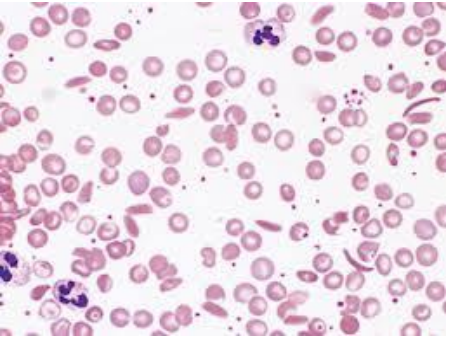
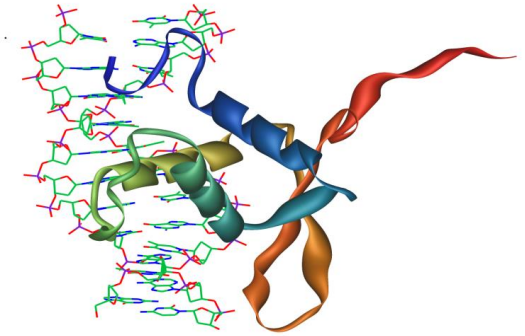




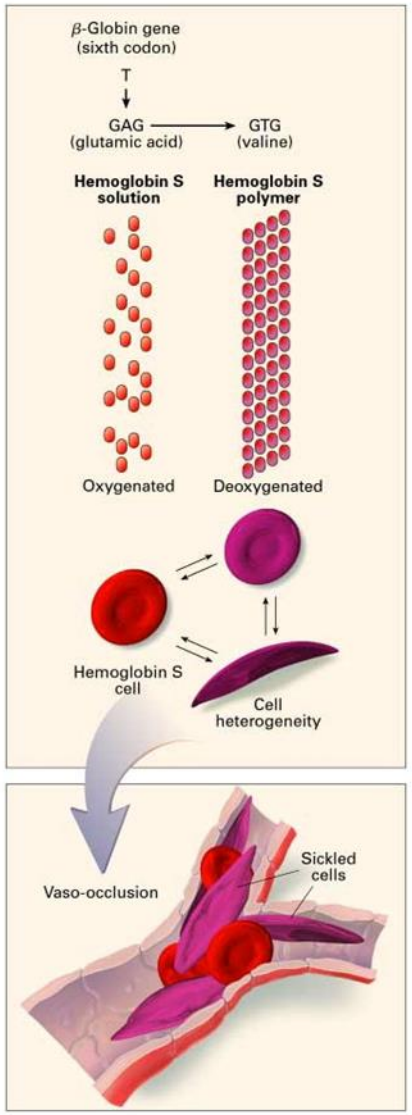
# FOXO3 Regulates Fetal Hemoglobin Levels in Sickle Cell Anemia

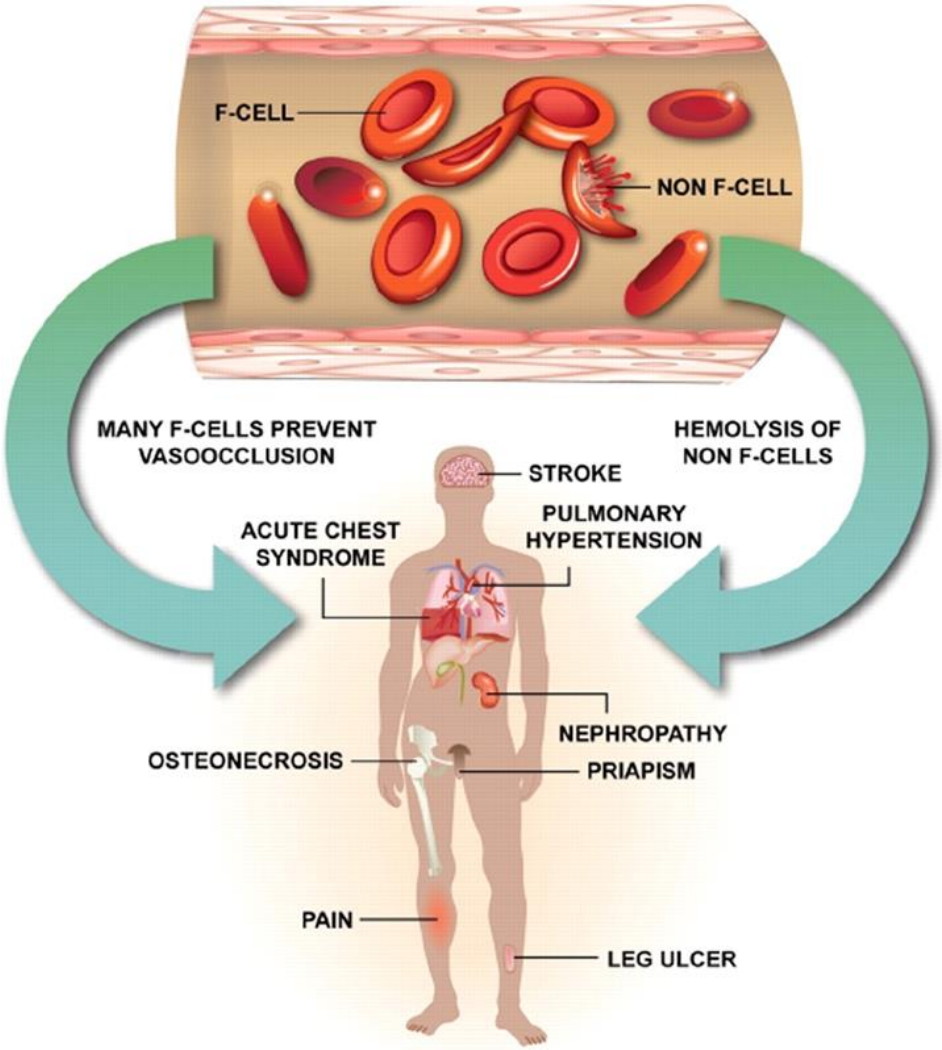
Yankai Zhang, Jacy R. Crosby,
Eric Boerwinkle, **Vivien A. Sheehan**

