# Molecular basis of the disease: functional and genetic validation of targets for therapeutic manipulation

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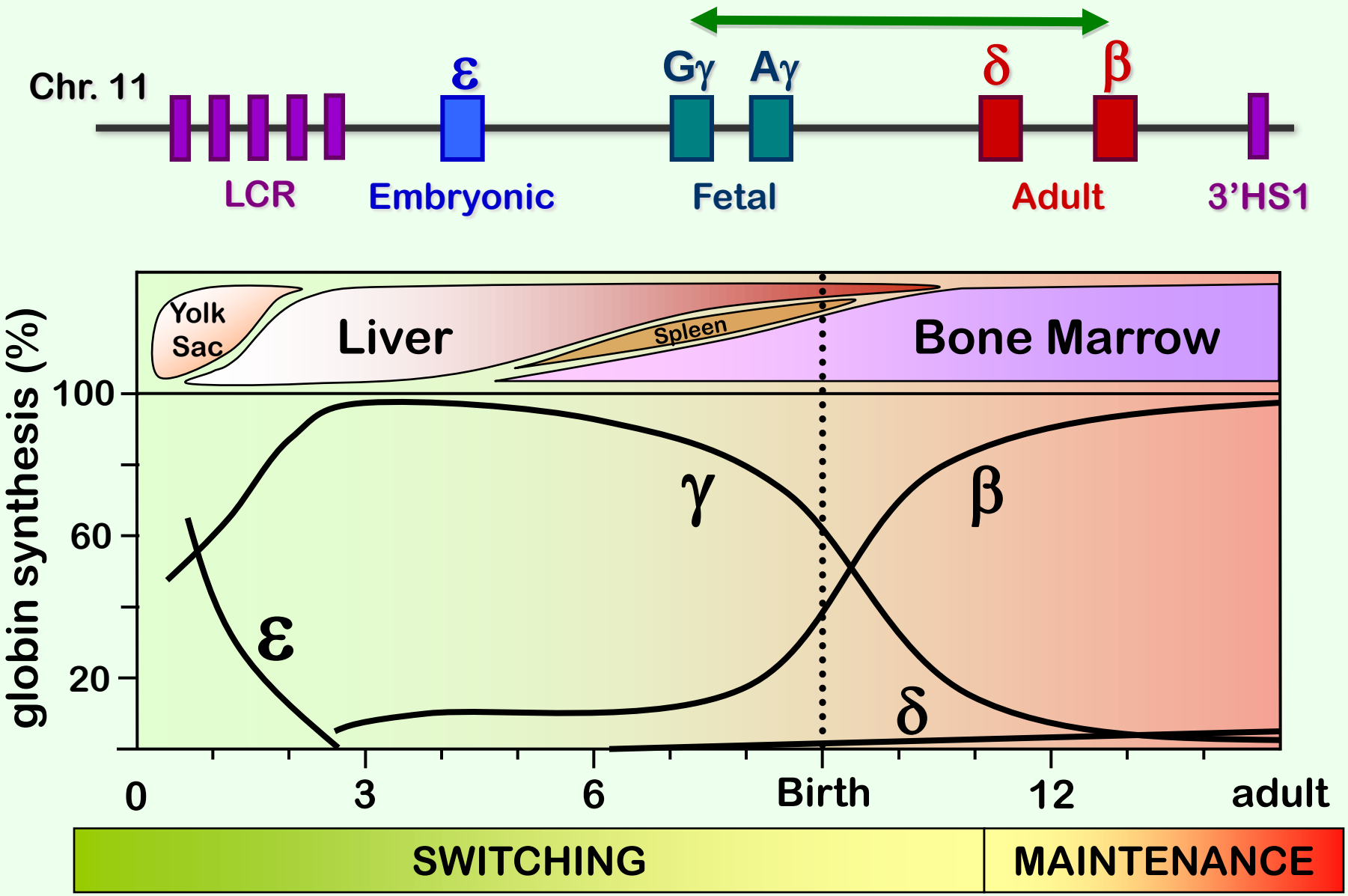
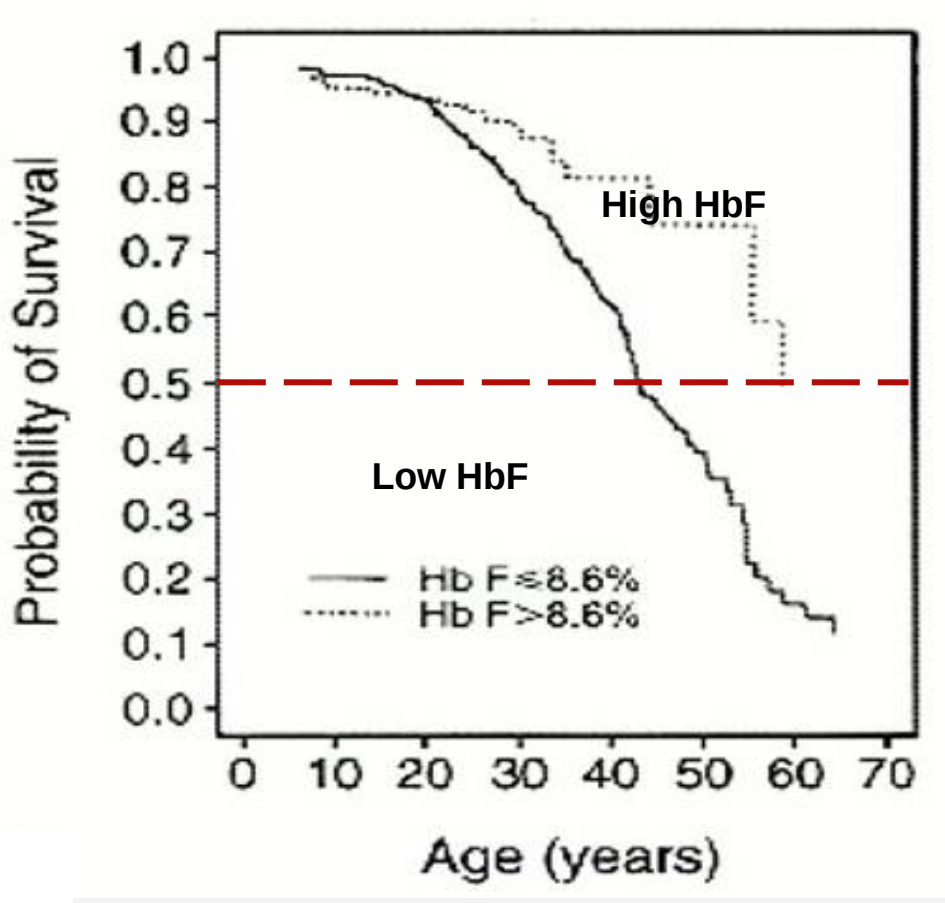
# Human Globin Switching



# SS disease survival and HbF

1. Increased HbF lessens severity of SCD
2. Relatively small increments provide substantial clinical benefit
3. No downside of 100% HbF




Platt et al. NEJM 1994

Need: > 300,000 SS babies/year in Africa alone; similar numbers for β-thalassemia

A major and increasing problem in underdeveloped regions

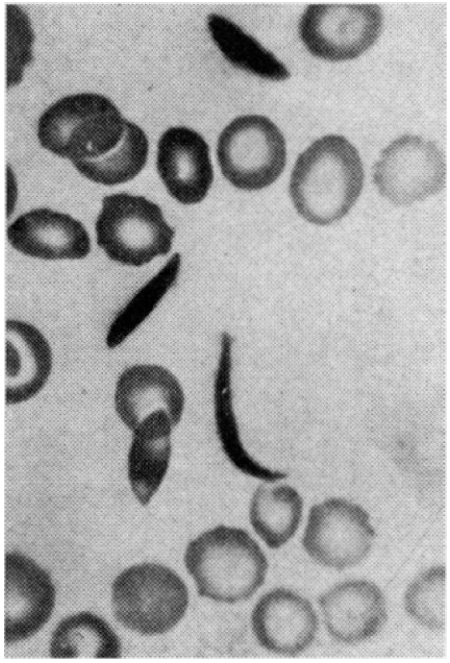
# Sickle Cell Anemia: the First "Molecular Disease"

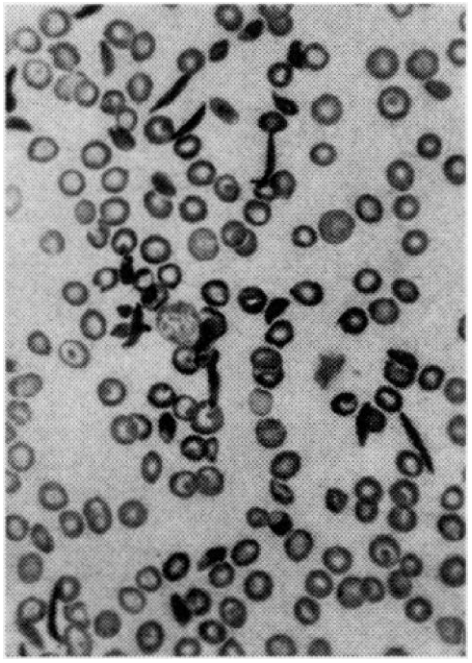
Arch. Int. Med. 5:517, 1910.

## Peculiar Elongated and Sickle-shaped Red Blood Corpuscles in a Case of Severe Anemia[a]

James B. Herrick, M.D.

*1013 State Street, Chicago, Illinois*





SCIENCE April 29, 1949, Vol. 109

## Sickle Cell Anemia, a Molecular Disease[1]

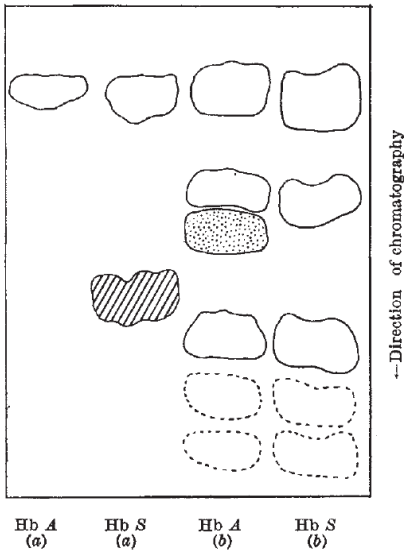
Linus Pauling, Harvey A. Itano,[2] S. J. Singer,[2] and Ibert C. Wells[3]

*Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California*[4]

## A SPECIFIC CHEMICAL DIFFERENCE BETWEEN THE GLOBINS OF NORMAL HUMAN AND SICKLE-CELL ANÆMIA HÆMOGLOBIN

By Dr. V. M. INGRAM

Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, University of Cambridge




October 13, 1956 NATURE

HbA Val-His-Leu-Thr-Pro-Glu-Glu-Lys ....

