Genetic Drivers of Hematologic Malignancies

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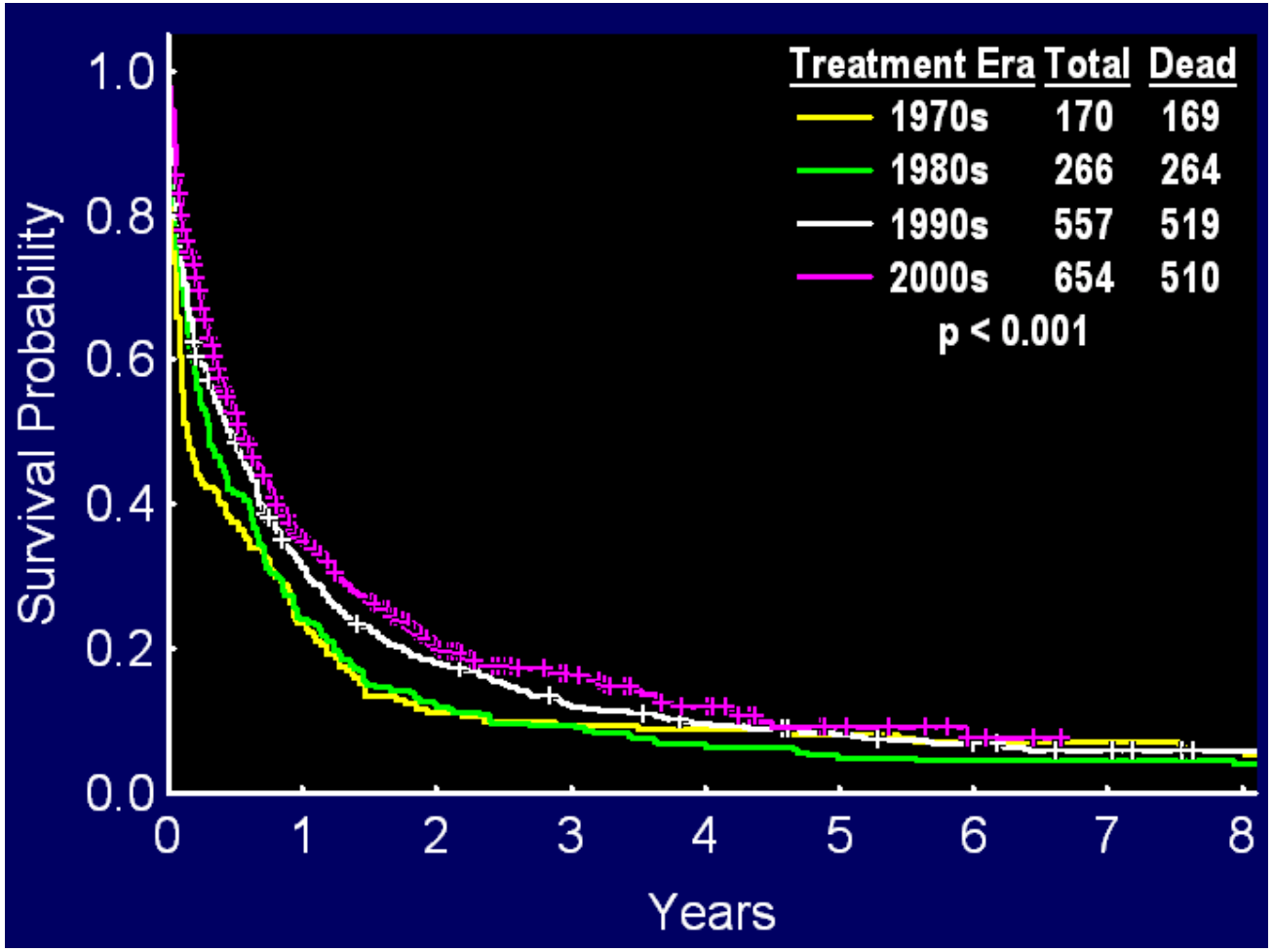
Hematologic Malignancies

- Hematologic malignancies are classified based on the lineage involved, bone marrow vs. lymph node involvement (leukemia versus lymphoma) and on the level of “acuity” (acute versus chronic leukemias)
  - Myeloid malignancies (Acute Myeloid Leukemia, Myeloproliferative Disorders, Myelodysplastic Syndromes)
  - Lymphoid Malignancies (Acute Lymphoblastic Leukemia, Chronic Lymphocytic Leukemias, Lymphomas)
  - Plasma cell malignancies (myeloma, plasma cell leukemia)

- In general, hematologic malignancies are systemic diseases, such that localized therapy is not a major component of therapy (except localized lymphoma)

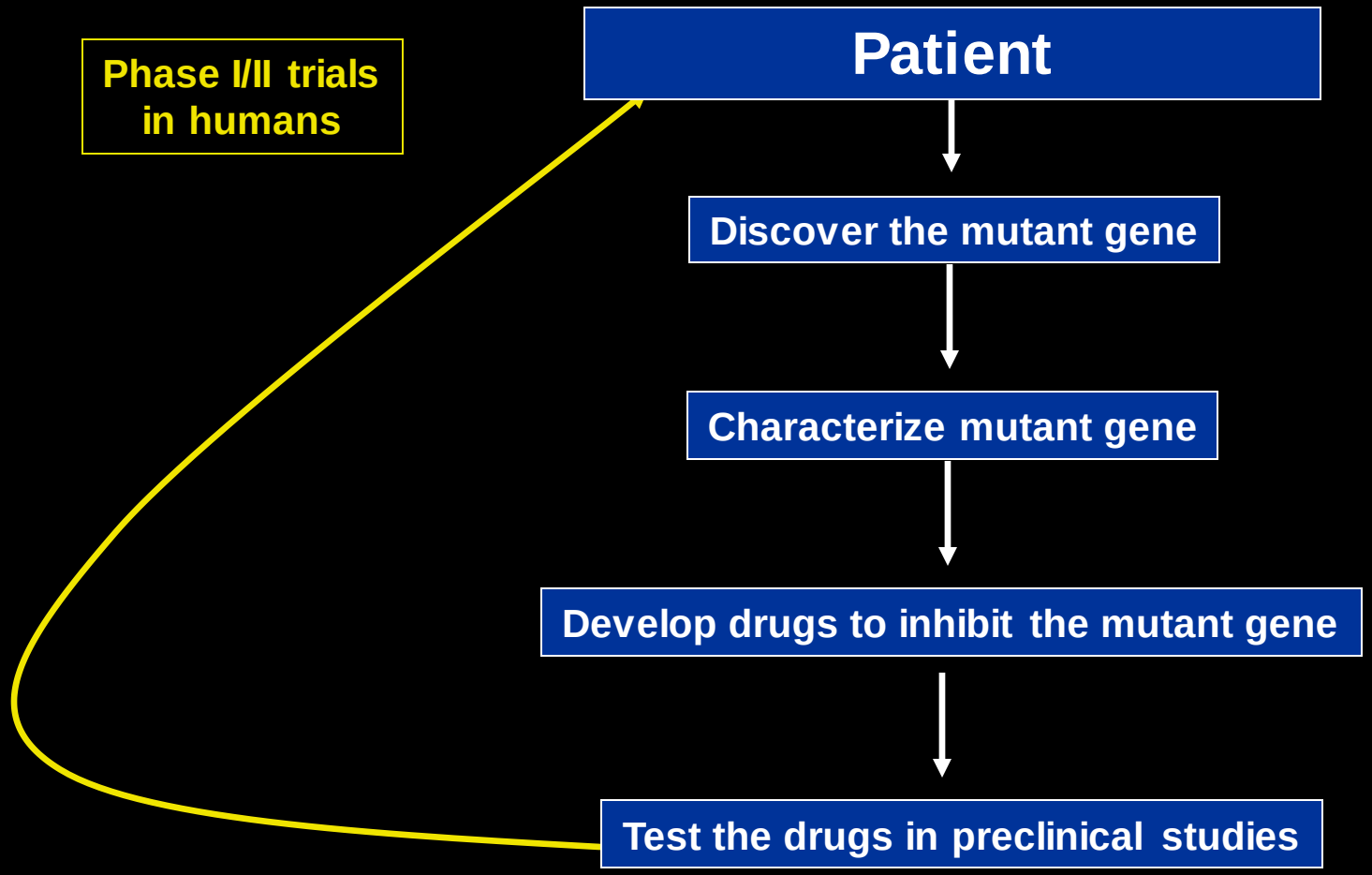
- We have learned a lot about cancer biology and therapy by investigating the mechanisms which drive hematopoietic transformation