Texas Children's Hematology Center

Baylor College of Medicine

# Sickle Cell Anemia

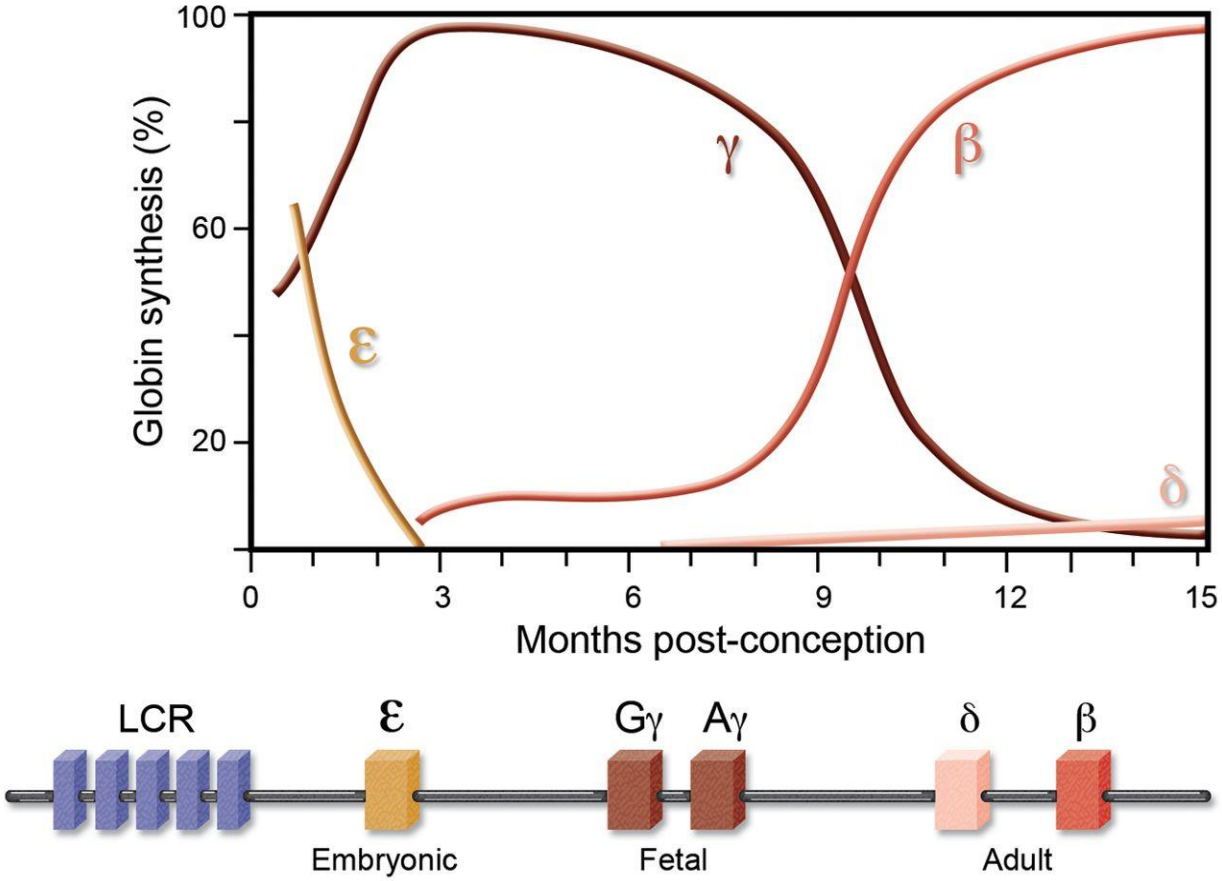


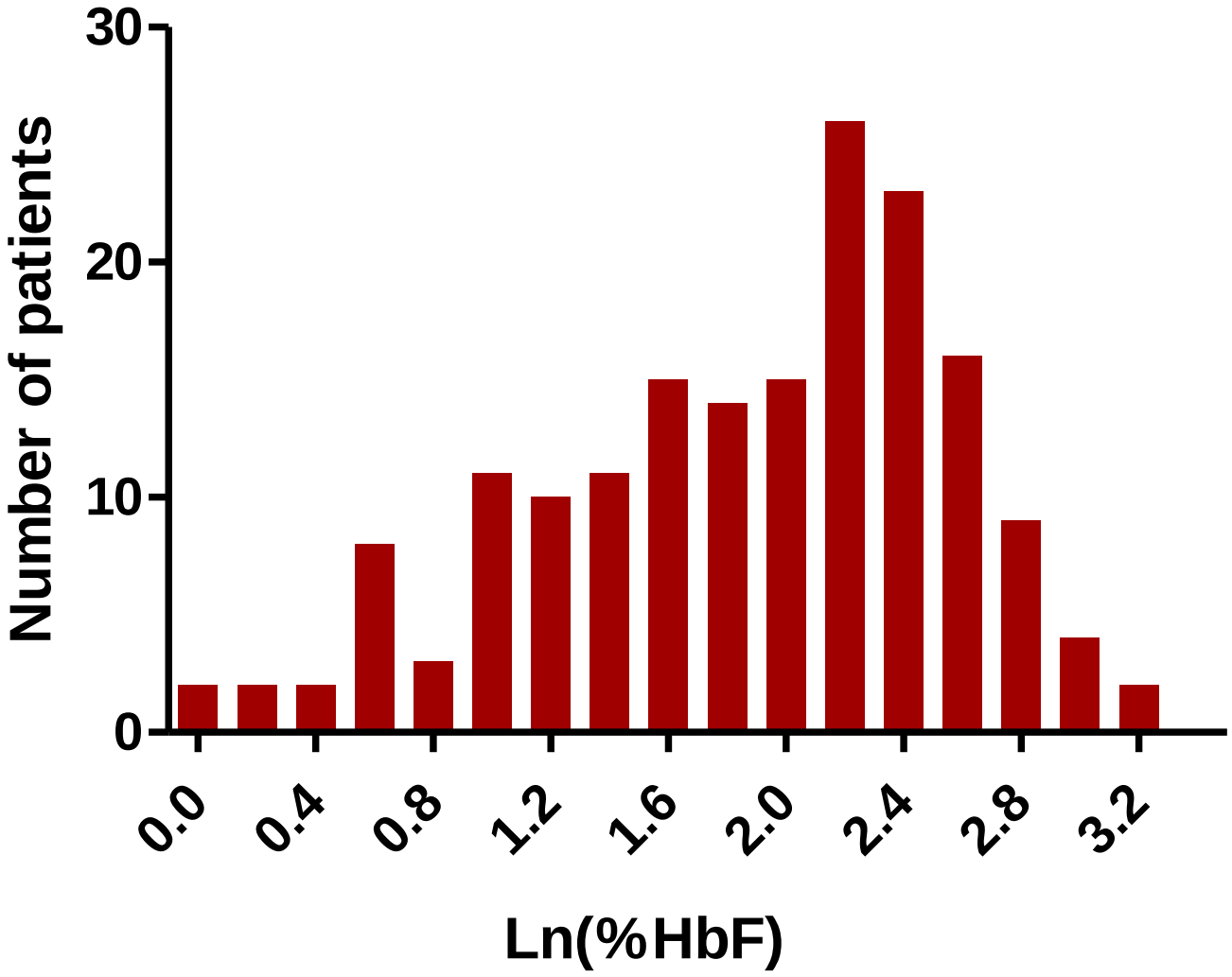

Steinberg MH. N Engl J Med 1999;340:1021-1030

Akinsheye I et al. Blood 2011;118:19-27

# Fetal Hemoglobin



# Variability of Endogenous HbF



# Next Generation Sequencing Methods

**Genome-wide association studies (GWAS)**

- Identified *BCL11A* as a regulator of endogenous HbF
- BCL11A is unlikely to be a good drug target
- *BCL11A* variants account for less than half of the observed variability of HbF

**Whole exome sequencing (WES)**

- Identifies all variants in protein coding regions
- Identifies rare variants with large effects
- Identifies causal variants
- Has not been applied to modifiers of endogenous HbF