HbS Val-His-Leu-Thr-Pro-Val-Glu-Lys ....

# Single goal

Replace defective adult $\beta^S$-globin chain
with fetal $\gamma$-globin chain

# Rationale for studying hemoglobin switching

1. Premise that underlying mechanisms of Hb switching and $\gamma$-gene silencing will identify targets for reactivation of HbF

2. Supposition that manipulation of a *single* target of the switching apparatus will lead to *sufficient* HbF reactivation for clinical benefit

3. The hypothesis has been untested

# Timeline of "switching" research

1950 1960 1970 1980 1990 2000 2010

HbF syndromes and genetics

Cloning globin loci

Transgenic assays--LCR

Transcription factor identification-GATA1

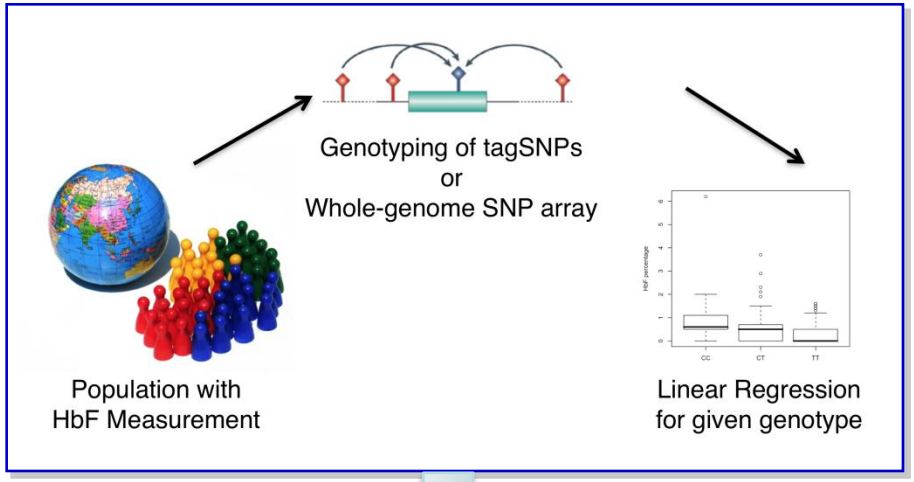
HbF linkage studies

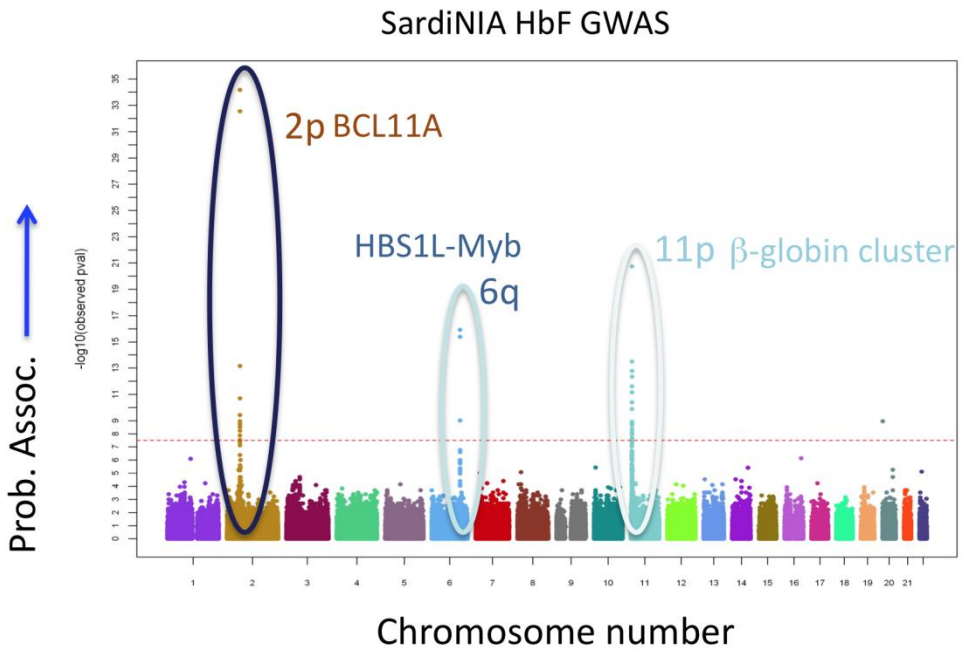
Chromosome dynamics

Looping of locus

**SPECIFIC gene(s) that regulate the level of HbF in humans**

# Genome-wide association of HbF levels




Σ GWAS peaks = ~ 50% genetic variation in HbF levels

(Menzel et al, Nat. Genet. 2007; Uda et al, PNAS, 2008)

# BCL11A: background and findings

1. Zinc-finger repressor protein, required for B-lymphoid cells in development (Liu et al, Nat. Immunol. 2003)

2. SNPs in BCL11A intron-2 associated with HbF levels by GWAS (Menzel et al, Nat. Genet. 2007; Uda et al, PNAS, 2008)

3. Required to maintain $\gamma$-globin silencing in primary human erythroid precursors generated from CD34+ progenitors (Sankaran et al, Science, 2008)

4. Required for developmental switching from embryonic to adult globin in mouse, and for developmental silencing of human $\gamma$ -gene in transgenic mice (Sankaran et al, Nature 2009; Xu et al, Genes Dev. 2010)

# BCL11A: background and findings

5. Required for complete silencing of human $\gamma$ -globin gene in adult mice (Xu et al, Science 2011)

6. Dispensable for red blood cell maturation and production (Xu et al, Science 2011)

7. Interacts with several corepressor protein complexes (that include enzymatic components, e.g. HDACs, Dnmt1) (Sankaran et al, Science 2008; Xu et al, unpublished data)

8. Effects of known HbF inducing agents (azaD and HDAC inhibitor) greatly augmented by loss of BCL11A (Xu et al, Science 2011)

9. Erythroid-restricted knockout of BCL11A rescues phenotype of SCD disease mouse models (Xu et al, Science 2011)

# Alternate approches to SCD

Primary defect in hemoglobin

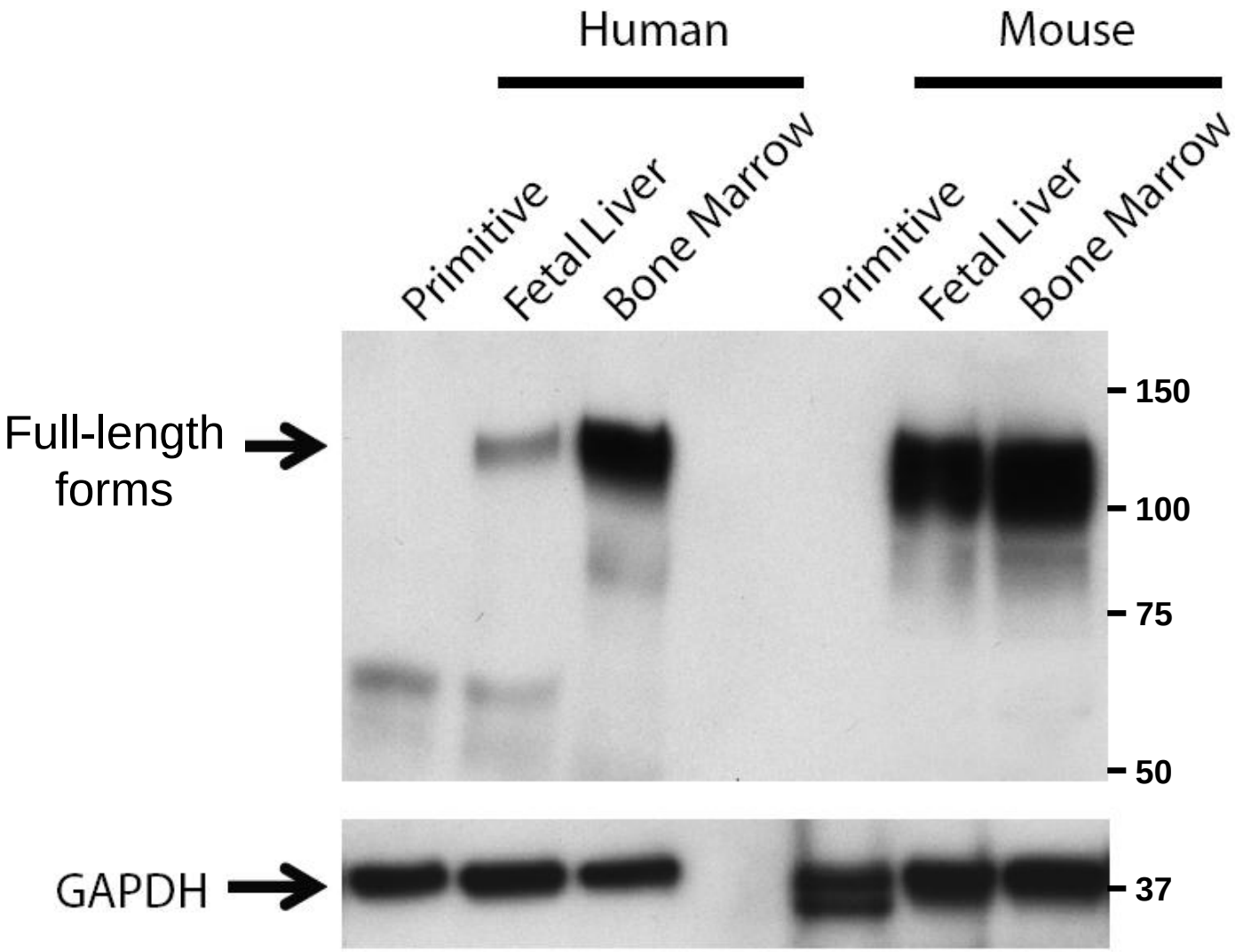
## HbS – directed

1. Impair HbS polymerization by small molecule strategies
2. Repair $\beta^S$-gene through iPS cells, gene correction, and *in vitro* production of HSCs for transplantation

## HbF – directed

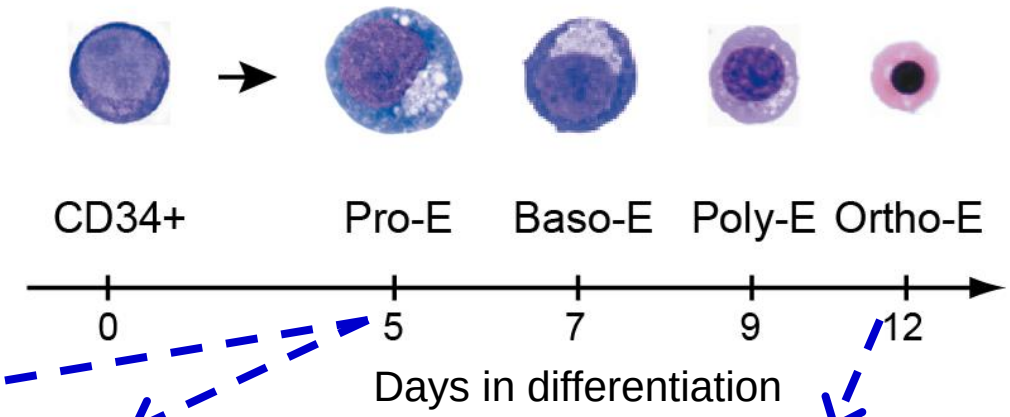
1. Interfere with $\gamma$-gene silencing through inhibitory RNAs to critical components of silencing apparatus
2. Interfere with $\gamma$ -gene silencing through impairment of silencing protein(s) or interactions with small molecules
3. Identify additional pathways, as yet unknown, regulating silencing