Lack of Progress in AML Outcomes



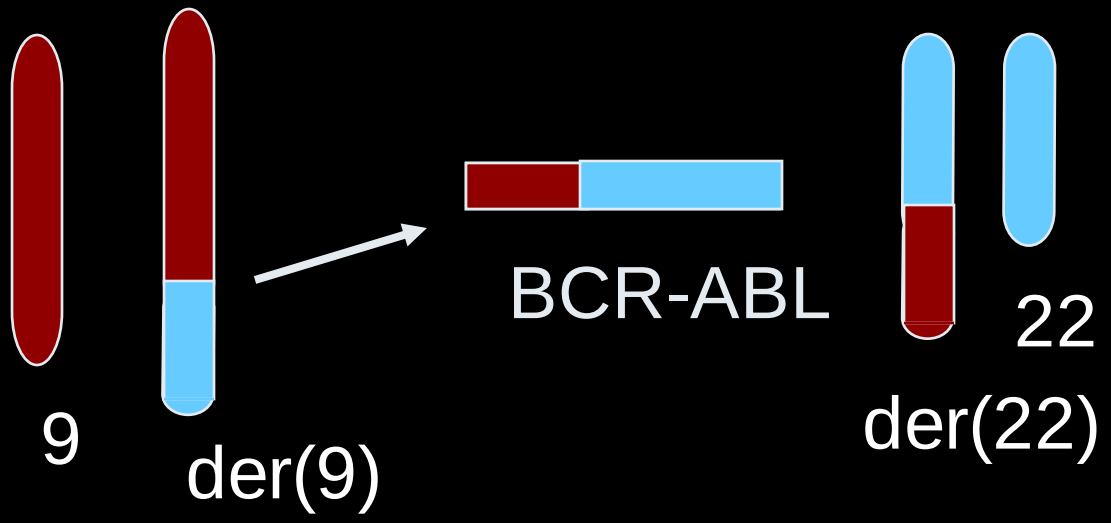
Issa, Kantarjian et al. Cancer 2008

Molecular Target Discovery

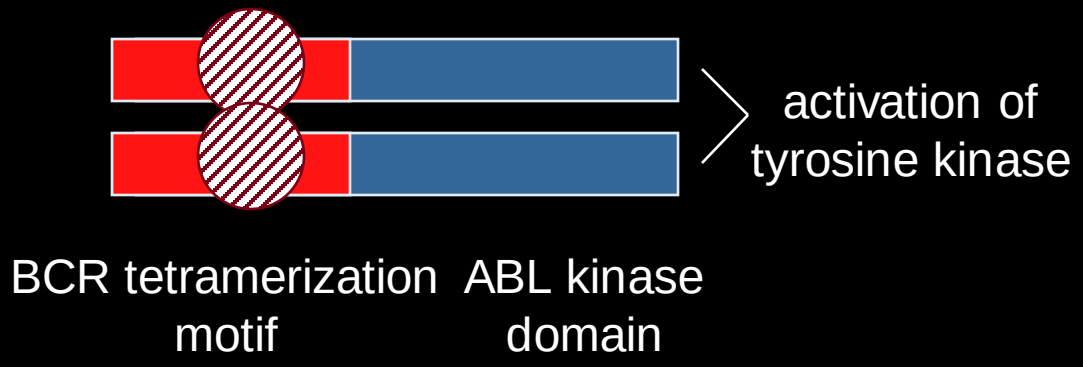


Entered clinical training as this paradigm began to be established

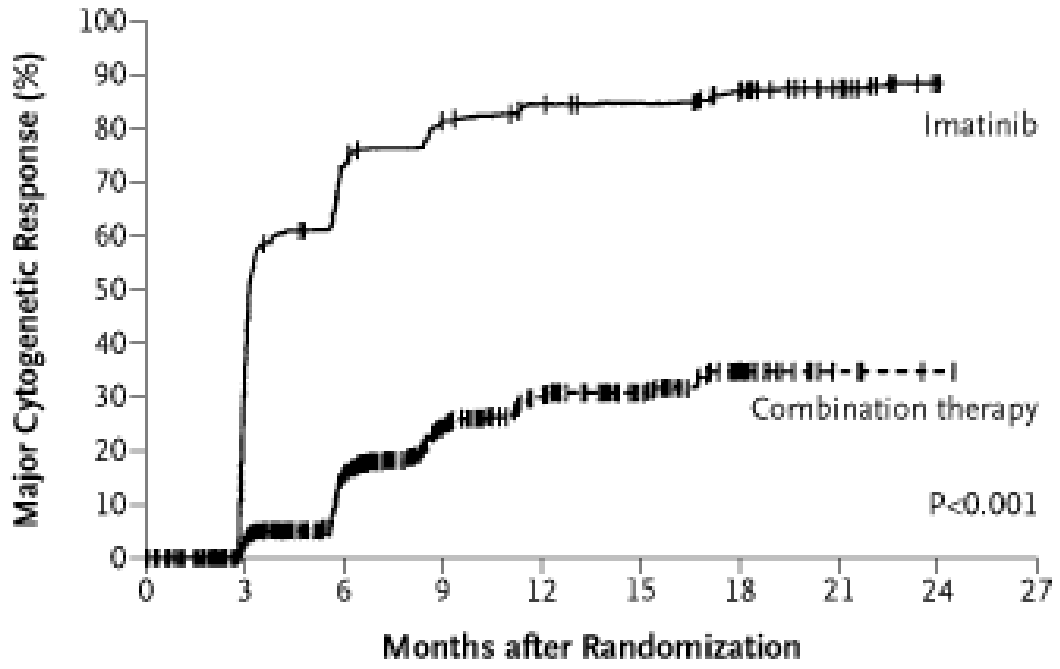
BCR-ABL gene rearrangement in chronic myeloid leukemia



- First genetic event identified in any human cancer (looking at chromosomes)
- Translocation/fusion of chromosomes 9 and 22
- Results in expression of a BCR-ABL fusion oncoprotein



Imatinib 400 mg po qd vs. Inteferon/Cytarabine

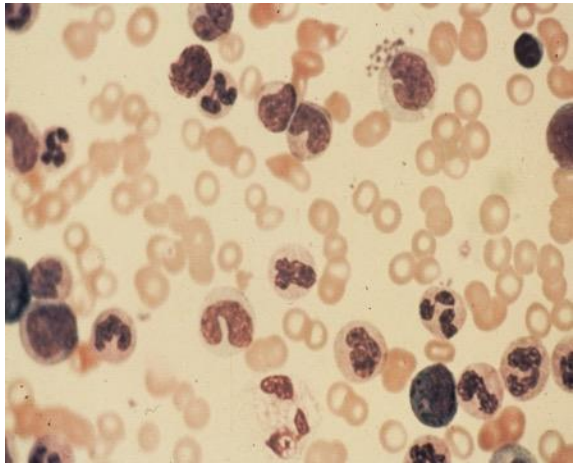


Druker et al. NEJM 2006

Myeloid Malignancies

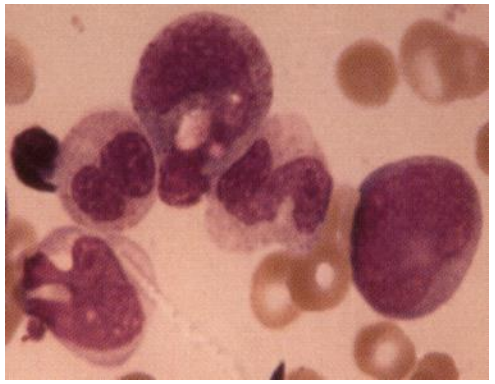
Myeloproliferative neoplasms

- enhanced proliferation/survival
- normal differentiation
- leukocytosis
- may progress to AML



