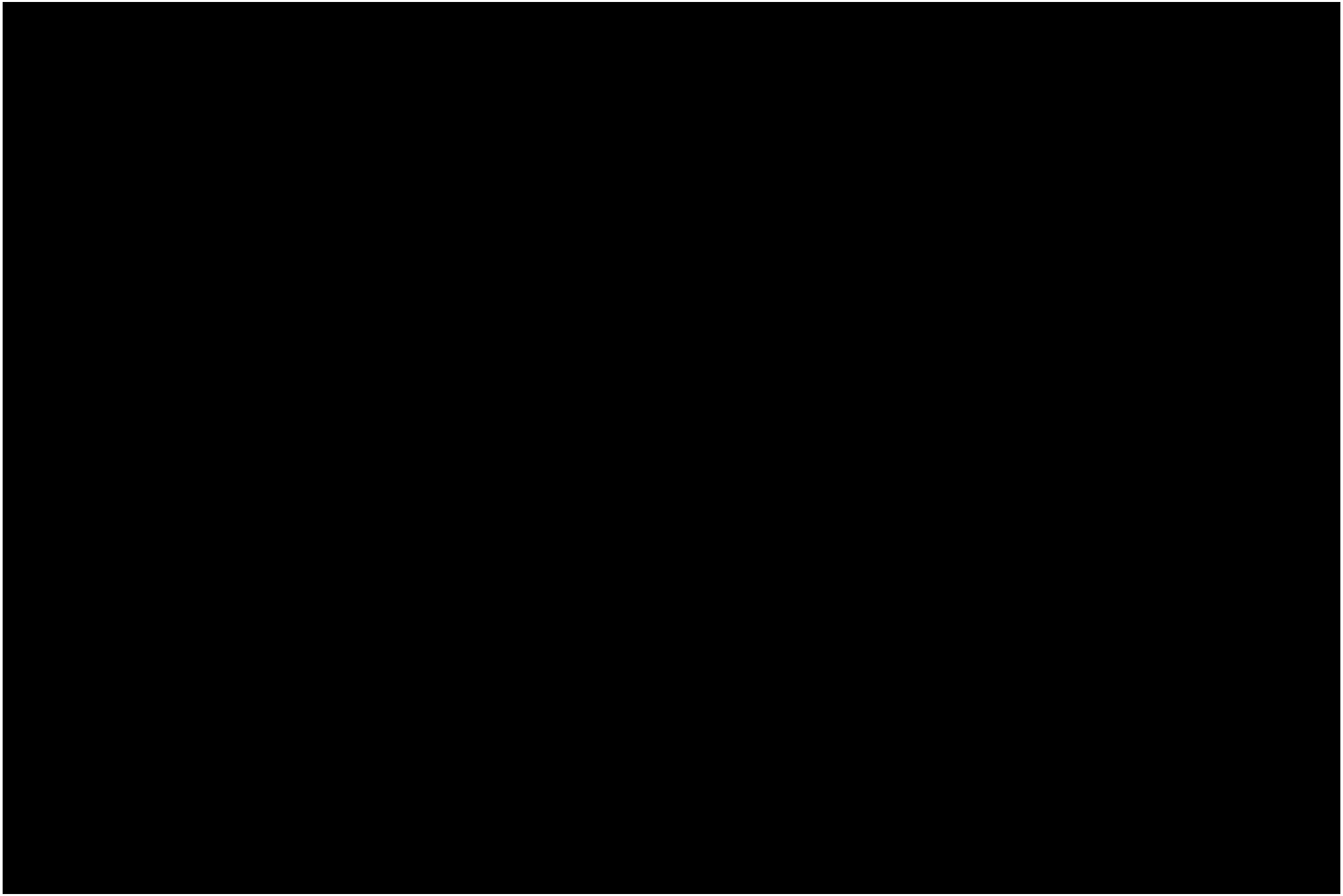
Great opportunities for great postdocs!
(and in my Harvard lab)

NUS
National University
of Singapore

CSI
SINGAPORE
Cancer Science
Institute of
Singapore

CSI @ Centre for Translational Medicine

That’s all, folks…



Acknowledgments: The Blind Men and the Elephant by John Godfrey Saxe

It was six men of Indostan,
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind.