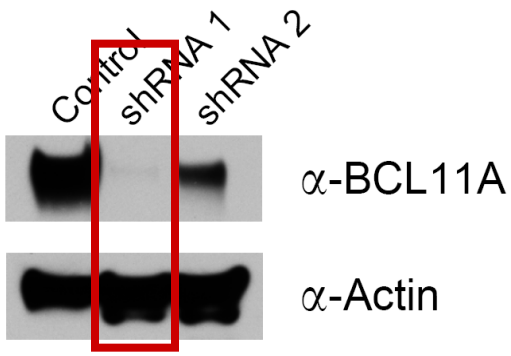
# Stage-specific expression of BCL11A



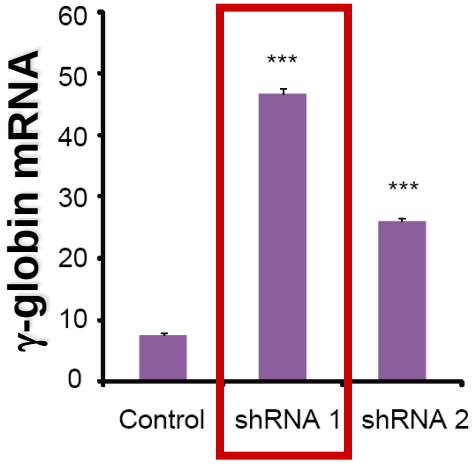
# Down-regulation of *BCL11A* Reactivates HbF Expression