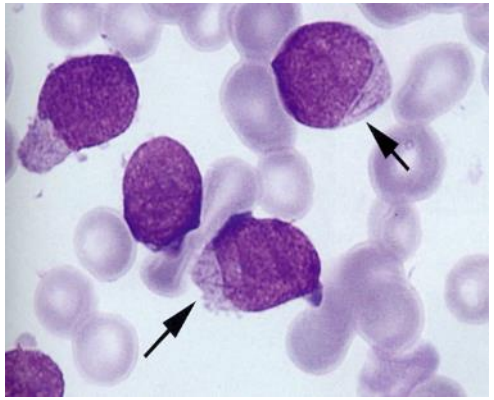
Myelodysplastic syndrome

- impaired differentiation
- cytopenia
- may progress to AML

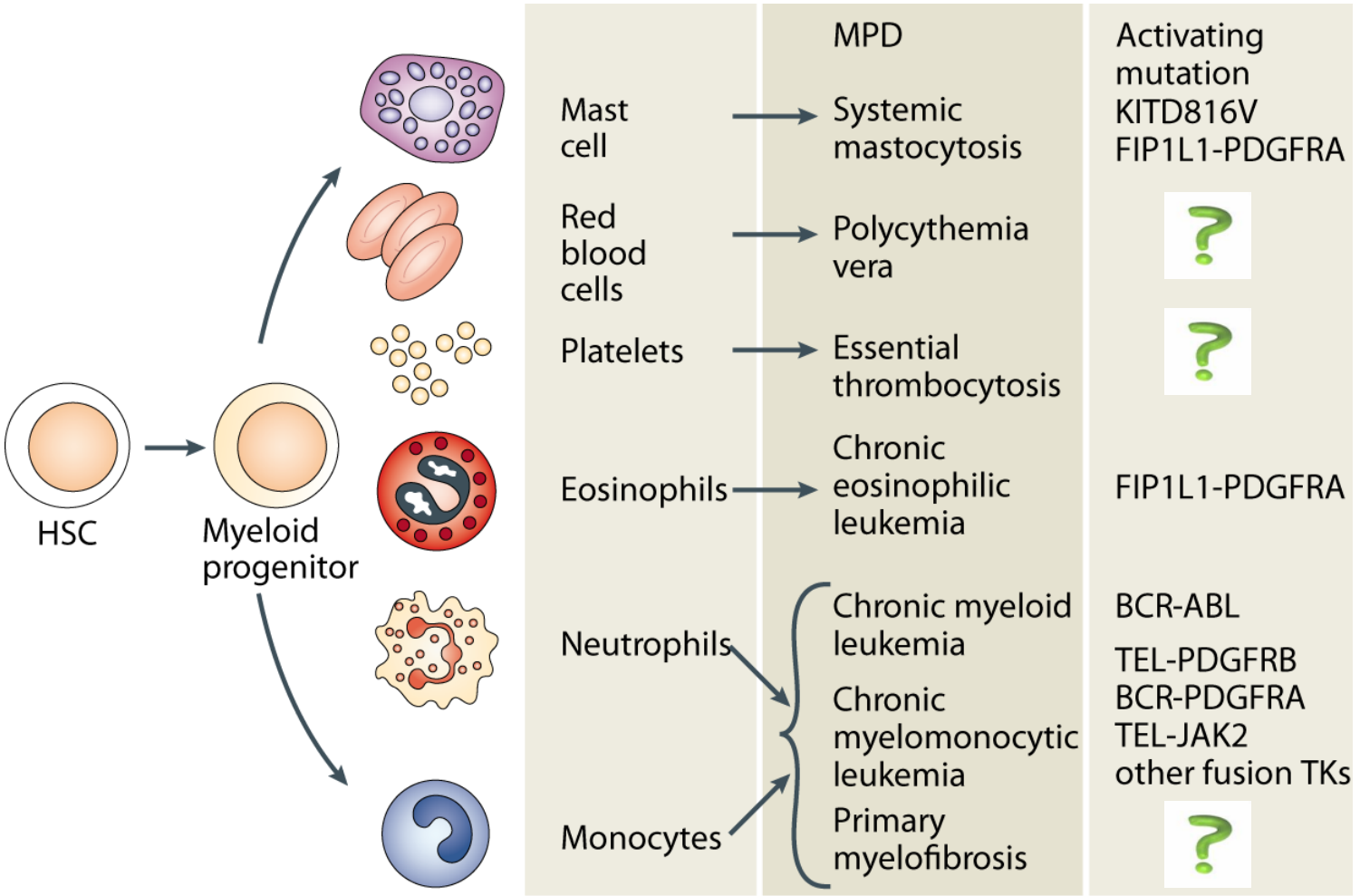


Acute myeloid leukemia

- enhanced proliferation and survival
- impaired differentiation
- limitless self-renewal



Myeloproliferative Disorders: 2004
Goal: Find the Mutant Gene…



Nature Reviews | Cancer

Investigation of the genetic basis of Myeloproliferative Neoplasms

- Mutations involving kinases are common in different myeloid leukemias (CML, others)

- Hypothesis: PV, ET, and PMF are caused by acquired somatic mutations that activate tyrosine kinases

- Sample collection: IRB approved protocol for collection of samples using the Internet

- High throughput resequencing of the tyrosine kinome in 325 MPN patients

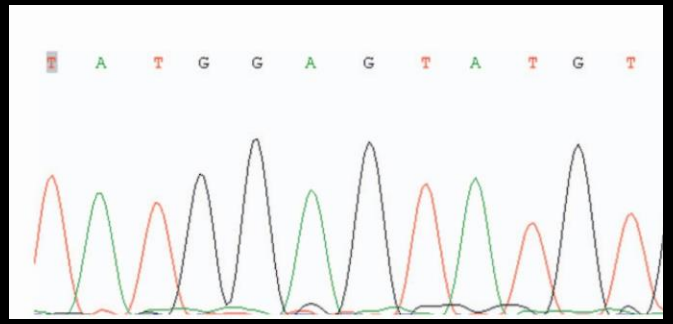
***Martha Wadleigh, Stephanie Lee, Gary Gilliland**

Tyrosine Kinase Sequencing in MPNs

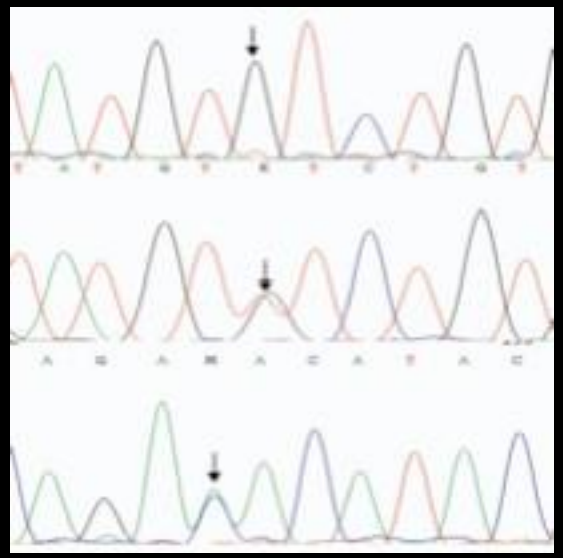
•Goal: analyze all tyrosine kinases for activating mutations