# WES Study Population

171 pediatric sickle cell anemia patients

HbSS

Aged 3-18 years

**HUSTLE**

Hydroxyurea Study of Long-Term Effects

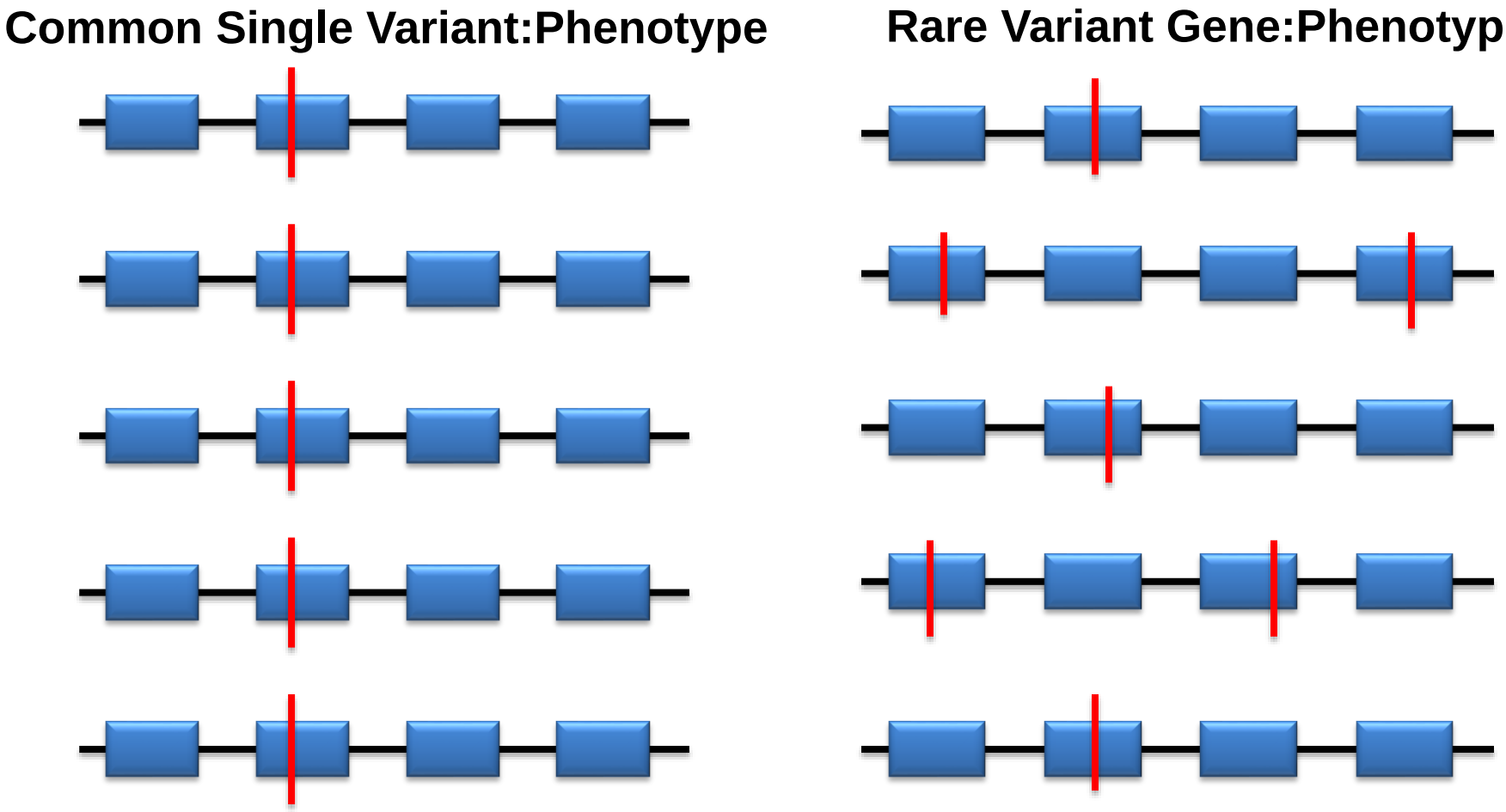
n=120

**SWiTCH**

Stroke with Transfusions Changing to Hydroxyurea

n=51

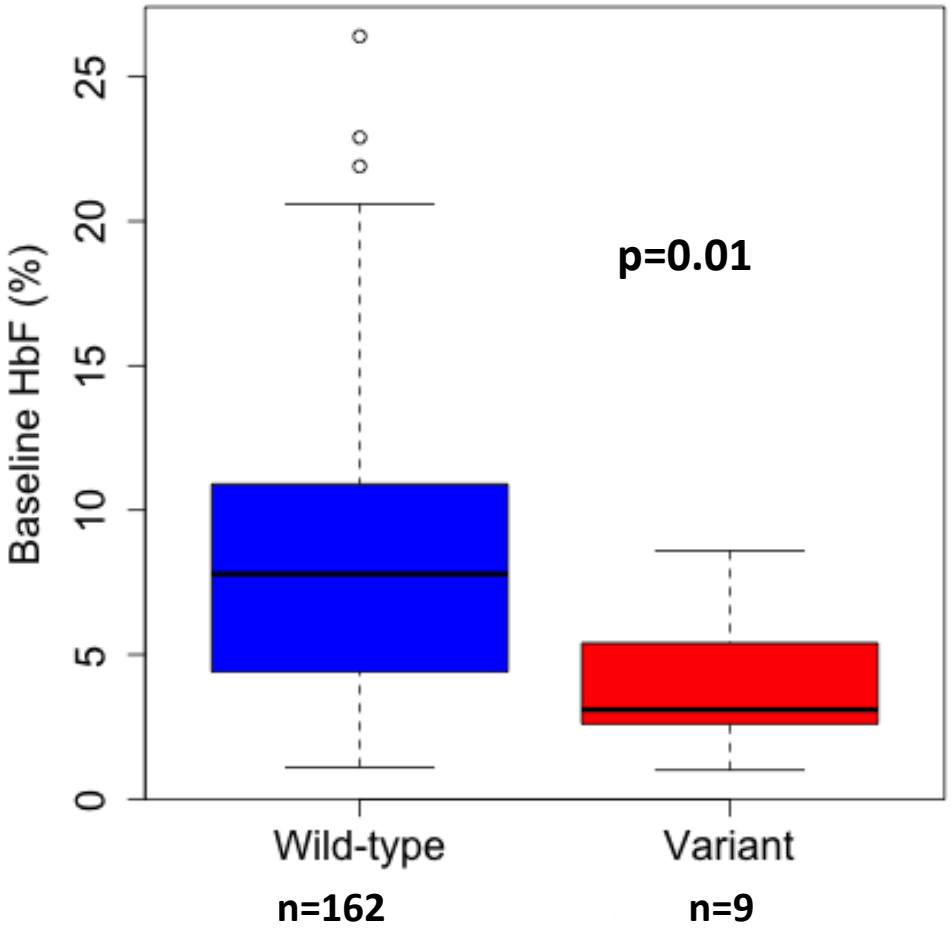
# Linear Regression vs Burden Analysis

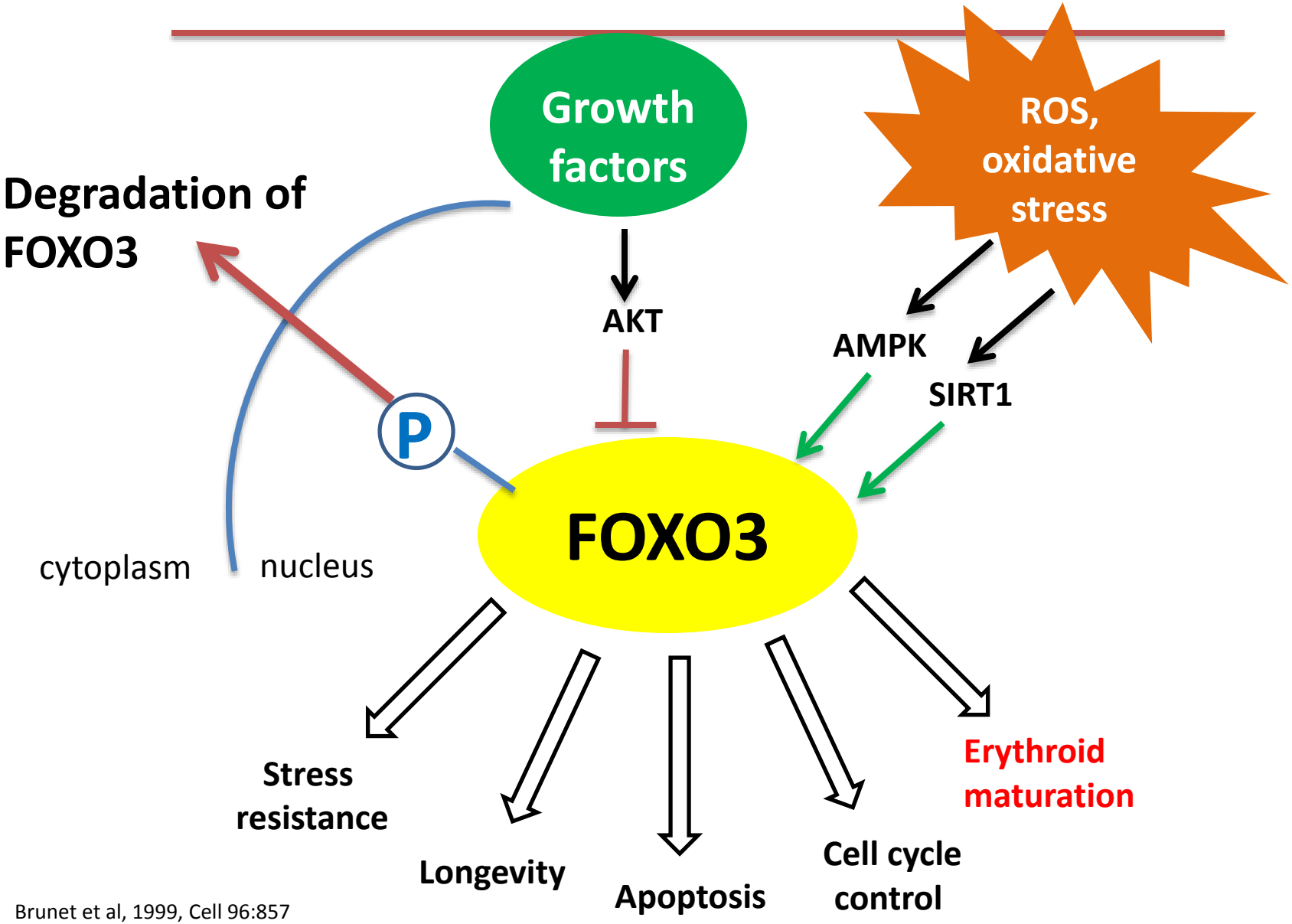
# T2 Burden Analysis Candidates

| Gene | Function | Number of nonsynonymous variants | Beta Value ln(%HbF) | P-value |
|---|---|---|---|---|
| *AMPK* | AMP-activated protein kinase | 2 | -1.5 | $1.5 \times 10^{-4}$ |
| *NKAIN3* | Na/K transport | 5 | -0.6 | $2.7 \times 10^{-4}$ |
| *TNFRSF9* | Tumor necrosis factor | 5 | 0.5 | $3.9 \times 10^{-4}$ |
| *FOXO3* | Transcriptional activator | 7 | -0.7 | $5.6 \times 10^{-4}$ |
| *EIF2AK1* | Heme-regulated inhibitor kinase | 7 | -0.3 | $6.9 \times 10^{-4}$ |