***Ex vivo* maturation of adult human CD34+ progenitors**

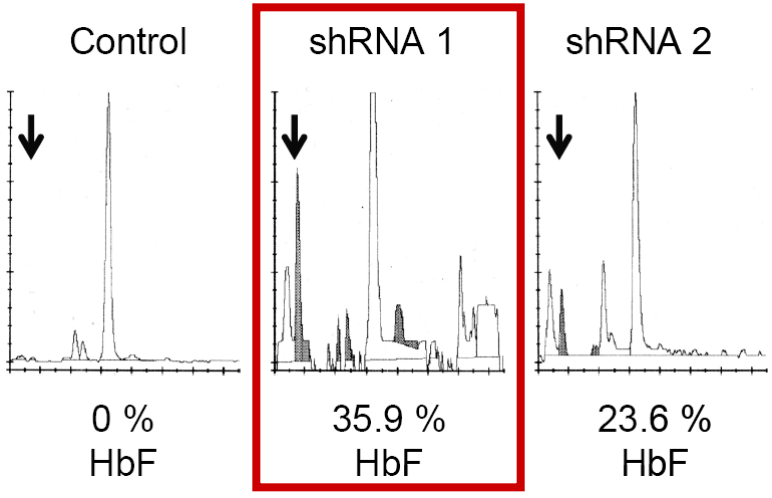



**Western Blot**




**qRT-PCR**

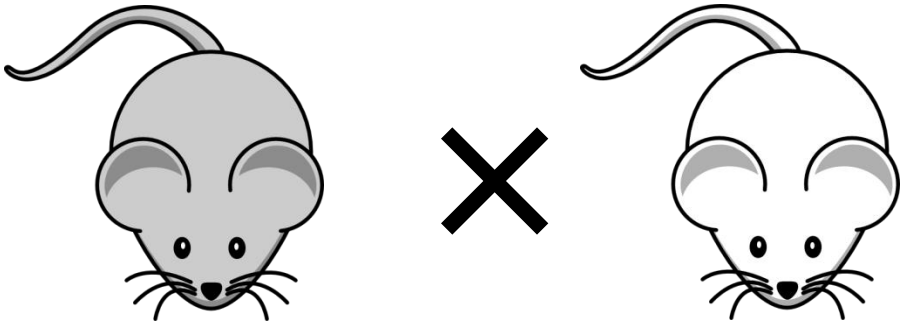



**HPLC**

**BCL11A maintains silencing of γ-globin expression in adult human erythroid cells**

Sankaran et al, Science 2008

# Testing Role of *BCL11A* in Developmental Switching

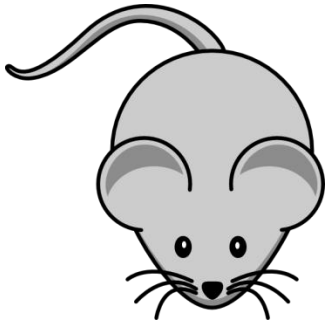


***Bcl11a* cKO**

*Ippolito GC et al.*

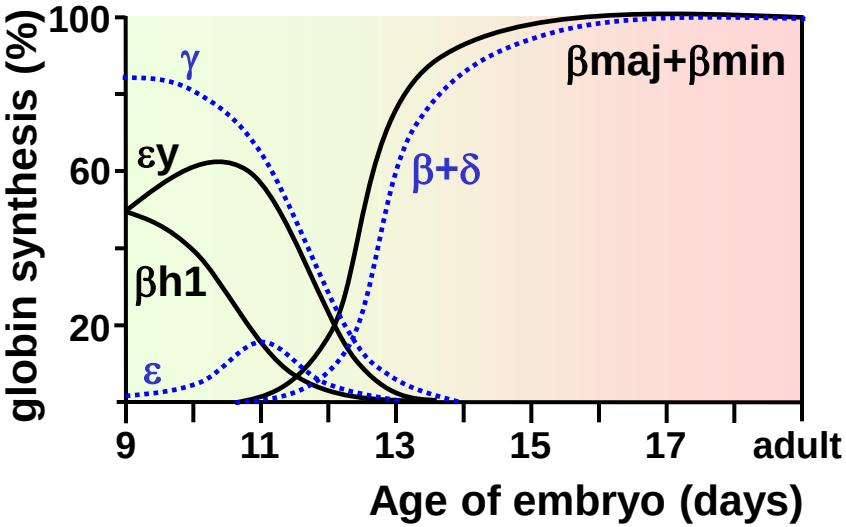
***β*-YAC**

*Peterson KR et al.*

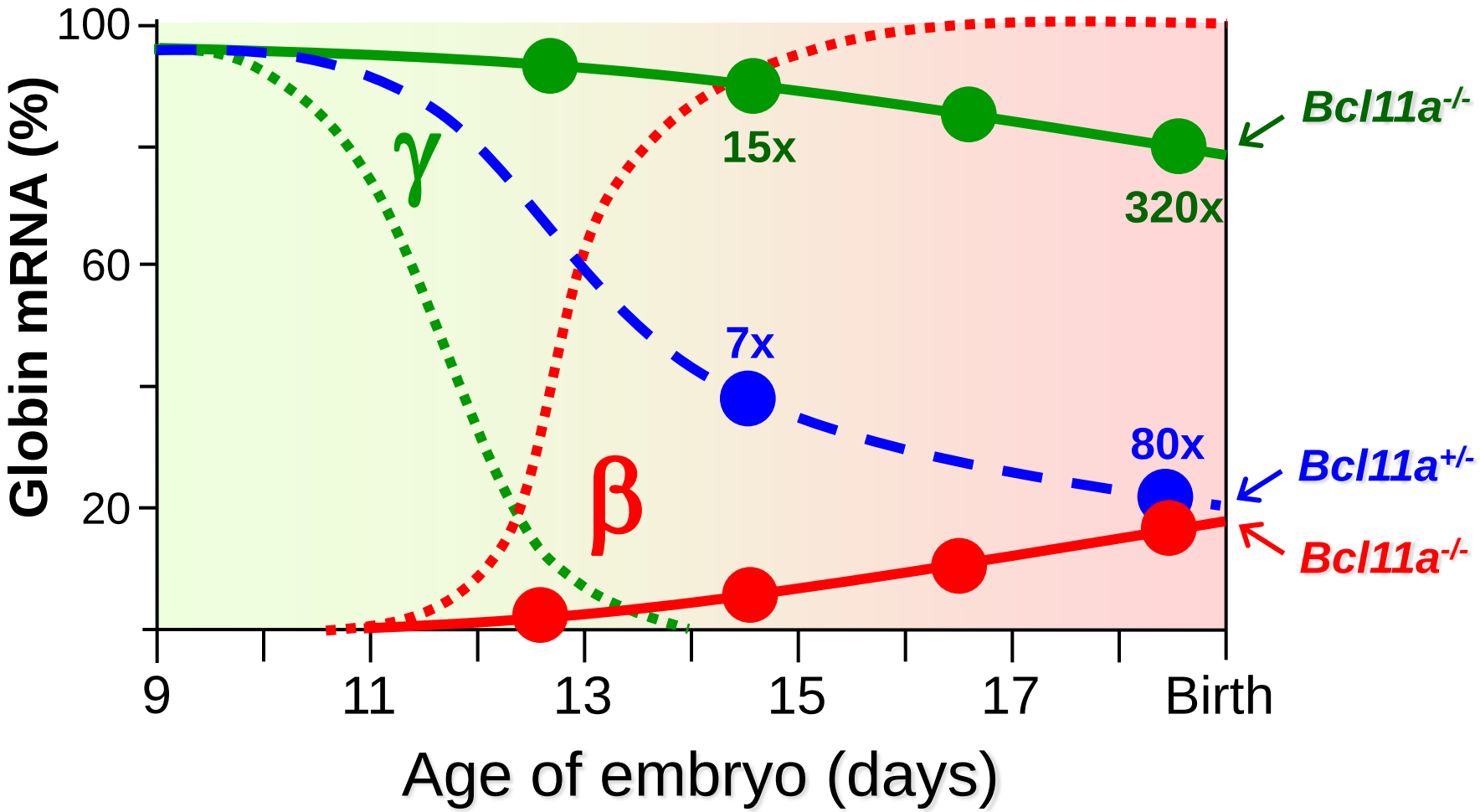


***β*-YAC; *Bcl11a*$^{-/-}$**
***β*-YAC; *Bcl11a*$^{+/-}$**
***β*-YAC; *Bcl11a*$^{+/+}$**

## Developmental Globin Switching

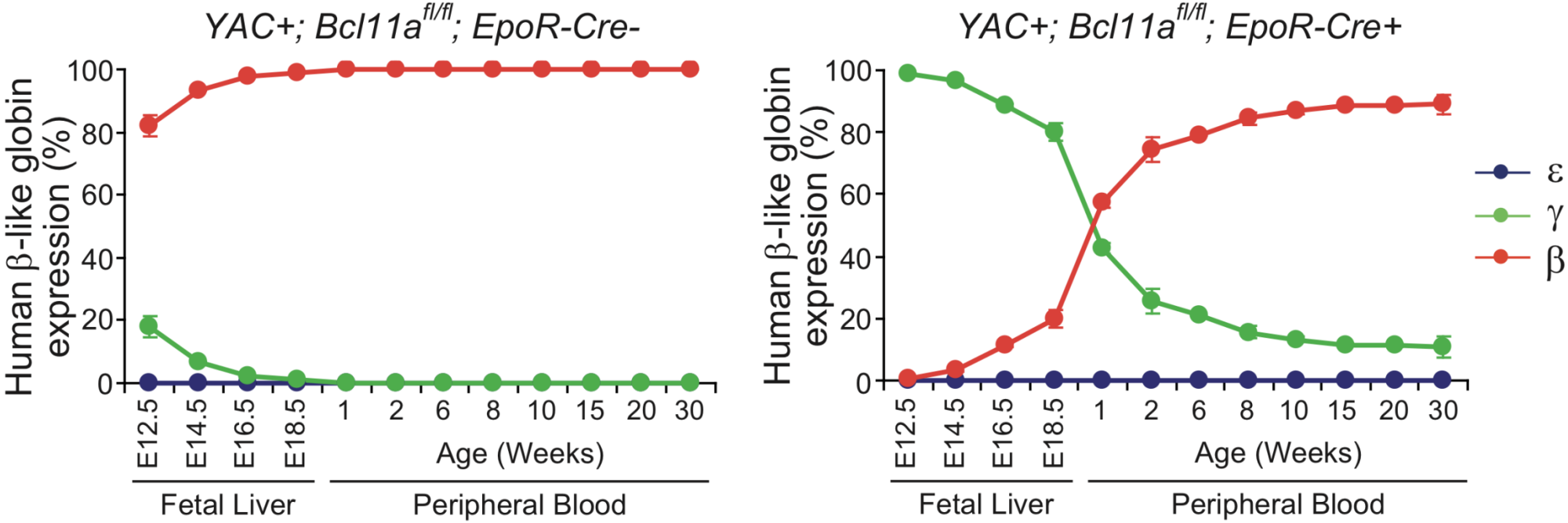


# BCL11A Controls Human Globin Switching in Dose-Sensitive Fashion



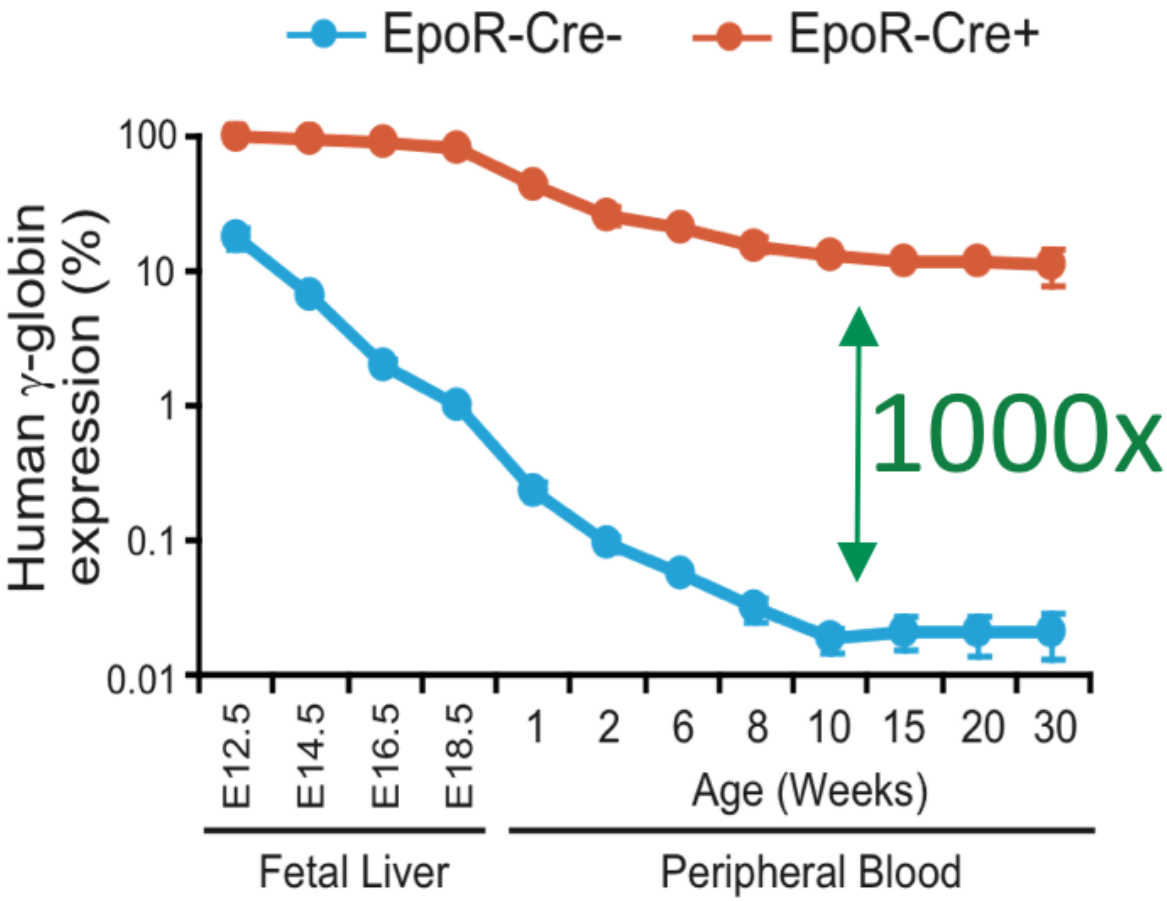

Sankaran & Xu *et al.* 2009 Nature

# Progressive, but incomplete, HbF silencing in adult mice lacking BCL11A




Xu *et al.* 2011 Science

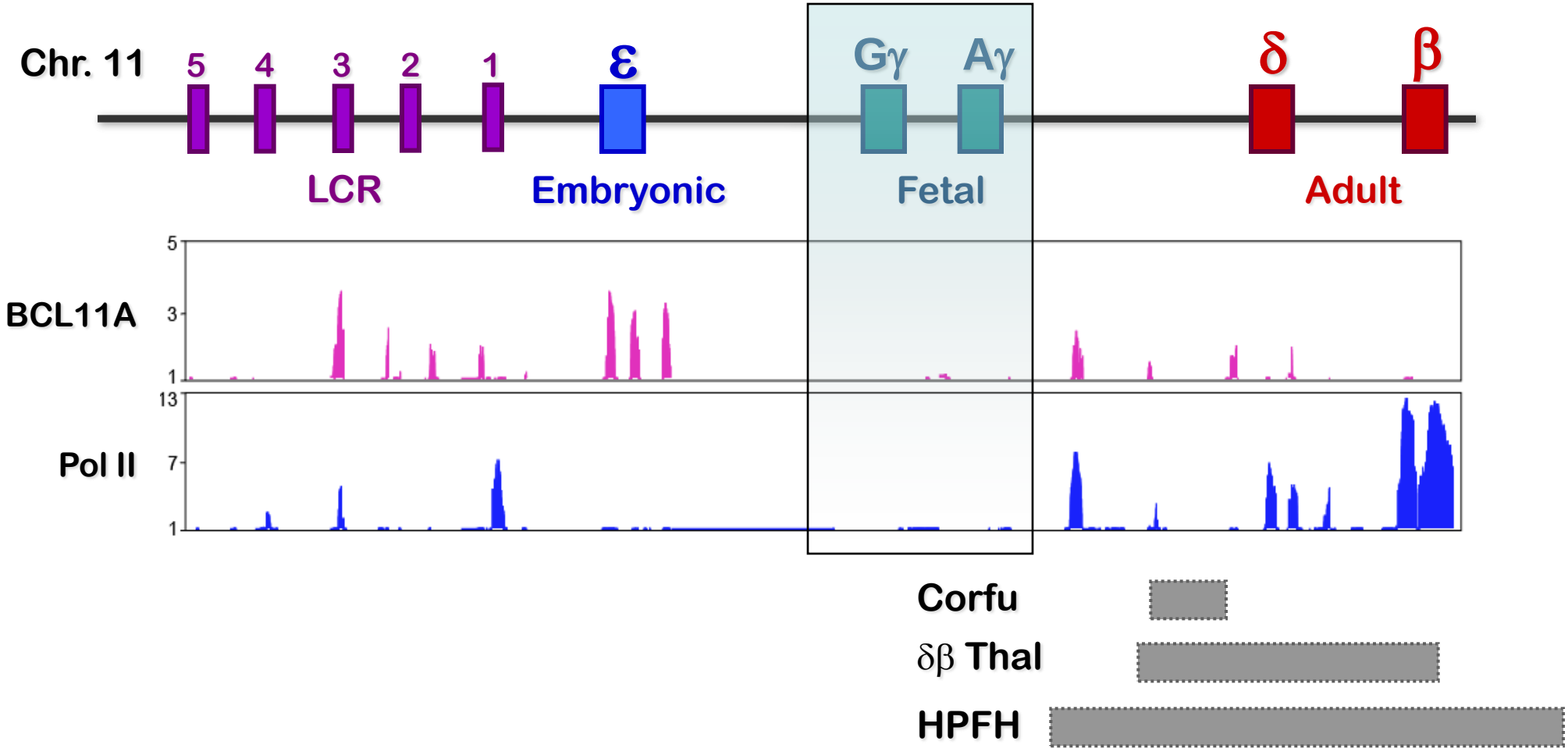
# Adult stage: 1000x derepression and partial epigenetic silencing