•Analysis of 325 patients
- All 90 tyrosine kinases
- 650 sequencing runs per patient
- 211,250 sequencing runs

•Analysis of samples using high throughput sequencing platform
  - •Robotic DNA amplification
  - •Automated DNA sequencing
  - •Computer-based high throughput sequence analysis



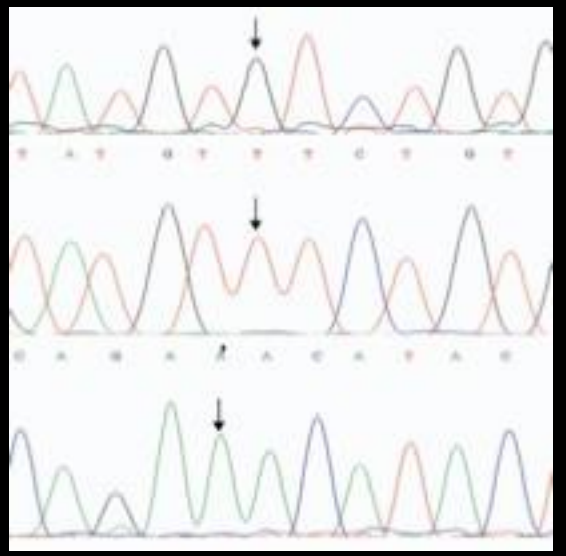
Homozygous *JAK2V617F* mutations in MPN



Matched Normal

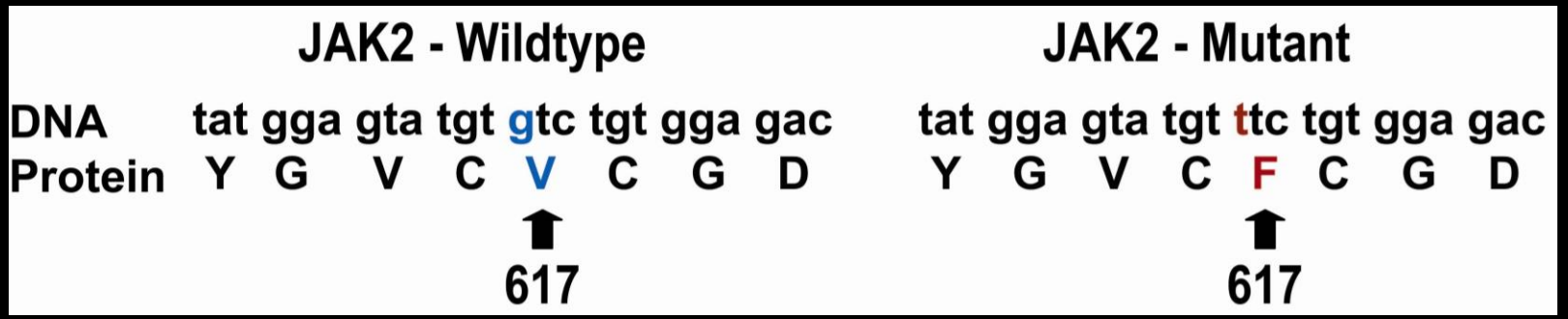
Forward

Reverse



Heterozygous

Homozygous



*Levine *et al.* Cancer Cell 2005
James *et al.* Nature 2005
Baxter *et al.* Lancet 2005
Kralovics *et al.* NEJM 2005

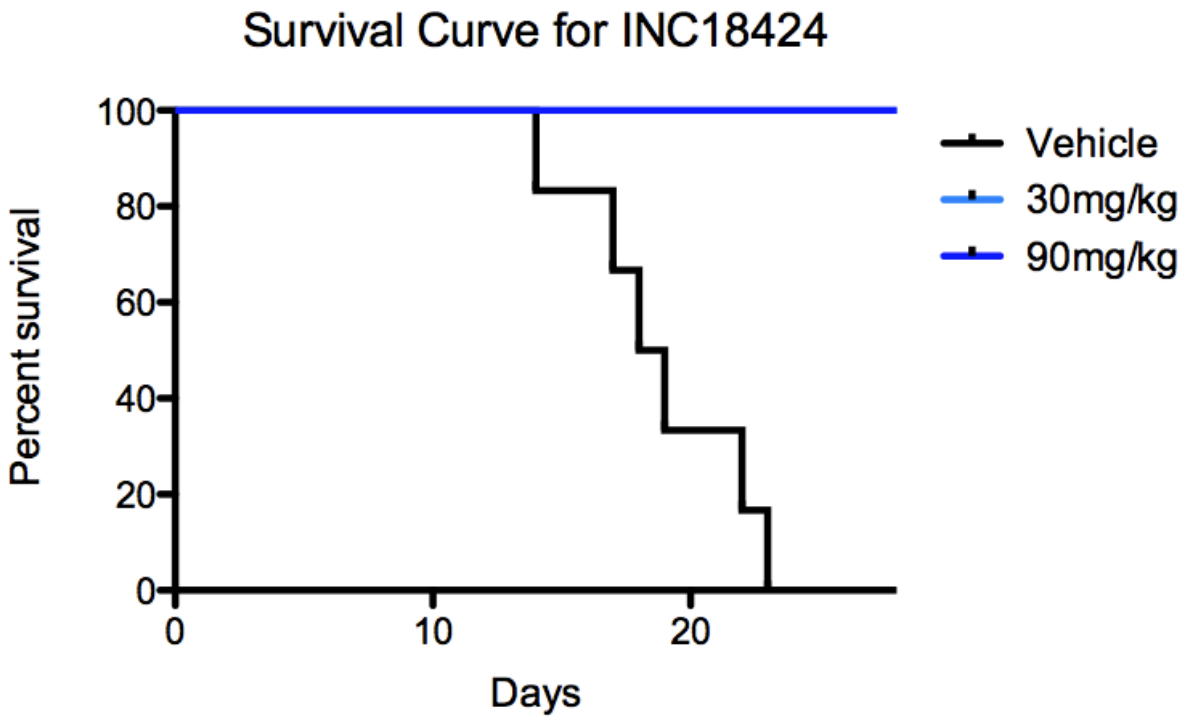
Frequency of JAK2V617F Mutation

PV	95%
ET	60%
PMF	50%
CML	0%
Normals	0%

Laboratory studies have shown that JAK2V617F, like BCR-ABL, is a mutant tyrosine kinase->tells cells to keep growing

*James *et al.* Nature 2005
Levine *et al.* Cancer Cell 2005
Baxter *et al.* Lancet 2005
Kralovics *et al.* NEJM 2005

Discovery of JAK2 mutations led to development of JAK inhibitors for MPN patients*