The First approached the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The Second, feeling of the tusk
Cried, "ho! what have we here
So very round and smooth and
sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear!“

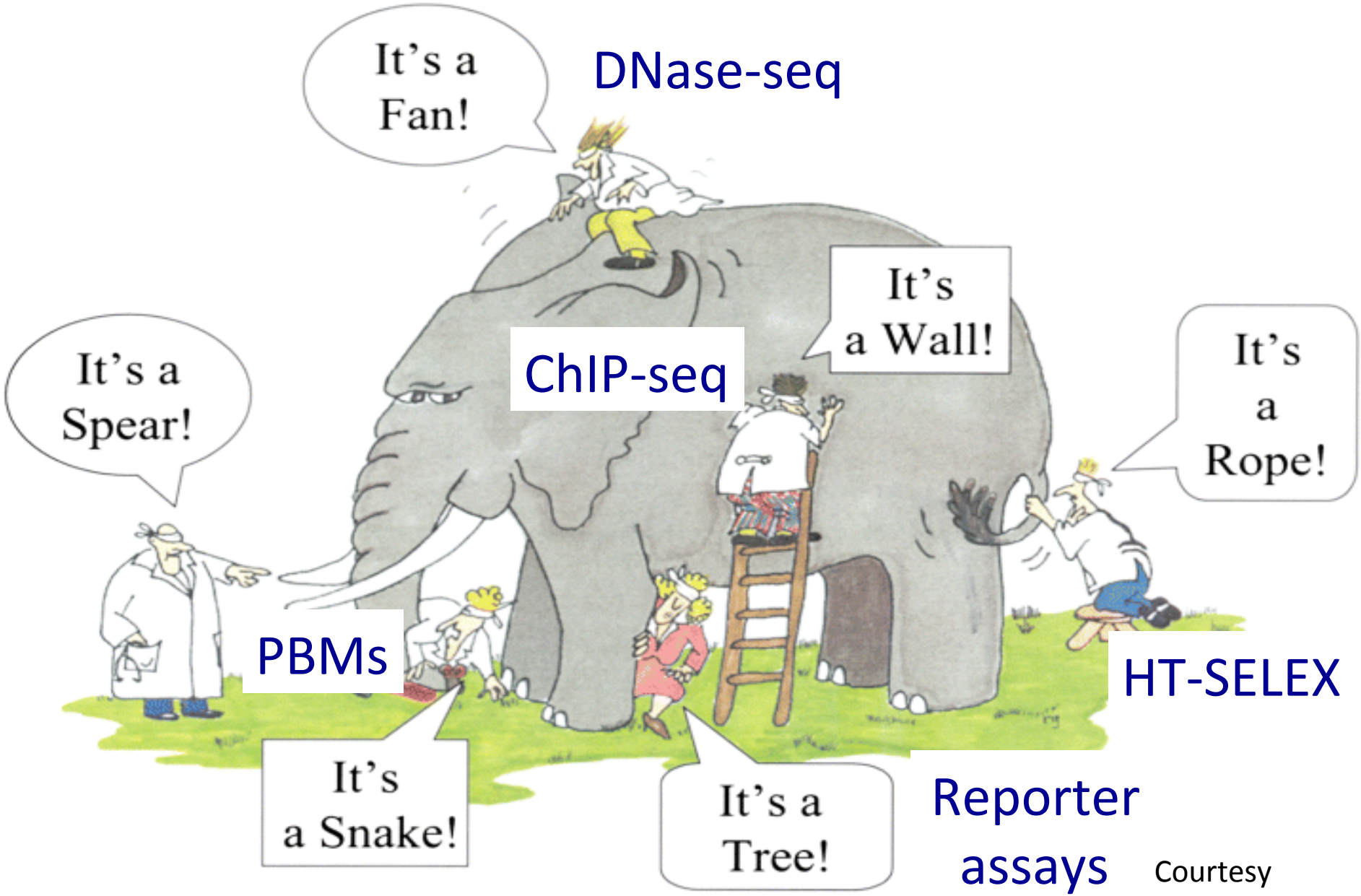
The Third approached the animal
And happening to take
The squirming trunk within his
hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!“

The Fourth reached out his eager
hand,
And felt about the knee.
"What most this wondrous beast is
like,
Is mighty plain," quoth he;
'"Tis clear enough the Elephant
Is very like a tree!"

The Fifth, who chanced to touch the
ear,
Said "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Than seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