Xu *et al.* 2011 Science

# BCL11A Occupies the Human β-Globin Locus

## Chromatin immunoprecipitation (ChIP)-on-chip analysis in adult human erythroid cells

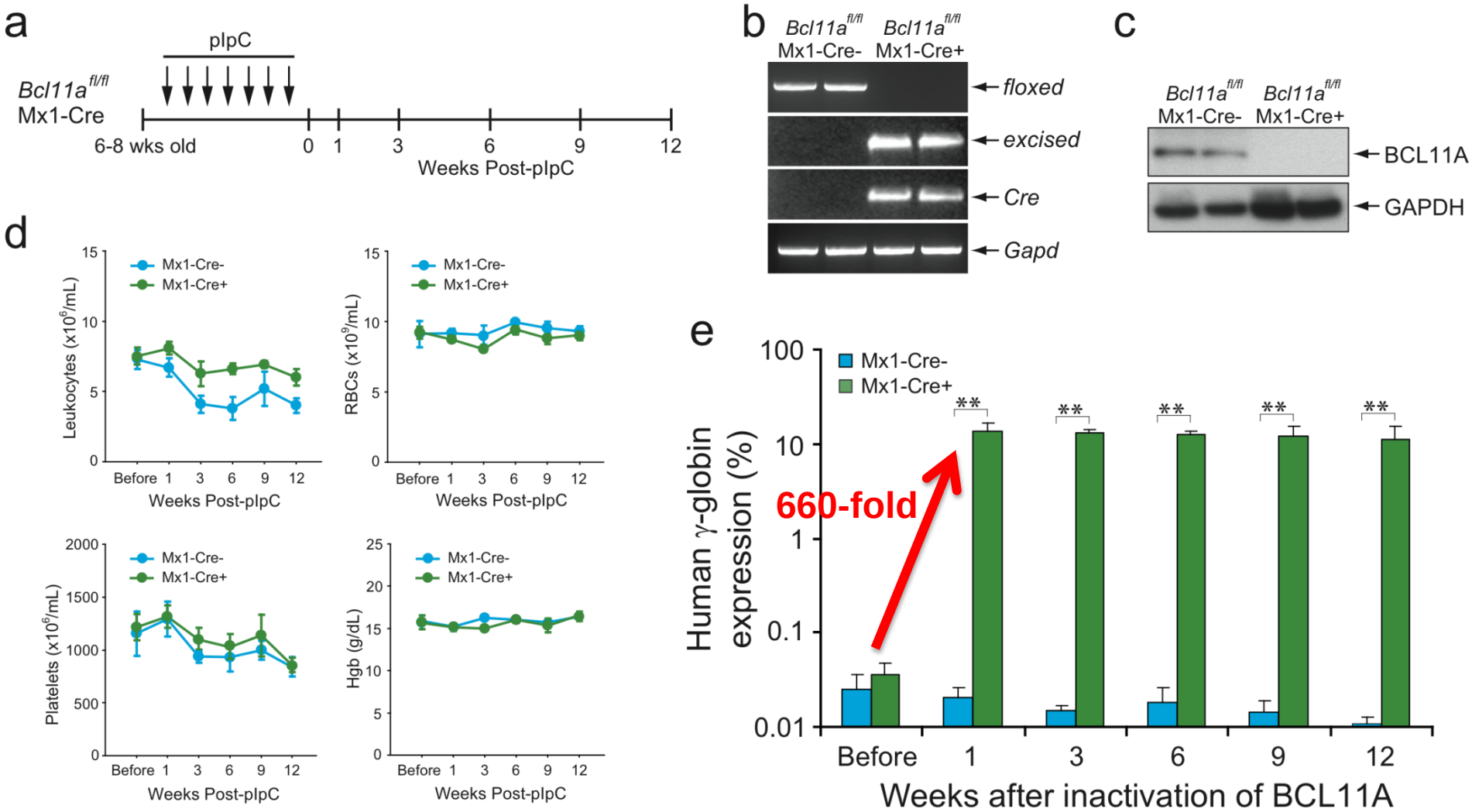


# In vivo reactivation of silenced genes?

1. Can it be accomplished?

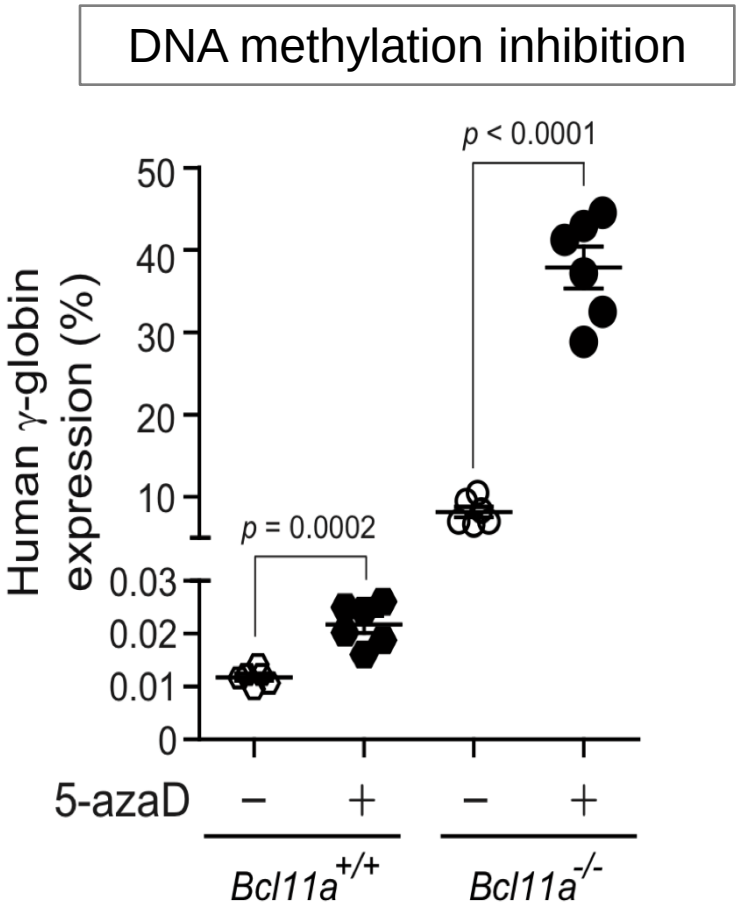
2. What are effects of drugs in combination with BCL11A loss?

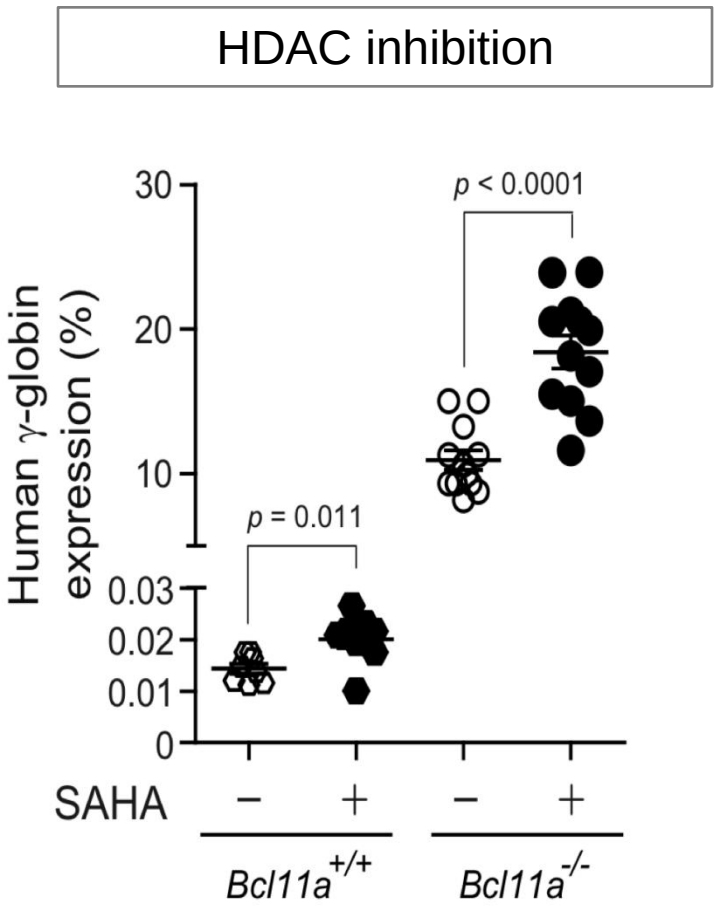
# Reinduction of previously silenced γ-genes upon inactivation of BCL11A