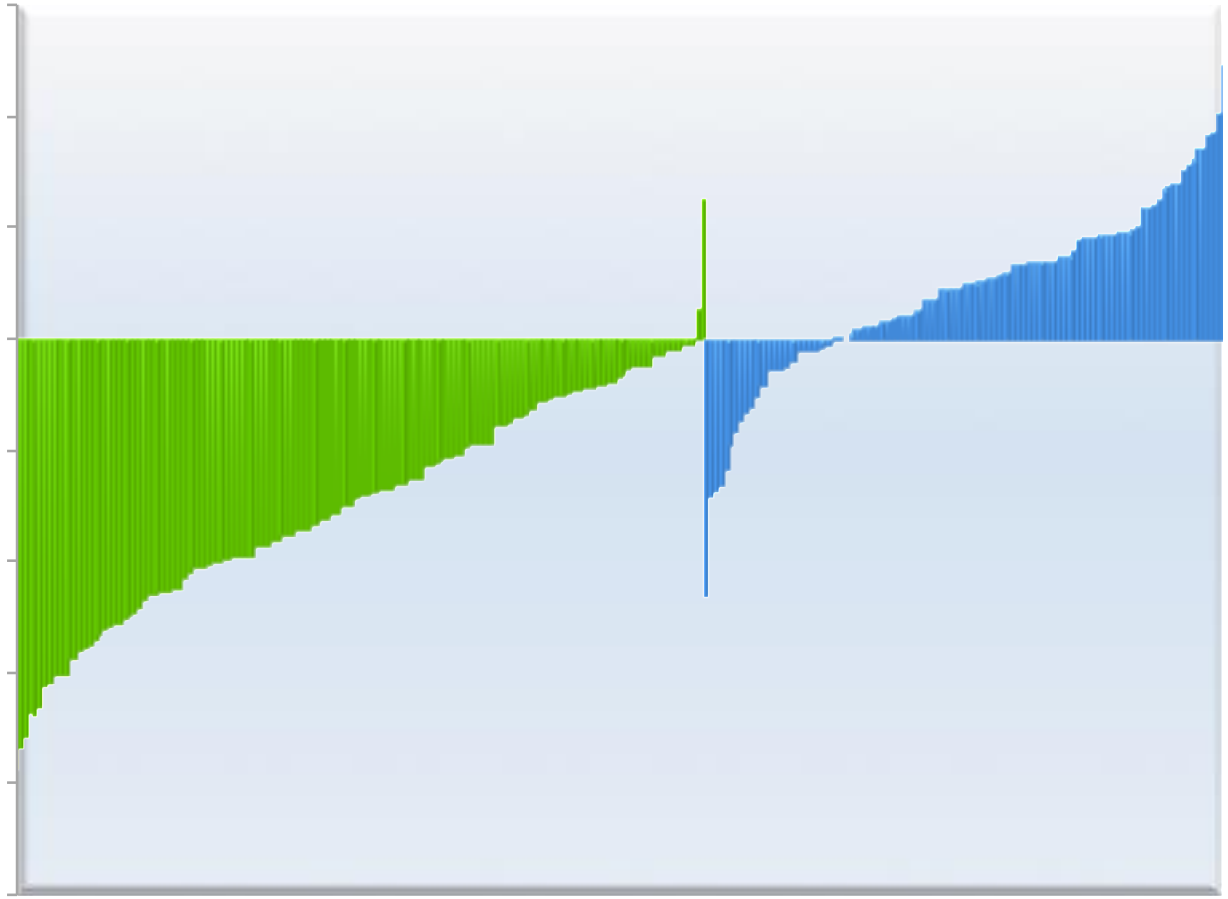


- Improved splenomegaly, thrombocytosis, leukocytosis, and myelofibrosis
- Approved November 2011

*Sachie Marubayashi, Priya Koppikar

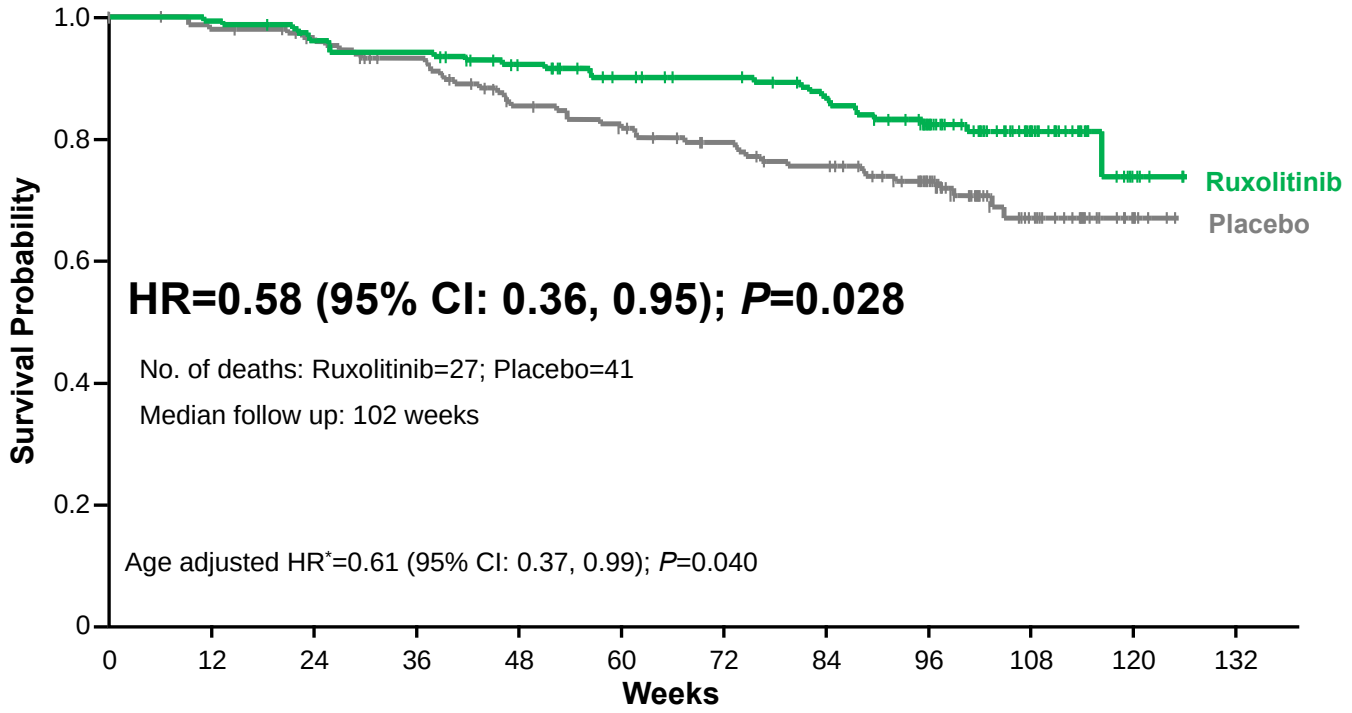
COMFORT I Trial: Spleen volume reduction with ruxolitinib vs. placebo

Spleen Size Before/After Therapy



Verstovsek et al. NEJM 2011

COMFORT I Trial – Overall Survival



- Improved survival –reduced progression/transformation to AML and reduced systemic complications
- Modest effect on MPN clone: JAK2 mutant cells persist despite long term JAK inhibitor therapy
- Dose limited by anemia/thrombocytopenia – though to be on-target effects

Verstovsek et al. Blood 2012

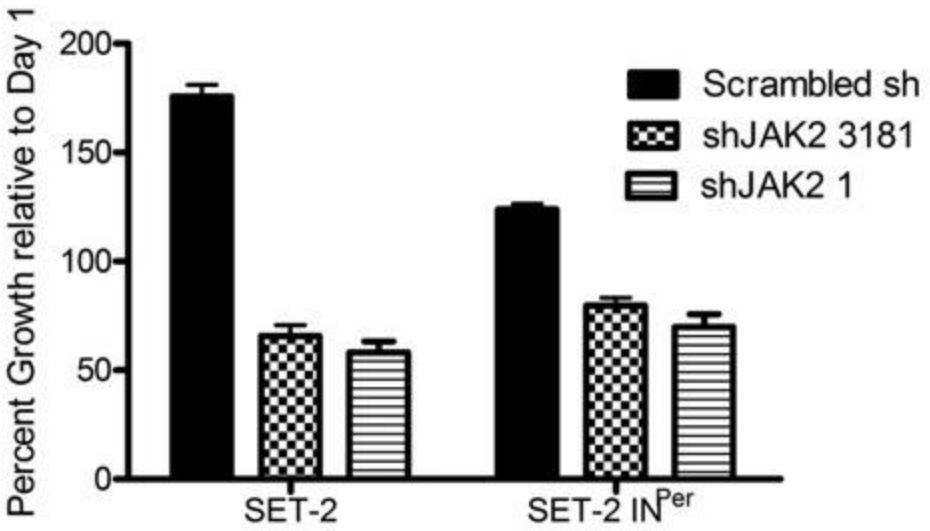
What have we learned so far?