# Effect of FOXO3 Variants on %HbF

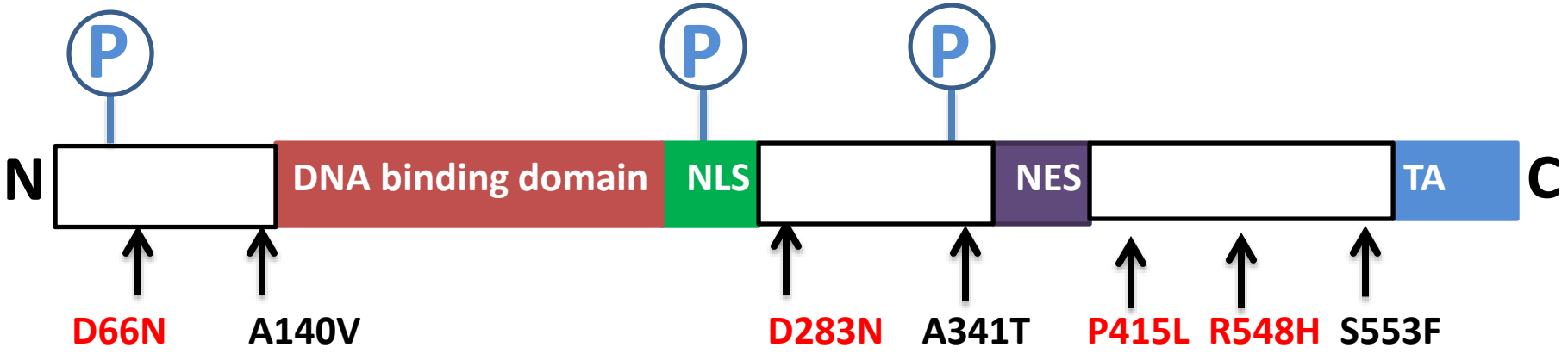


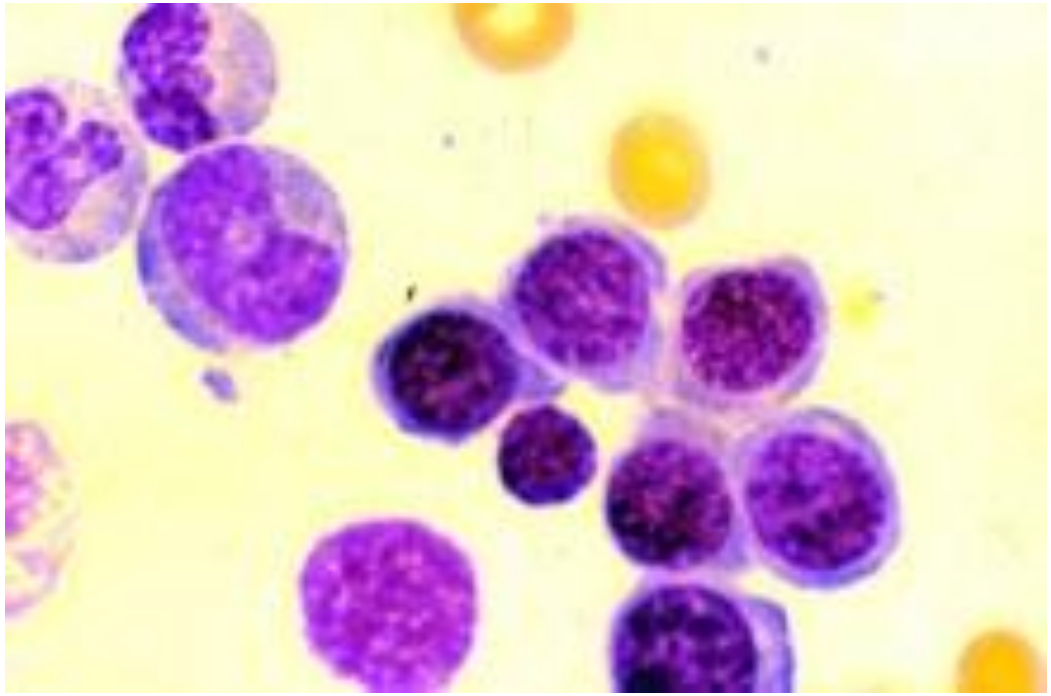
# Forkhead box O3




Brunet et al, 1999, Cell 96:857

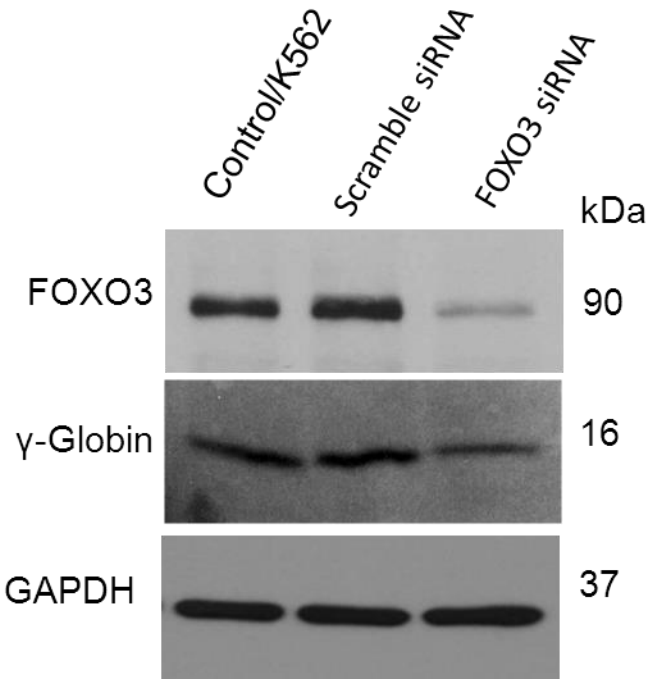
# Location of FOXO3 Variants

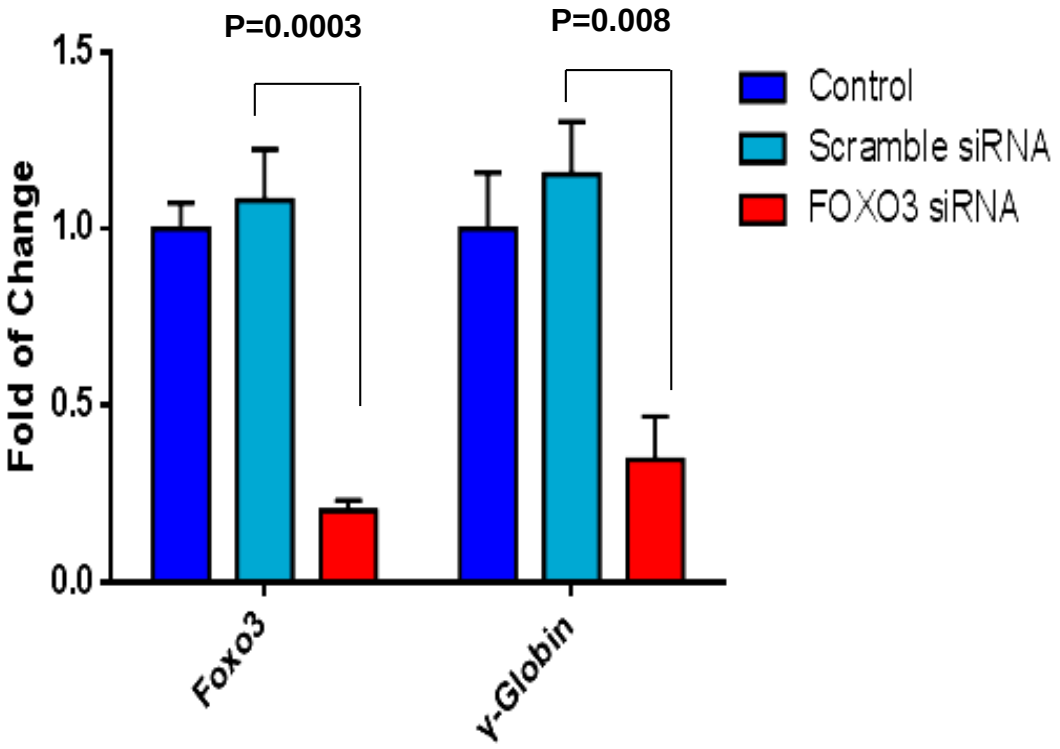




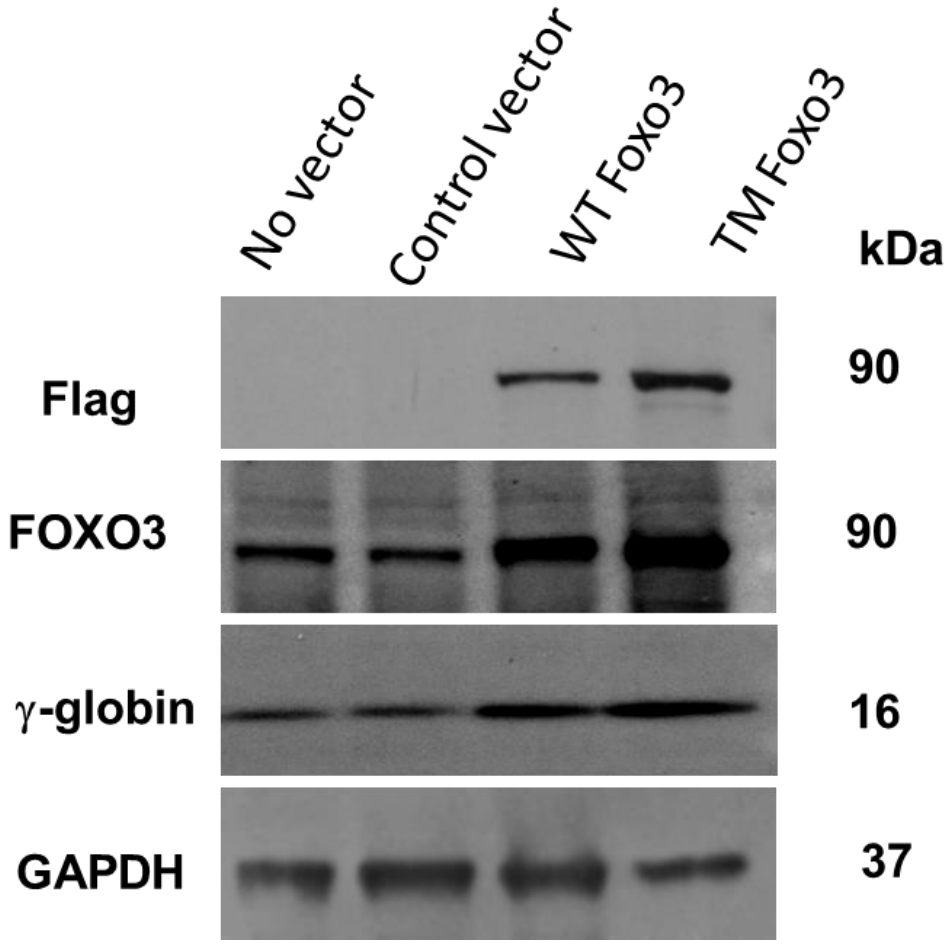
# FUNCTIONAL STUDIES

# *FOXO3* siRNA knockdown reduces HbF levels in K562 cells

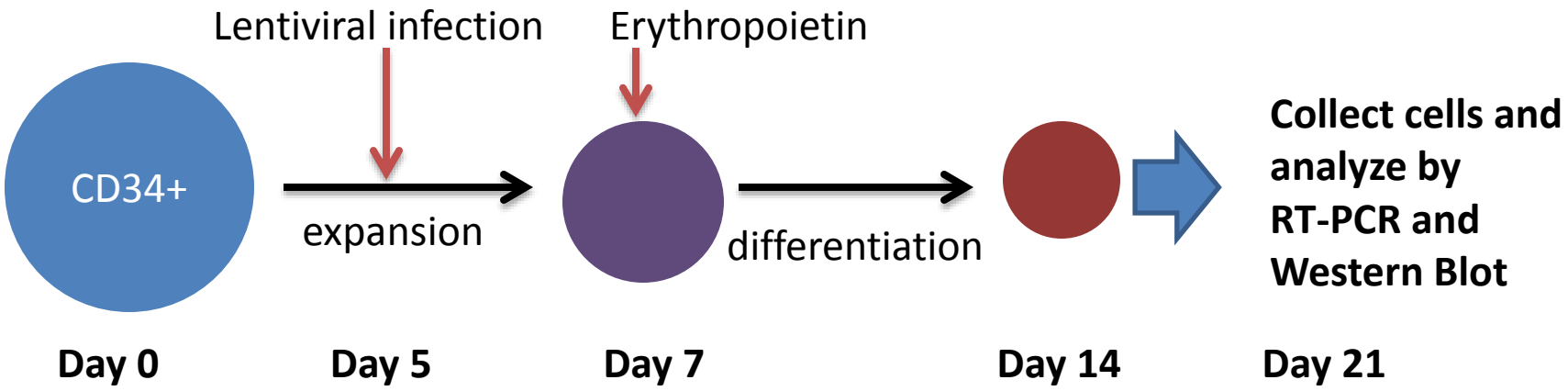