Xu *et al.* 2011 Science

# Cooperative induction of HbF by Bcl11A loss and known HbF inducers



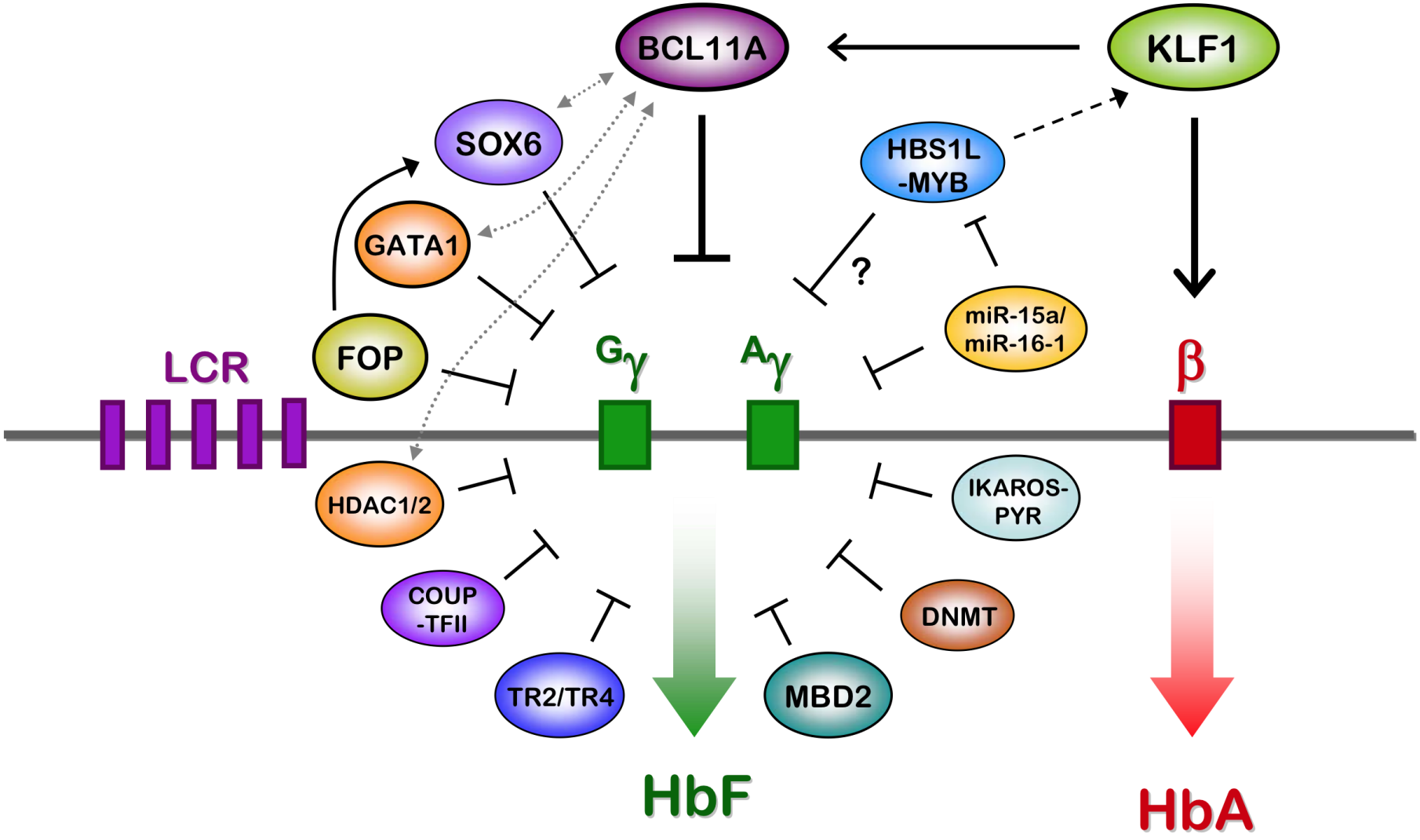

Xu *et al.* 2011 Science

# Any target for future therapy?

Validation of possible targets for therapeutic manipulation

Criteria: both function and genetic

# Potential HbF regulators

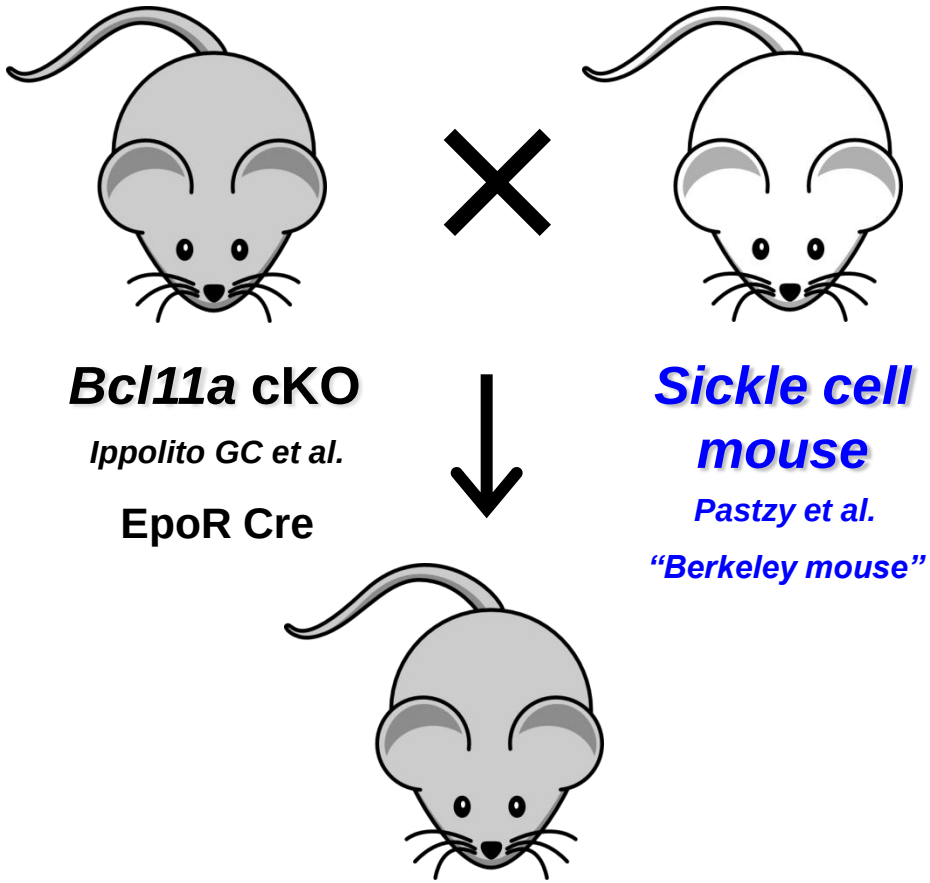


# Rationale for studying hemoglobin switching

Can manipulation of a *single* target of the switching apparatus lead to *sufficient* HbF reactivation for clinical benefit?

Alternatively, each component only contributes quantitatively for a small portion of switching and silencing *in vivo*.

# “Proof of principle” testing in preclinical model




Monitor phenotype: hematology, pathology, HbF

# "Berkeley" SCD mouse

• SCIENCE • VOL. 278 • 31 OCTOBER 1997

## Transgenic Knockout Mice with Exclusively Human Sickle Hemoglobin and Sickle Cell Disease

Chris Pászty,* Catherine M. Brion, Elizabeth Manci, H. Ewa Witkowska, Mary E. Stevens, Narla Mohandas, Edward M. Rubin

To create mice expressing exclusively human sickle hemoglobin (HbS), transgenic mice expressing human α-, γ-, and $\beta^S$-globin were generated and bred with knockout mice that had deletions of the murine α- and β-globin genes. These sickle cell mice have the major features (irreversibly sickled red cells, anemia, multiorgan pathology) found in humans with sickle cell disease and, as such, represent a useful in vivo system to accelerate the development of improved therapies for this common genetic disease.

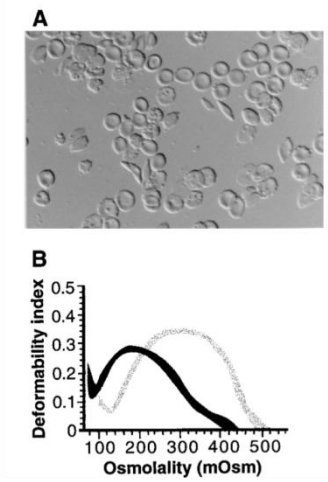
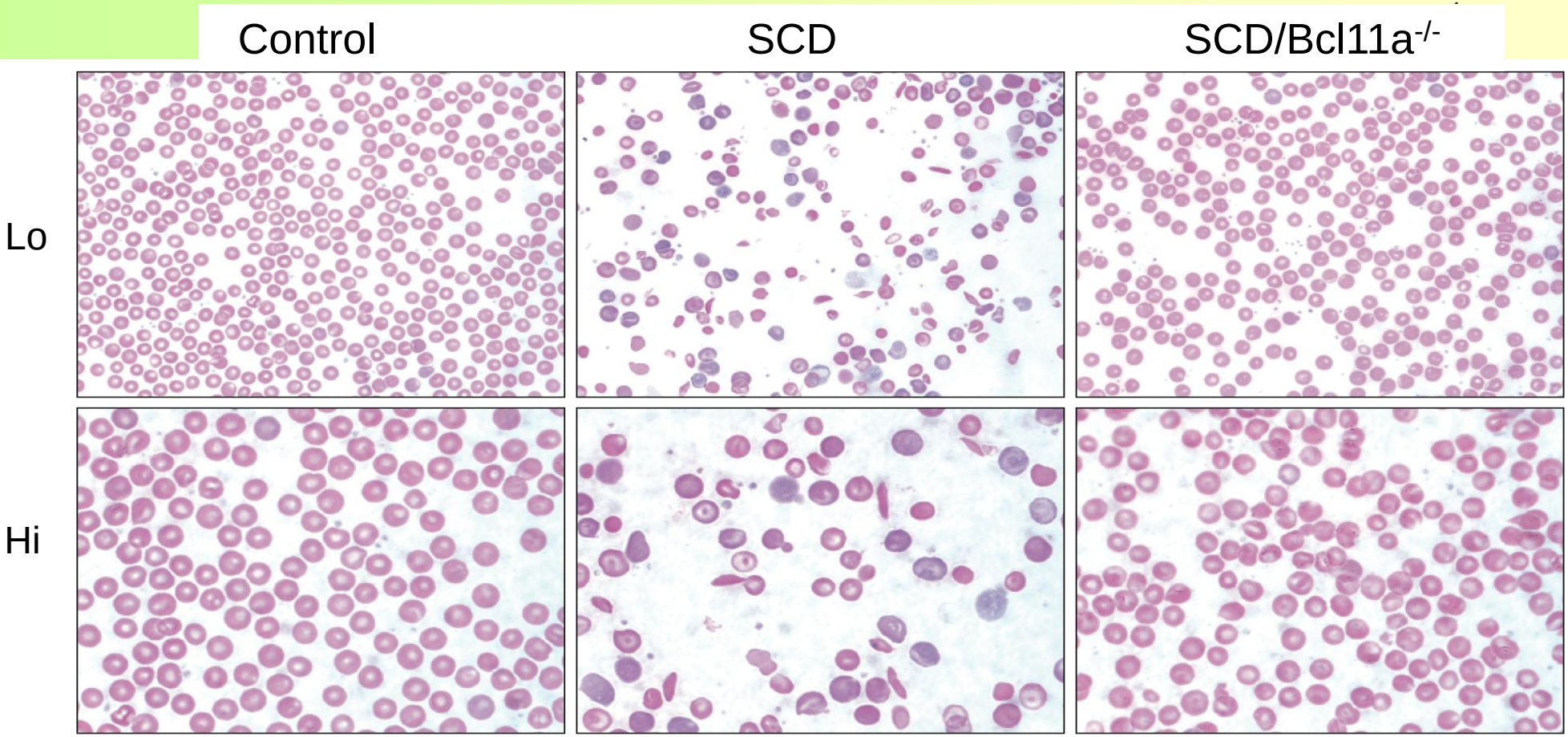



**Fig. 2.** Morphology and cellular characteristics of erythrocytes from adult sickle cell mice. (**A**) Oxygenated sickle cell mouse blood showing ISCs (elongated cells). (**B**) Osmotic deformability profiles of erythrocytes from wild-type (stippled curve) and sickle cell (solid curve) mice.