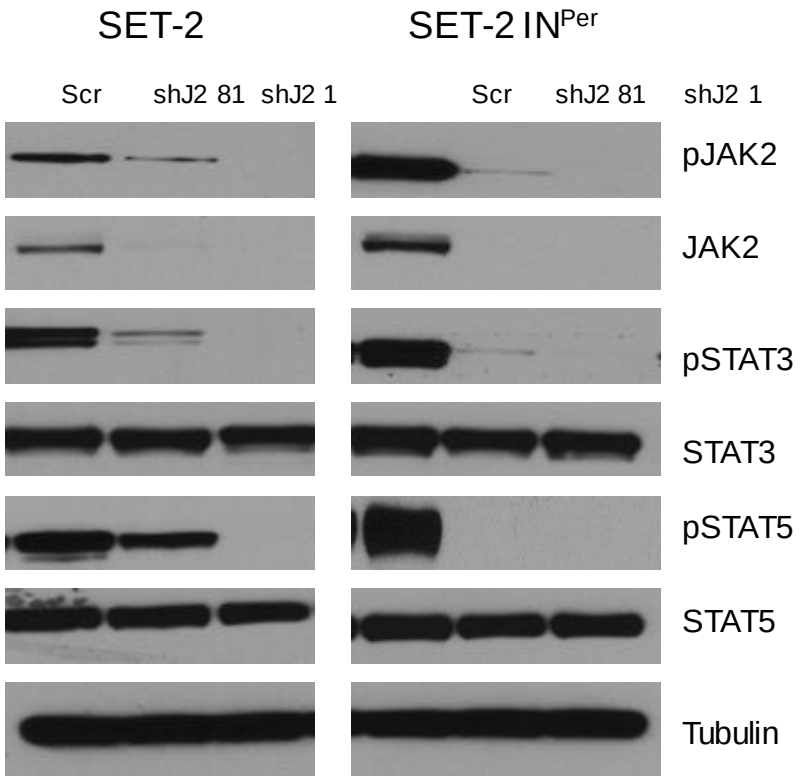
- JAK2 inhibitors improve spleen size, clinical symptoms in PMF patients, but do not improve cytopenias
  - Is this due to effects on the malignant clone?

- Different JAK2 inhibitors have non-overlapping toxicities
  -Is this due to differences in potency, pharmacokinetics, off-target effects?

- To date we have seen minimal effects on mutant allele burden
  -Is this due to incomplete dependence on JAK2, short treatment duration, additional mutations, or emergence of resistance?

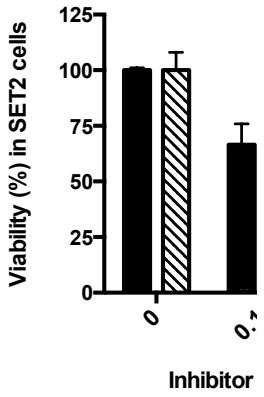
JAK2 dependence in JAK-inhibitor persistent/resistant cells

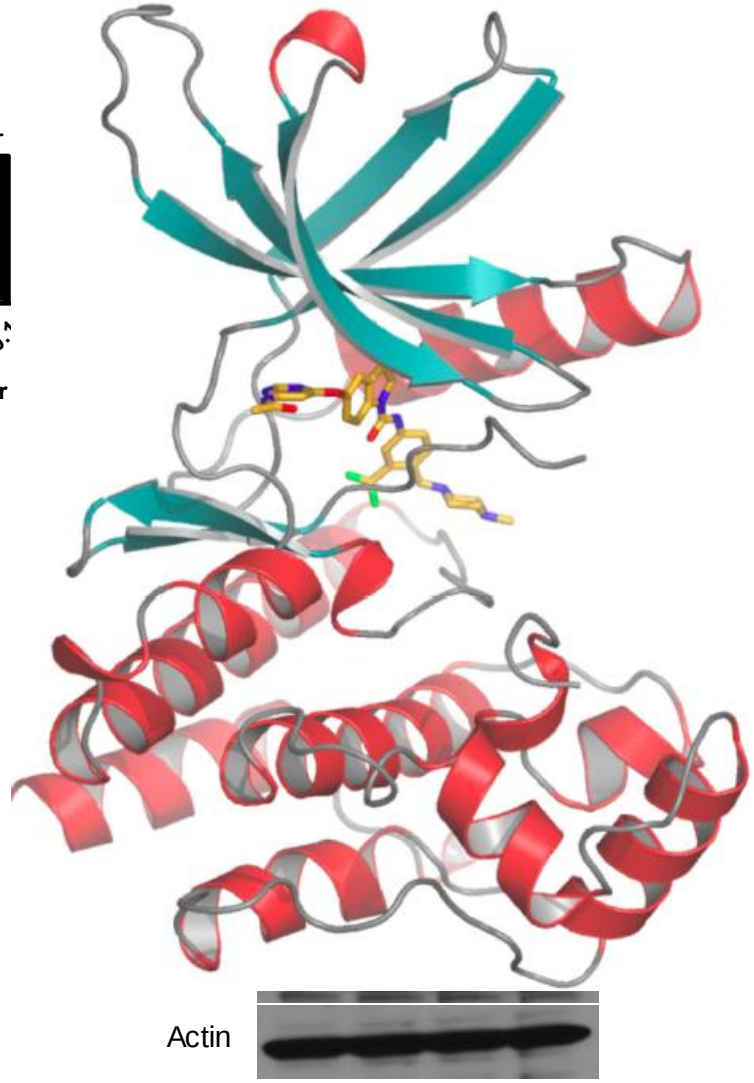


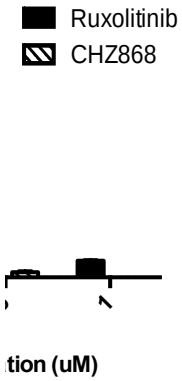


- Stable knockdown of JAK2 inhibits growth and signaling in persistent cells
- Can this be leveraged therapeutically?

Type II JAK2 inhibitor CHZ868 inhibits JAK-STAT signaling in JAK2V617F mutant cells