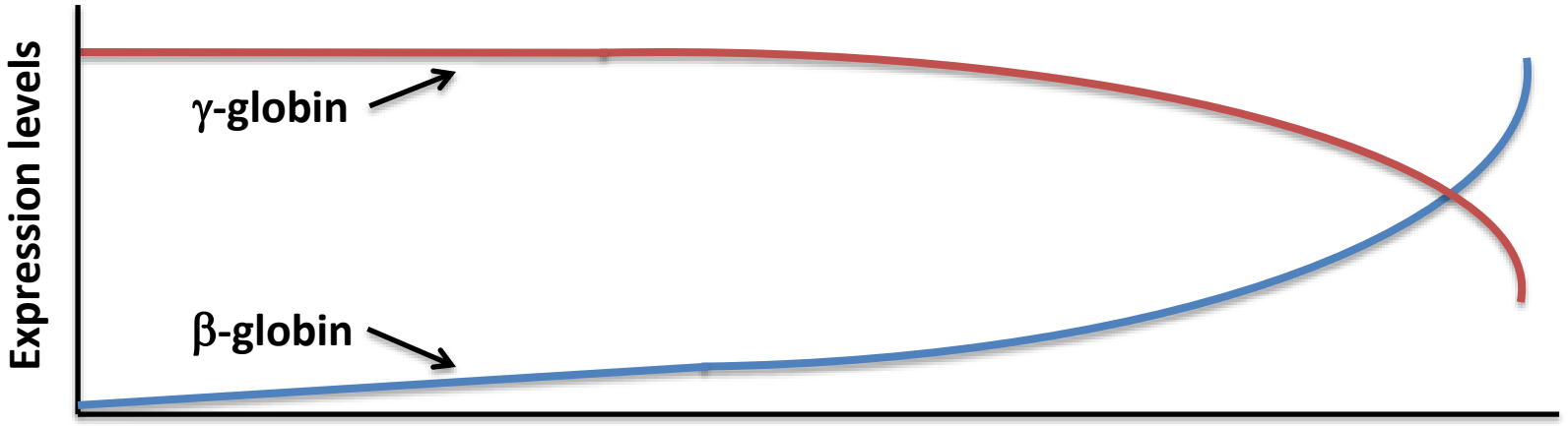



# FOXO3 overexpression increases HbF in K562 cells

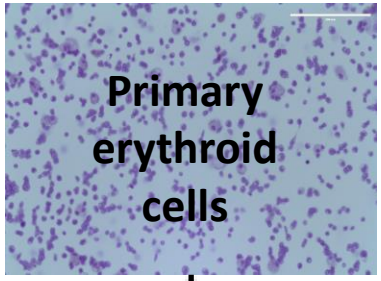


# Primary Erythroid Culture

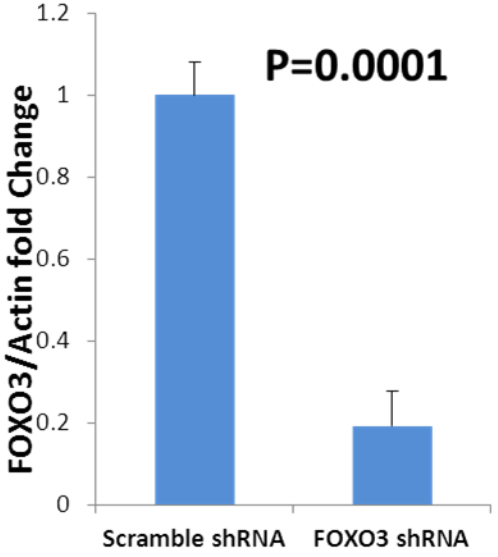


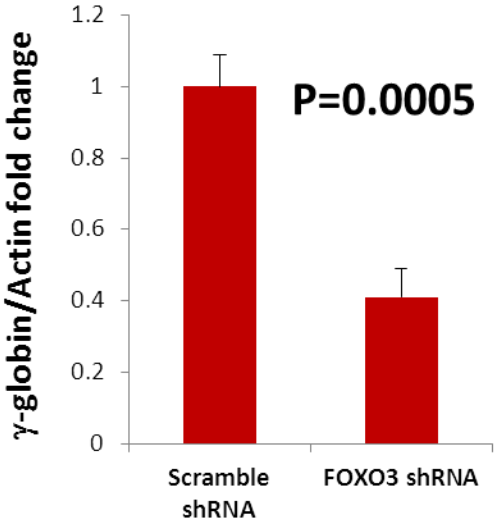



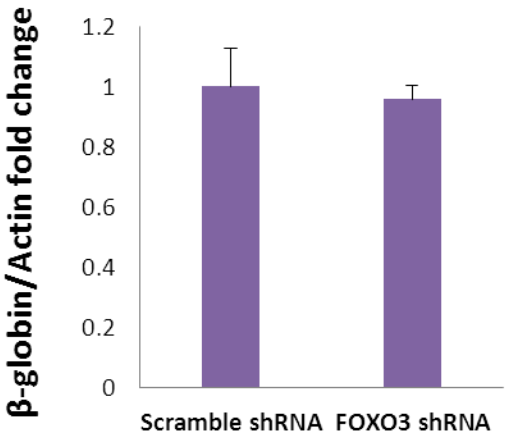
# shRNA knockdown of *FOXO3* reduces HbF in primary erythroid cells