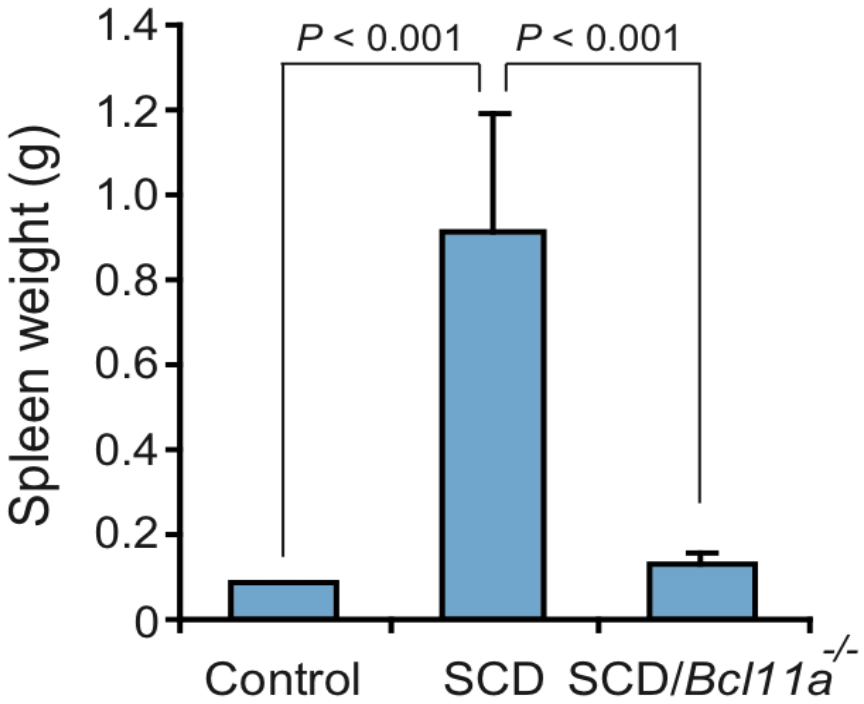
| | Hct (%) | Reticulocytes (%) | MCH (pg) | MCV (fl) | MCHC (g/dl) | HDW (g/dl) |
|---|---|---|---|---|---|---|
| Wild type | 43.6 ± 1.2 | 3.4 ± 0.5 | 13.2 ± 0.3 | 40.3 ± 0.2 | 33.8 ± 0.7 | 4.2 ± 0.2 |
| Sickle cell | 28.7 ± 2.5 | 26.8 ± 2.2 | 8.3 ± 0.4 | 34.0 ± 1.1 | 26.5 ± 0.7 | 8.3 ± 0.1 |

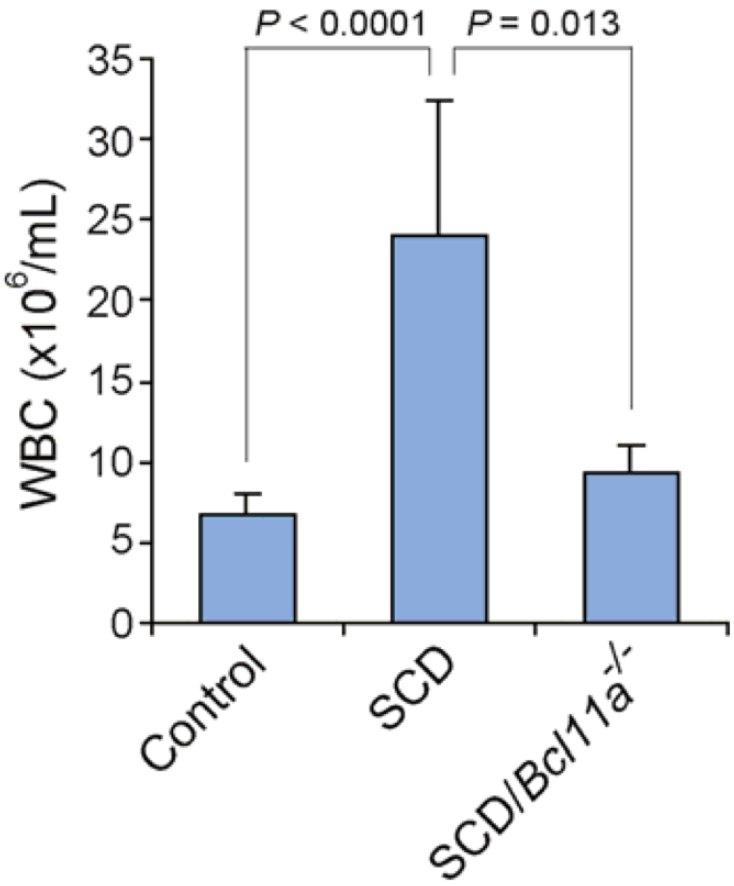
# Rescue of SCD by inactivation of BCL11A



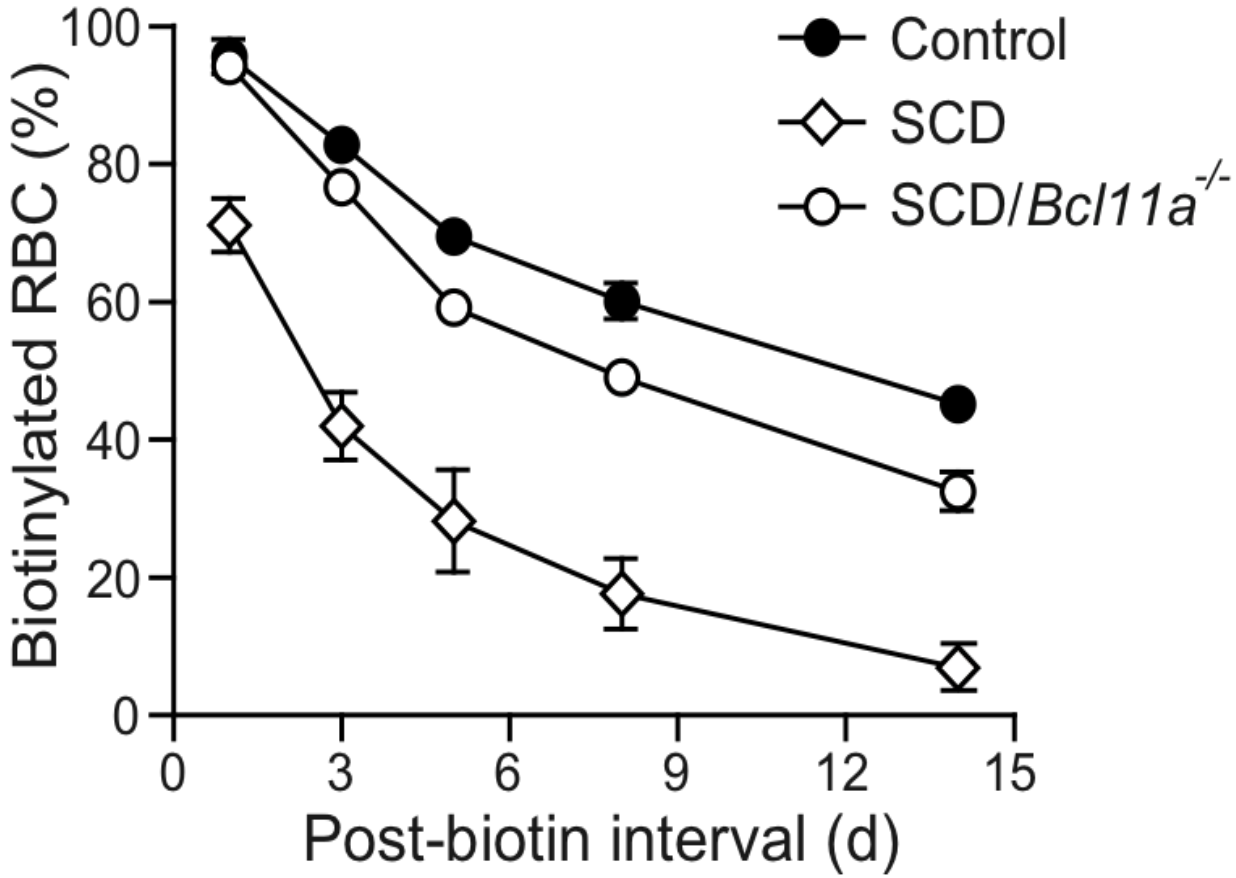

| Mice | RBC $x10^6/\mu l$ | Hb g/dl | Hct % | MCV fl | MCH pg | MCHC g/dl | Retic % | RDW % | Urine concentration mOsm |
|---|---|---|---|---|---|---|---|---|---|
| Control | 10.1 ± 0.2 | 13.1 ± 0.3 | 44.2 ± 1.0 | 44.1 ± 1.4 | 13.0 ± 0.4 | 29.7 ± 0.6 | 3.1 ± 0.6 | 19.0 ± 0.7 | 2440 ± 213 |
| SCD | 6.4 ± 0.5 | 7.8 ± 0.6 | 28.3 ± 1.9 | 44.8 ± 1.6 | 12.3 ± 0.6 | 27.5 ± 0.6 | 38.2 ± 3.9 | 26.9 ± 0.5 | 1037 ± 82 |
| SCD/Bcl11a$^{-/-}$ | 9.8 ± 0.4* | 13.6 ± 0.7** | 46.2 ± 1.4** | 47.2 ± 0.9 | 13.8 ± 0.2 | 29.3 ± 0.8 | 7.0 ± 0.3* | 23.4 ± 0.4* | 2133 ± 333* |

# Normalization of spleen size and WBC counts

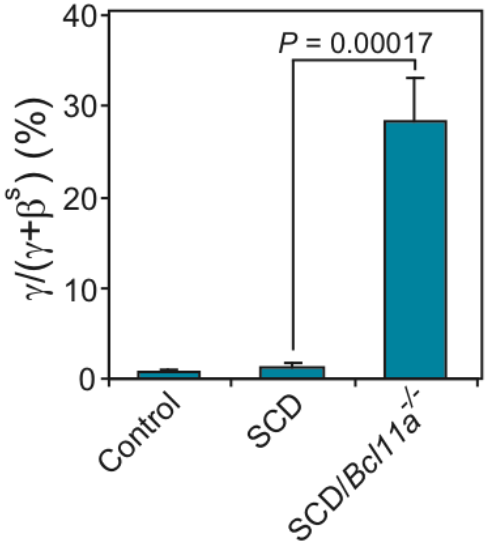


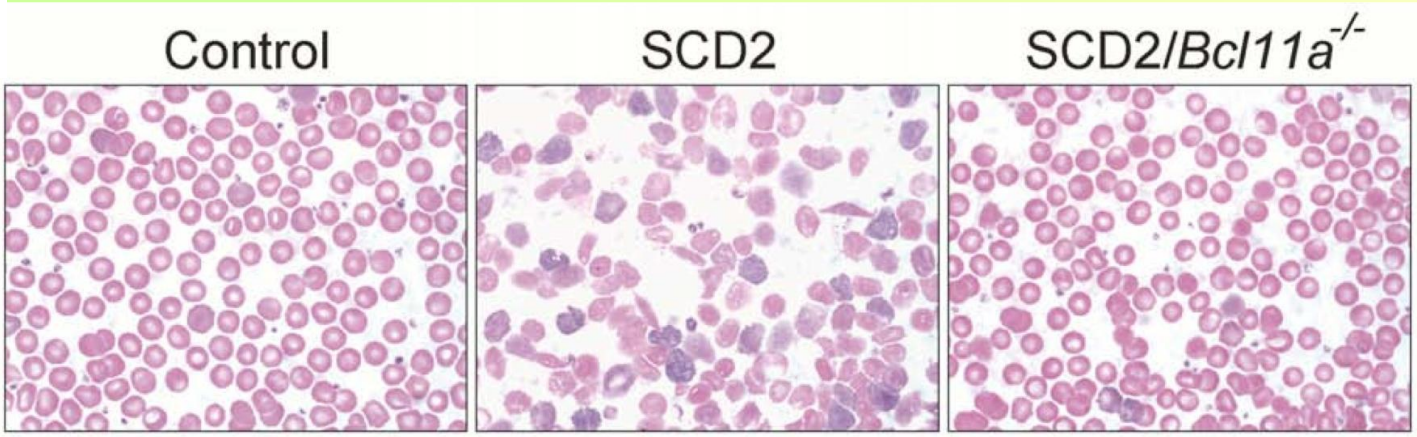
# Improvement in RBC survival in rescued SCD mice