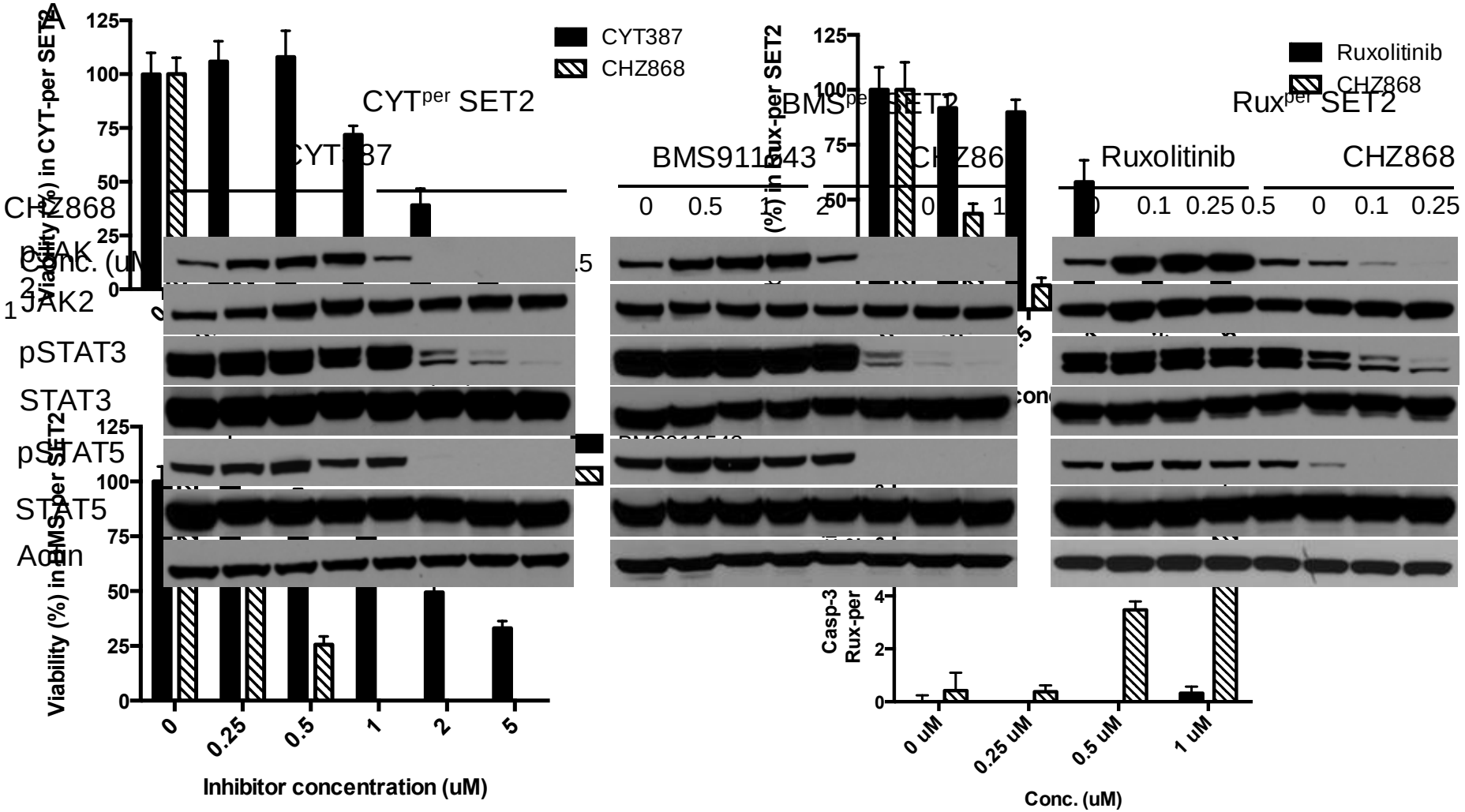




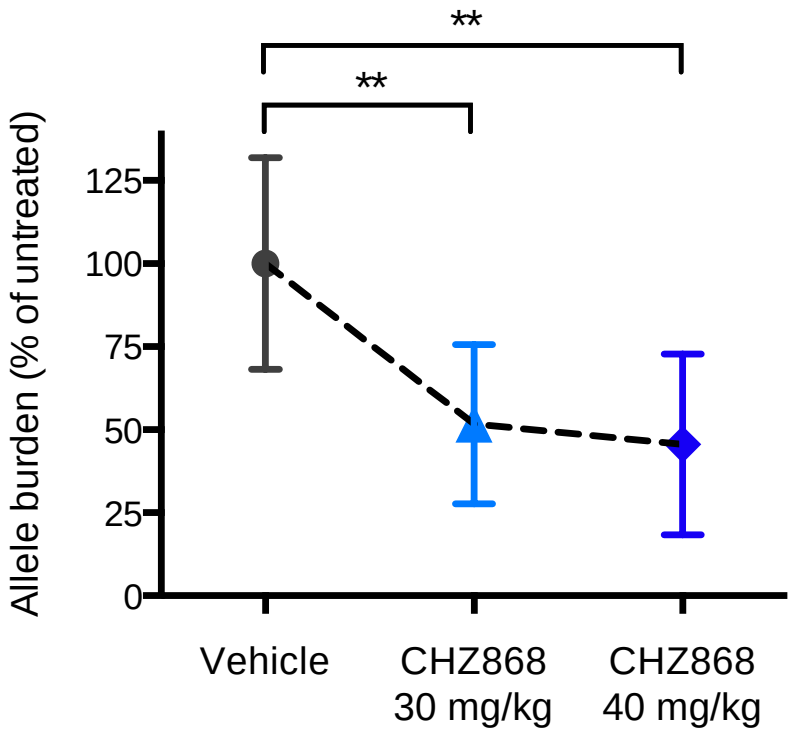


Meyer et al. Cancer Cell 2015

CHZ868 inhibits JAK-STAT signaling in JAK2-mutant cells which are resistant to type I inhibitors



Type II inhibition by CHZ868 reduces allele burden in a *Jak2*V617F knock-in model

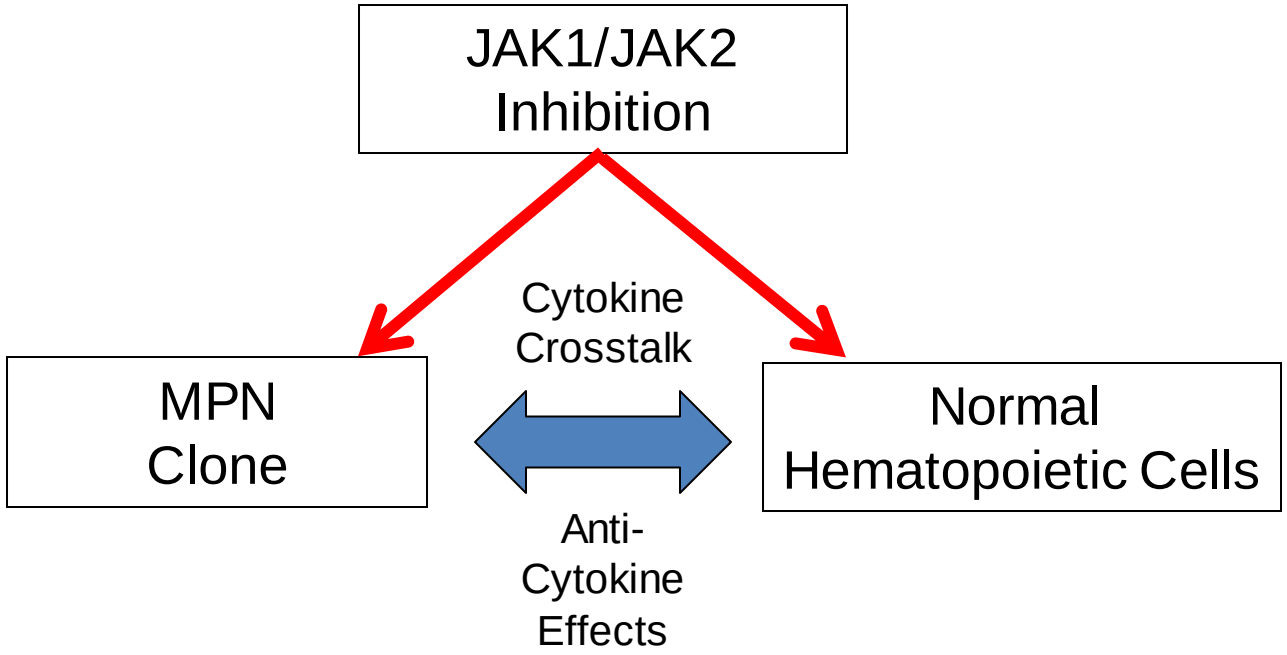


- Similar data from Weinstock lab in JAK2-mutant ALL (Li *et al.* Cancer Cell 2015

- Initiated new effort (academic->biotech) aimed to develop novel JAK2 inhibitor
    - Clinical type II JAK inhibitors
    - Approaches to specifically target mutant JAK2

Inflammatory cytokine signaling in normal and malignant hematopoiesis

How can inflammatory cytokine signaling be targeted in MPN/AML?