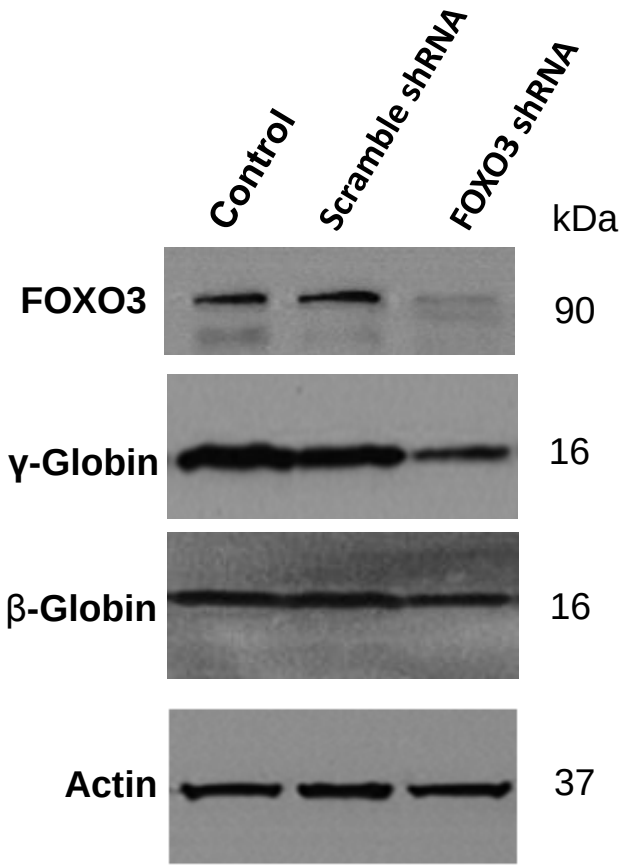



shRNA



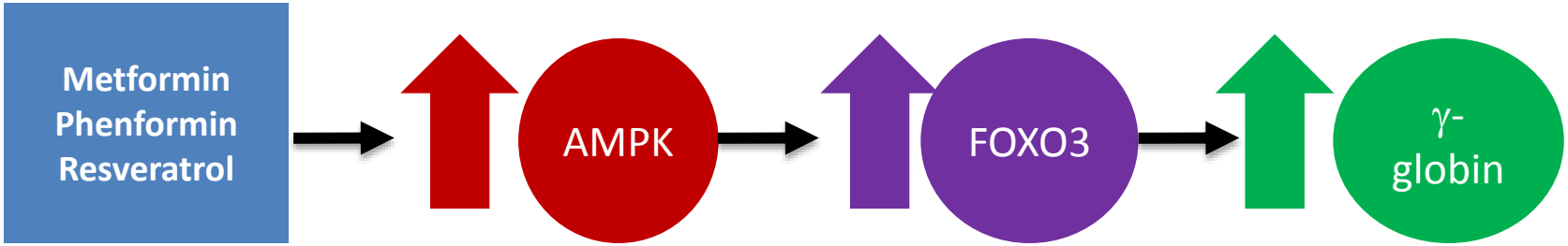



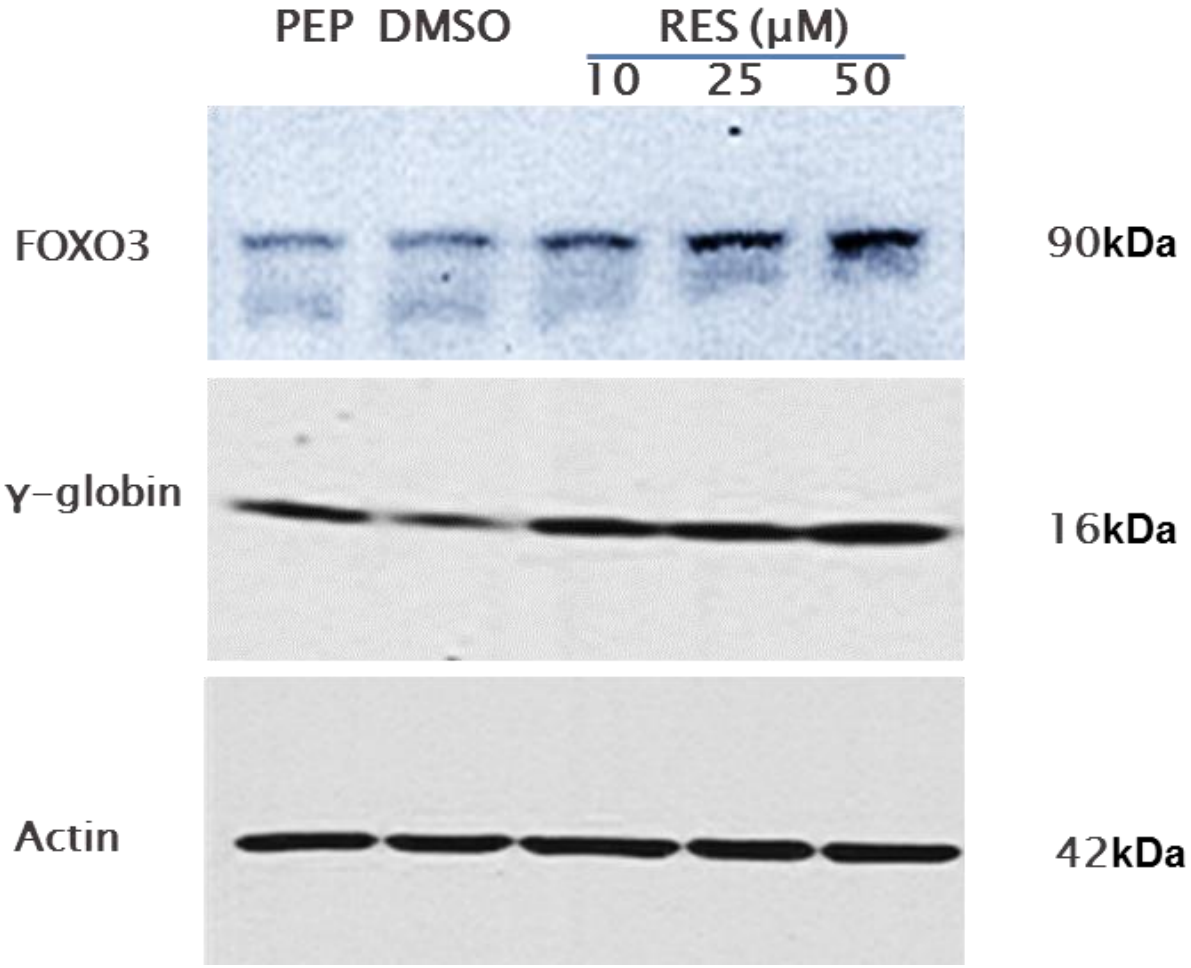



# FOXO3 Inducing Agents May Increase HbF Levels

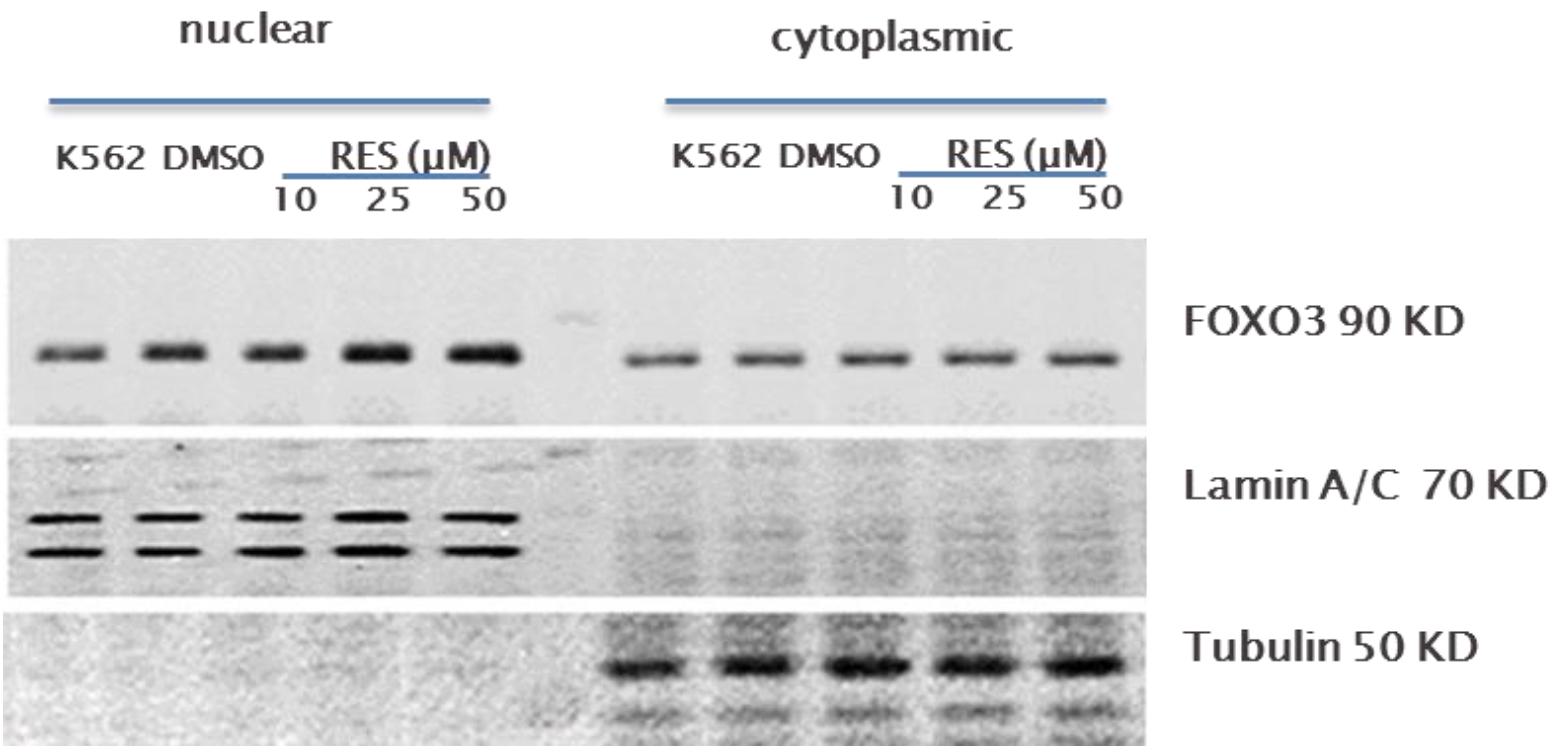
- AMPK activates FOXO3 through phosphorylation
- Variants in AMPK were also associated with lower HbF levels in our WES study
- Metformin, phenformin, and resveratrol increase AMPK expression levels, and may increase γ-globin through FOXO3