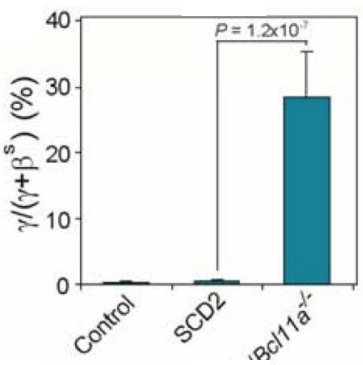


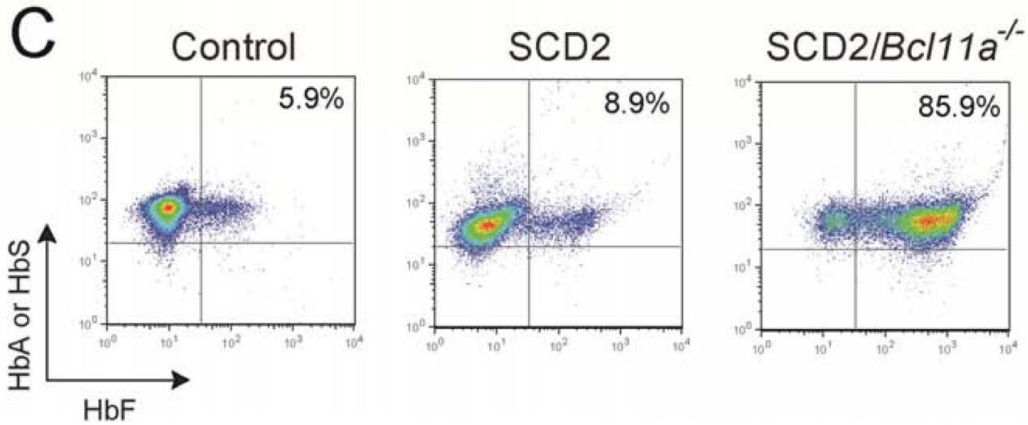
# HbF expression and distribution in rescued SCD mice

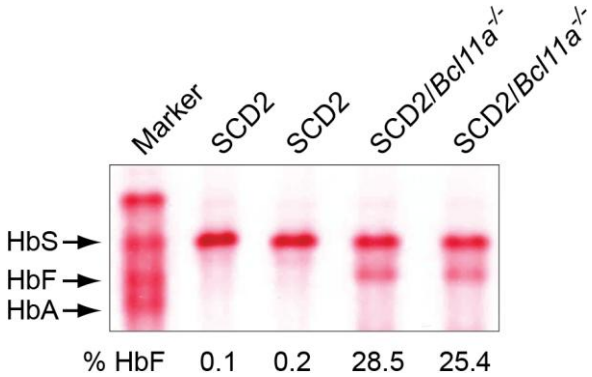


# Rescue of "Townes" SCD mice



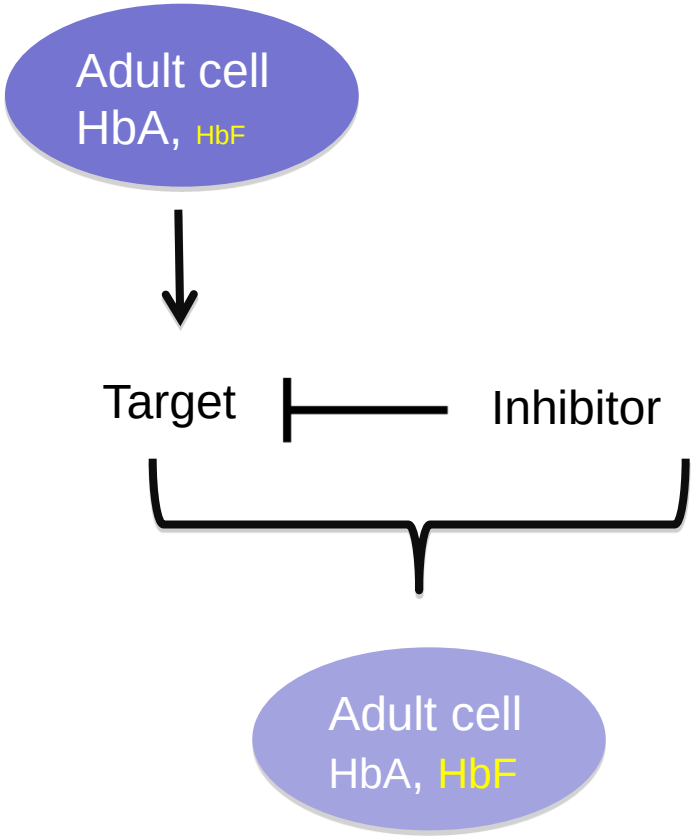




| Mice | RBC $x10^6/\mu l$ | Hb g/dl | Hct % | MCV fl | MCH pg | MCHC g/dl | Retic % | RDW % | WBC $x10^3/\mu l$ |
|---|---|---|---|---|---|---|---|---|---|
| Control | 9.9 ± 0.5 | 13.3 ± 0.5 | 46.8 ± 1.1 | 47.7 ± 2.9 | 13.6 ± 1.0 | 28.5 ± 0.7 | 3.2 ± 0.5 | 19.1 ± 1.5 | 11.2 ± 2.1 |
| SCD2 | 6.7 ± 0.6 | 8.4 ± 0.7 | 37.6 ± 1.9 | 51.5 ± 2.1 | 12.8 ± 0.8 | 24.9 ± 1.0 | 56.5 ± 2.4 | 30.0 ± 1.9 | 53.4 ± 16.8 |
| SCD2/*Bcl11a* $^{-/-}$ | 9.6 ± 0.8* | 12.9 ± 1.3** | 45.7 ± 4.2* | 47.5 ± 0.7 | 13.4 ± 0.4 | 28.0 ± 0.3* | 10.9 ± 4.2** | 24.0 ± 1.5* | 20.2 ± 6.0 |

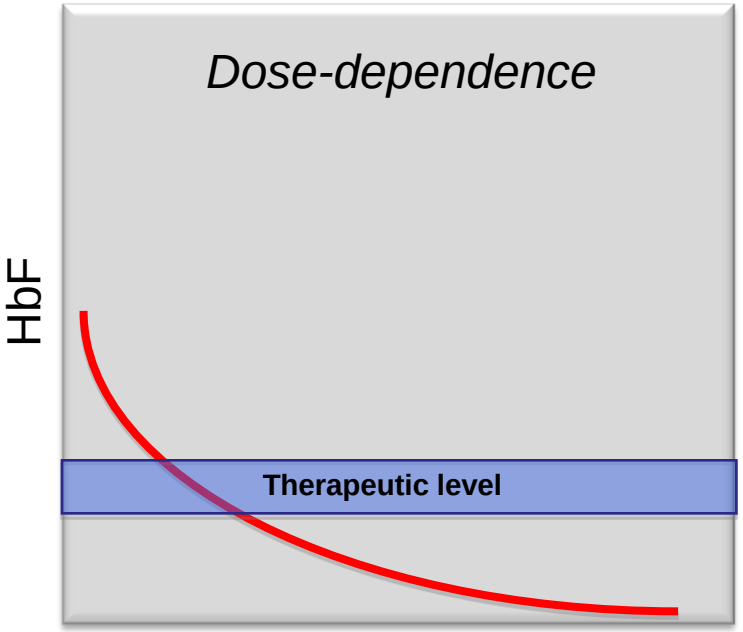
# Conclusions

1. Recent genetic approaches have transformed the current understanding of HbF regulation.
2. BCL11A is a central mediator of developmental globin switching and silencing.
3. BCL11A is a major brake on HbF expression, and elimination (or reduction) of BCL11A facilitates derepression by other agents.
4. Inactivation of BCL11A alone is *sufficient* to rescue phenotype of mouse model of sickle cell disease.
5. These studies are the first validation of the underlying premise that addressing a single target may achieve phenotypic correction of sickle cell disease (or β-thalassemias).

# Targeted therapy for Hb reactivation




No resistance




Functional
BCL11A conc

# “Drugging the undruggable”

Transcription factors: considered poor drug targets

1. RNA inhibition: shRNA by gene transfer; systemic modified RNAi
2. High-throughput screens for small molecules that bind BCL11A and disrupt function
3. High-throughput screens for small molecules that disrupt interaction BCL11A with associated proteins
4. Screens for additional pathways regulating BCL11A expression or function

# Some unknowns

1. What level of knockdown of BCL11A function/activity is needed to achieve adequate HbF reactivation in humans (as opposed to mice)? How do we assess? What is the quantitative relevance of data in CD34 derived cells or mice?

2. Are there other cell/organ systems that require BCL11A (beyond B-cells)? And how might this affect dosing/therapy?

3. Are there BCL11A-associated proteins that are more accessible targets (e.g. enzymes) and also contribute major portion of *in vivo* activity?

4. What are the prospects for identification of unknown silencing components through searches for rare variants (by DNA sequencing)?

# Future goals

1. Understand in detail molecular biology/dynamics of the globin switch and $\gamma$-globin silencing

2. Pursue BCL11A as therapeutic target--simultaneously explore genetic and chemical approaches

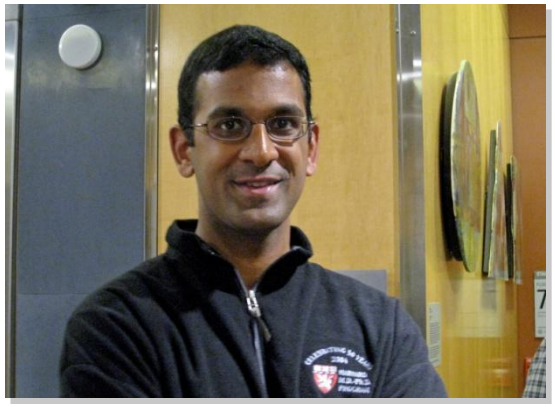
3. Intensify efforts to drug the undruggable (a new frontier ?)

4. Ultimately bring molecular biology/genetics to bear on the management of $\beta$-hemoglobin disorders

# Thanks to

Jian Xu

Cong Peng



Vijay Sankaran

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HARVARD MEDICAL SCHOOL

BROAD INSTITUTE

HHMI HOWARD HUGHES MEDICAL INSTITUTE

HSCI HARVARD STEM CELL INSTITUTE