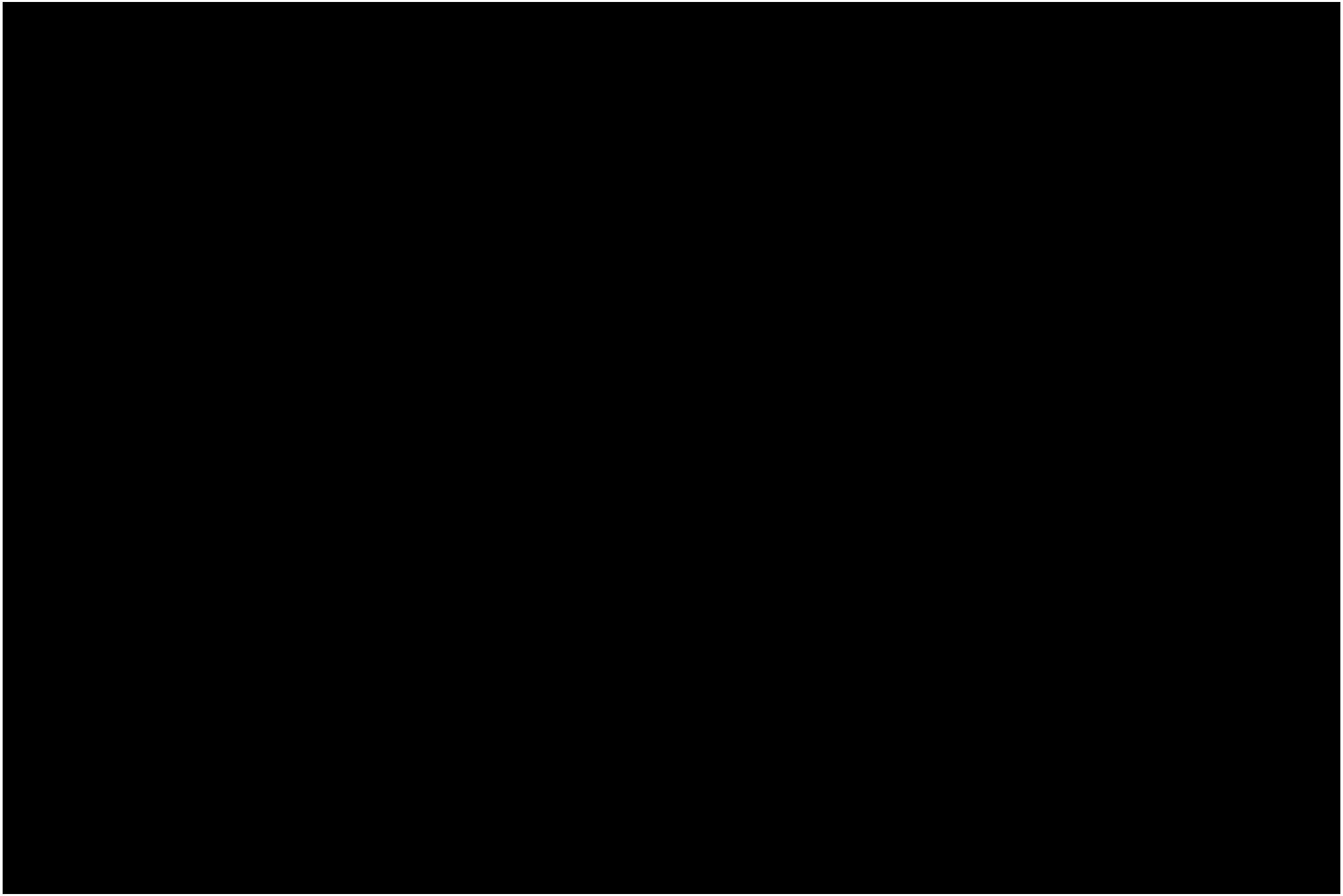
And so these men of
Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and
strong,
Though each was partly
in the right,
And all were in the wrong!



Courtesy
Marian Walhout

I just try to do science;
only the Swami of Venice Beach knows the real truth!