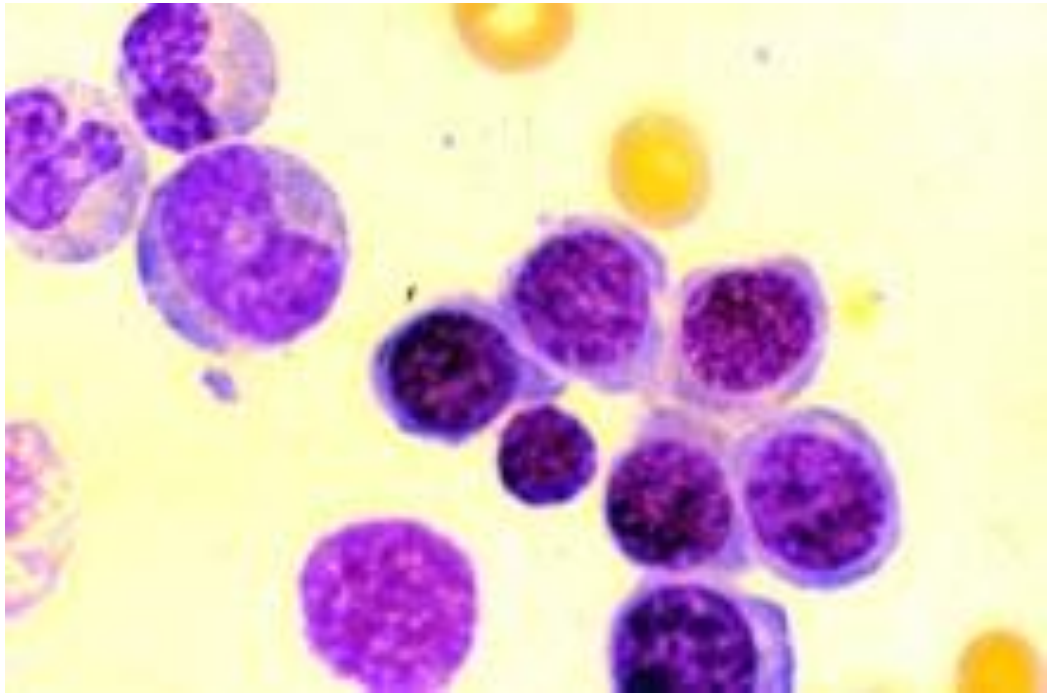


# Resveratrol Induces γ-Globin in PEP

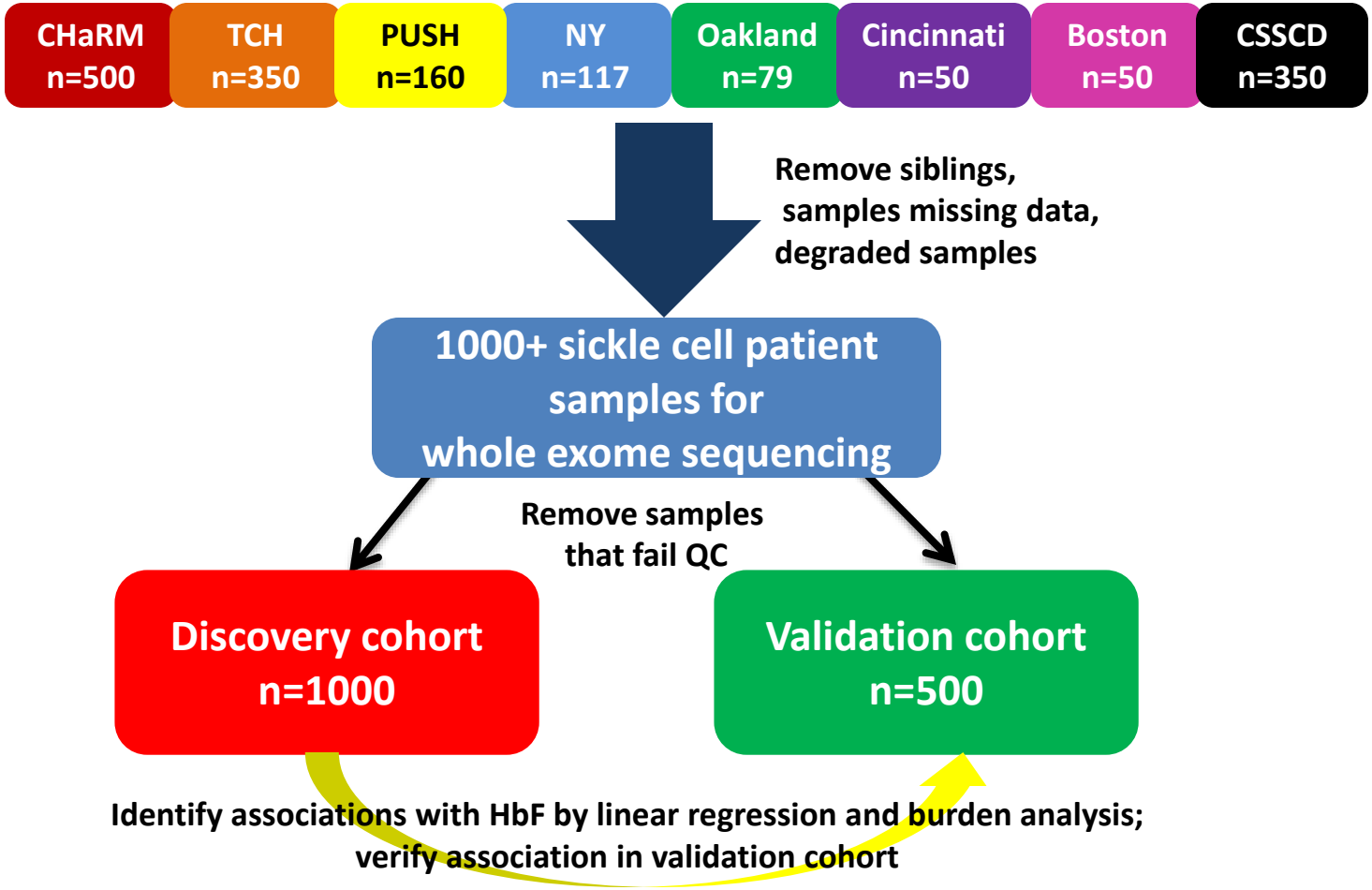


# FOXO3 Accumulates in Nucleus with Resveratrol Treatment

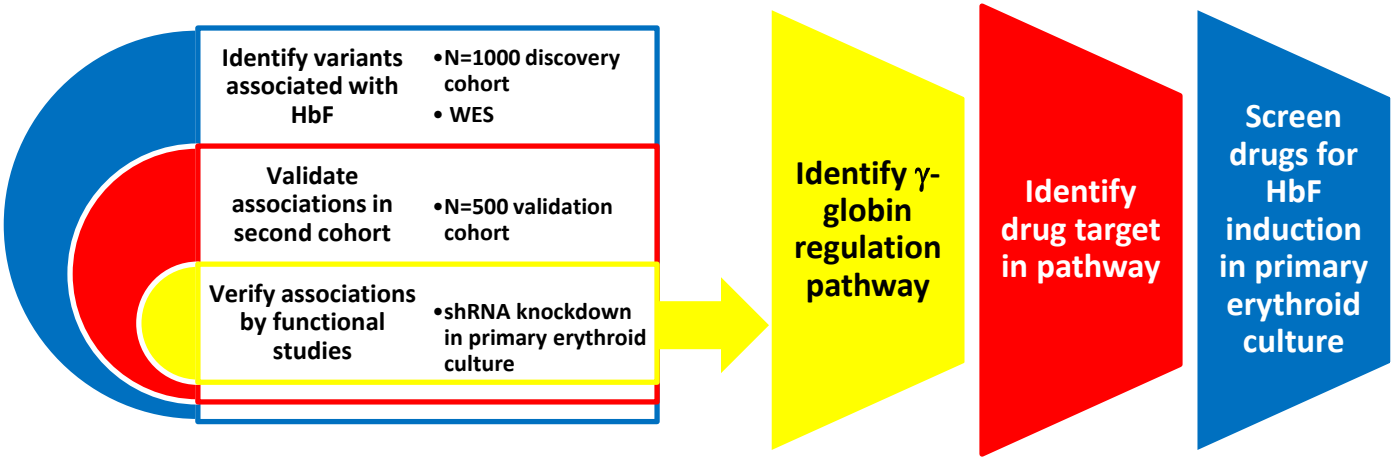




# FUTURE DIRECTIONS


CHaRM n=500
TCH n=350
PUSH n=160
NY n=117
Oakland n=79
Cincinnati n=50
Boston n=50
CSSCD n=350
Remove siblings, samples missing data, degraded samples
1000+ sickle cell patient samples for whole exome sequencing
Remove samples that fail QC
Discovery cohort n=1000
Validation cohort n=500
Identify associations with HbF by linear regression and burden analysis; verify association in validation cohort

# Future Plans



# Future Analyses

**Analyze WES data on a new cohort of 1000 SCD patients to investigate further relationships between *FOXO3* and γ-globin expression.**

**a.** Identify all non-synonymous *FOXO3* gene variants that are associated with reduced HbF levels.

**b.** Use gene based testing and pathway analysis to determine whether variants in FOXO3 regulatory genes (*AMPK, SIRT1*) are associated with HbF levels.

**c.** Use nonbiased SNP and gene based testing to identify all variants that segregate with HbF level in the WES cohort.

# Future Analyses

**Determine the mechanisms by which *FOXO3* regulates γ-globin expression.**

**a.** Analyze primary human erythroid cells by chromatin immunoprecipitation-sequencing (ChIP-seq) to determine whether FOXO3 binds the γ-globin locus or other loci that regulate HbF (*BCL11A*, *MYB, KLF1*).

**b.** Analyze primary human erythroid cells with and without FOXO3 knockdown by RNASeq to identify genes altered by FOXO3 knockdown.

**c.** Use Gene Set Enrichment Analysis (GSEA) to combine WES, ChIP-Seq and RNASeq analyses.

# Conclusions

- Burden analysis of WES data identified seven *FOXO3* variants associated with lower endogenous HbF in pediatric sickle cell patients.
- In K562 cells and primary erythroid cells, knockdown of *FOXO3* reduced γ-globin levels.
- Overexpression of FOXO3 increased γ-globin levels.
- FOXO3 may be a viable drug target.
- Further work is needed to elucidate the role of *FOXO3* in γ-globin regulation

# Acknowledgments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