That's all, folks

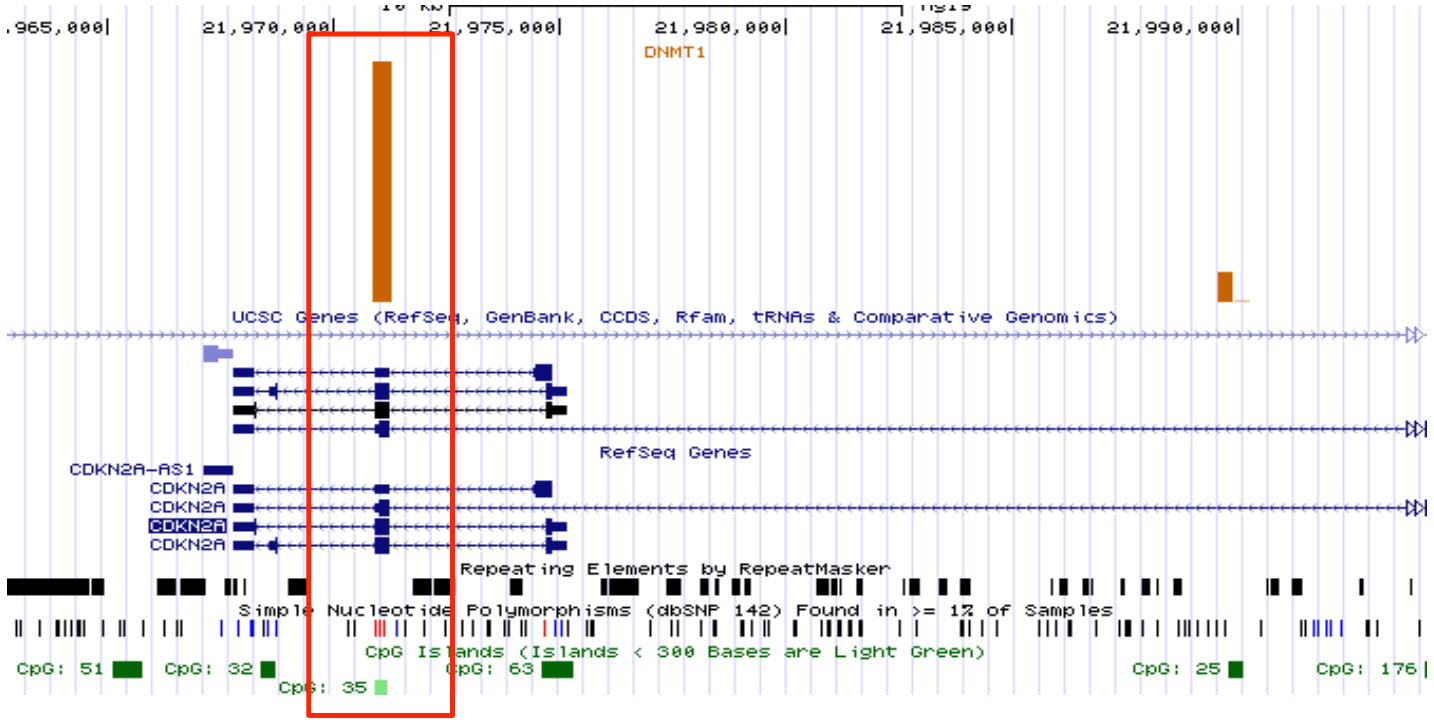


A typical Tenen lab meeting:
sleeping behind eye goggles to pretend to be awake

Special thanks
to
Gerhard Behre
Hematology/
Oncology
Leipzig

DNMT1 binds RNA within the p16 gene

DNMT1 Rip-Seq in HL-60 (myeloid) cells,
in which it is unmethylated and expressed



How are these DNMT1 interacting RNAs generated?

- nuclear, poly A-, 5' capped
- pol III dependent (at early time points)
- cell cycle regulated
- cannot detect splicing
- the ecRNA 5' end is frequently methylated in cancer
  (and the C/EBPα promoter is *not)*

Questions:

- what transcription factors are involved?
- what is the relationship between pol III and pol II?
- what is the expression in normal cells and disease states (MDS)?

C/EBPα noncoding RNA (ncRNA)

- Almost all nuclear, almost all poly A-
- Levels of the ncRNA correlate with levels of mRNA
- Levels of ncRNA are 50 fold less than mRNA (total RNA), *BUT*
- Levels of nuclear ncRNA are comparable to nuclear mRNA
- Unlike the mRNA, the ncRNA is pol III dependent
- Knocking down ncRNA with shRNAs leads to decreased mRNA and increased methylation
- Overexpressing a portion of the ncRNA can induces C/EBPα mRNA and demethylation in nonexpressing lines (a gene selective effect)

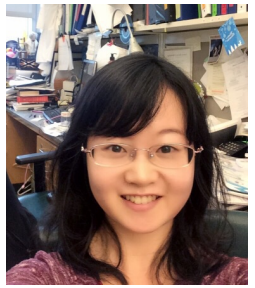
Original question: what noncoding RNAs are in the C/EBPα locus?
But the final question became:

(1) Does DNA methylation silence genes? or…
(2) Does DNA methylation follow silencing?

For many genes (? thousands), DNA methylation enforces silencing

- RNA (transcription) protects DNA from methylation
- Mechanism: DNMT1 binds RNA greater affinity than DNA and inhibits DNA methylation
- Supports the model that RNA protects DNA from methylation, and that genes shut down first, then are methylated, rather than being shut down by methylation
- RNA can induce gene selective demethylation of tumor suppressors
- Almost all DNMT1-binding RNAs are nuclear and poly A-
  (a new RNA "space")

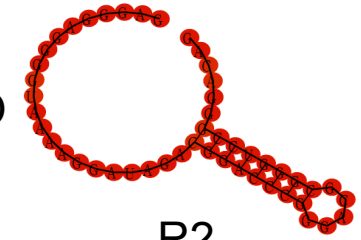
Yanjing Liu



Using RNA to target methylation and activate silenced p16

5’GAGGGAGGGGTAAAAGGATAGACGGACCCGGGACGCGGGTCCGGGACAG 3’

TFO (reverse Hoogsteen Base)
at -594 to -569 in p16 promoter



R2 from ecCEBPA)