- **JAK inhibitors have effects on malignant and non-malignant cells- contribute to disease pathogenesis and therapeutic efficacy**
- **Similar mechanisms relevant to other hematopoietic and epithelial malignancies- role of JAK inhibitors in other pathologic contexts**

What other mechanisms drive the chronic inflammatory state in MPNs?

What is the role of JAK1/2-mediated inflammatory cytokine signaling in normal hematopoietic cells?

Not all screens for mutations work so well…

• 37% of patients with acute myeloid leukemia have mutations in the FLT3 tyrosine kinase

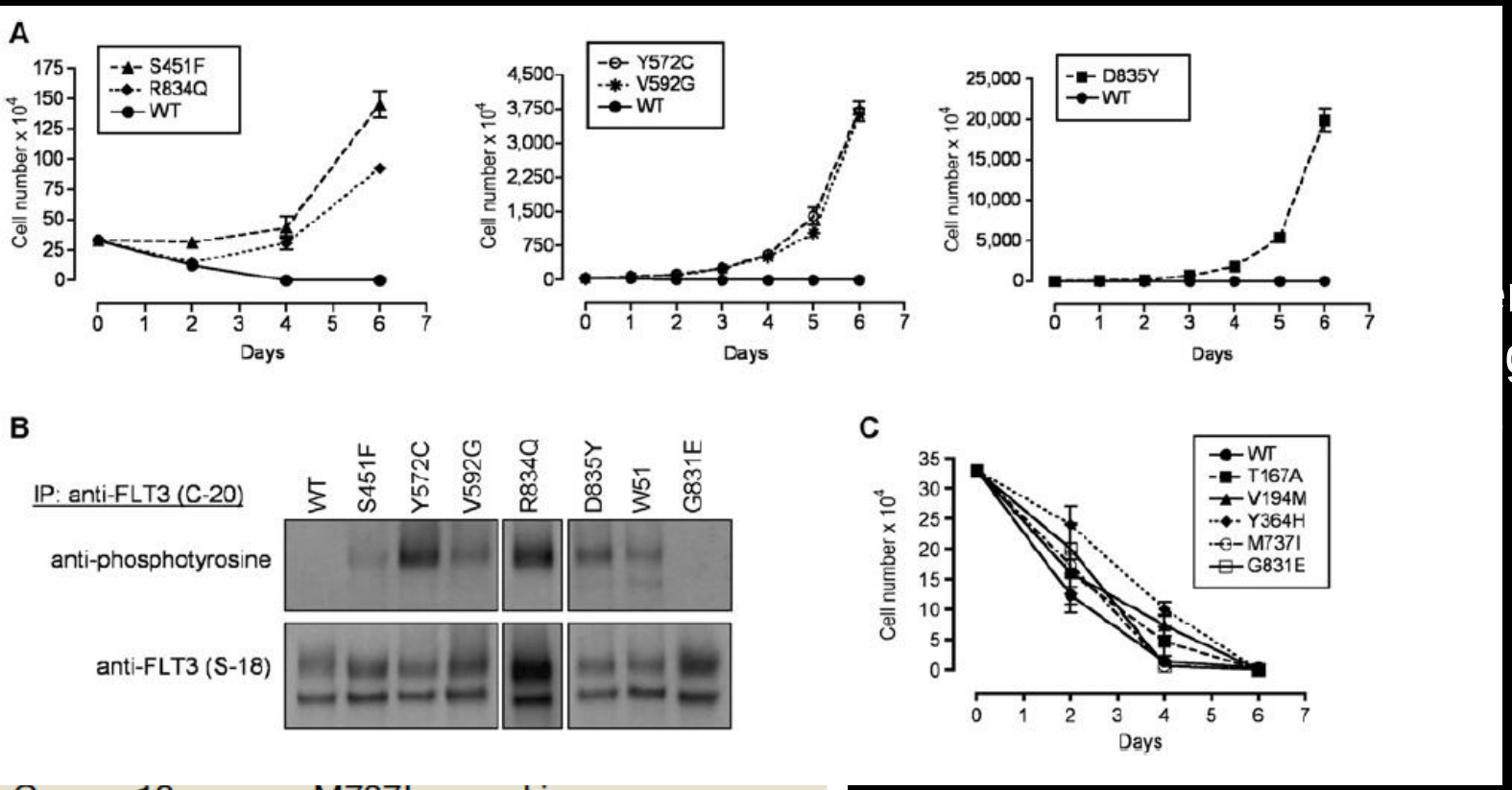
• Another 20% have mutations in other kinases or in other signaling pathways

•Hypothesis – sequence analysis of the tyrosine kinome will lead to identification of additional novel disease alleles

•However, the screen only identified additional, novel mutations in FLT3, no other kinases were recurrently mutated

Frohling et al. Cancer Cell 2007

Novel somatic FLT3 mutations in AML

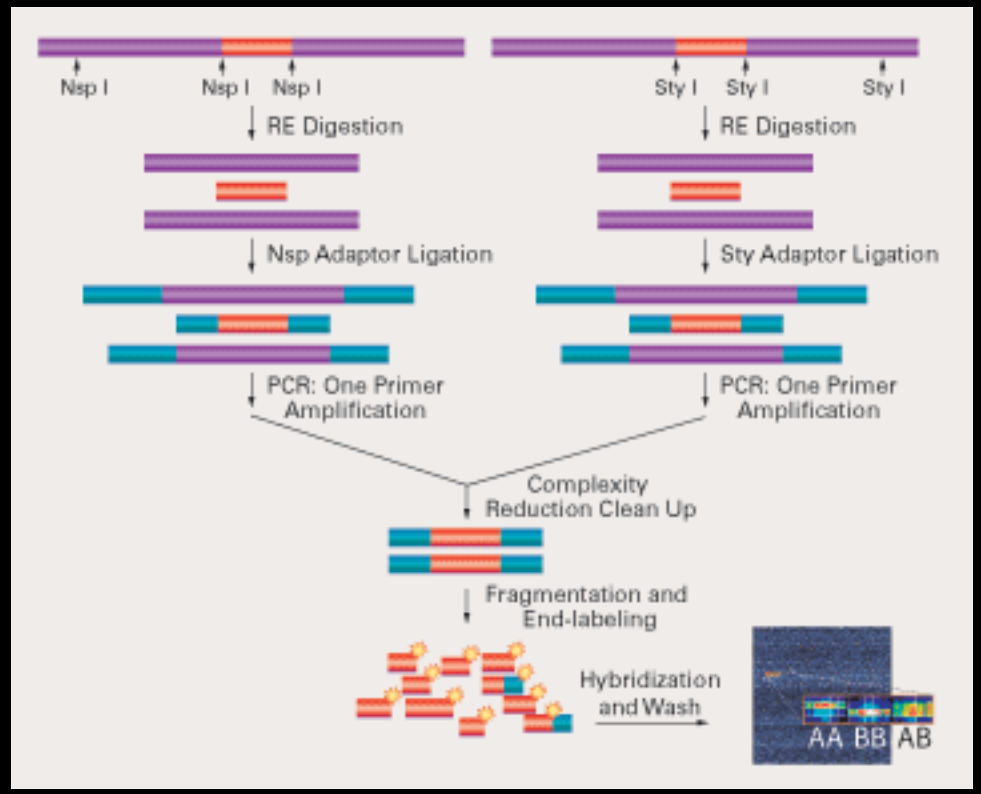


2293G > C	18	M737I	kinase
2574G > A	20	G831E	activation loop
2583G > A	20	R834Q	activation loop

Some, but not all mutations score as oncogenic – drivers and passengers in the same gene!

Frohling et al. Cancer Cell 2007

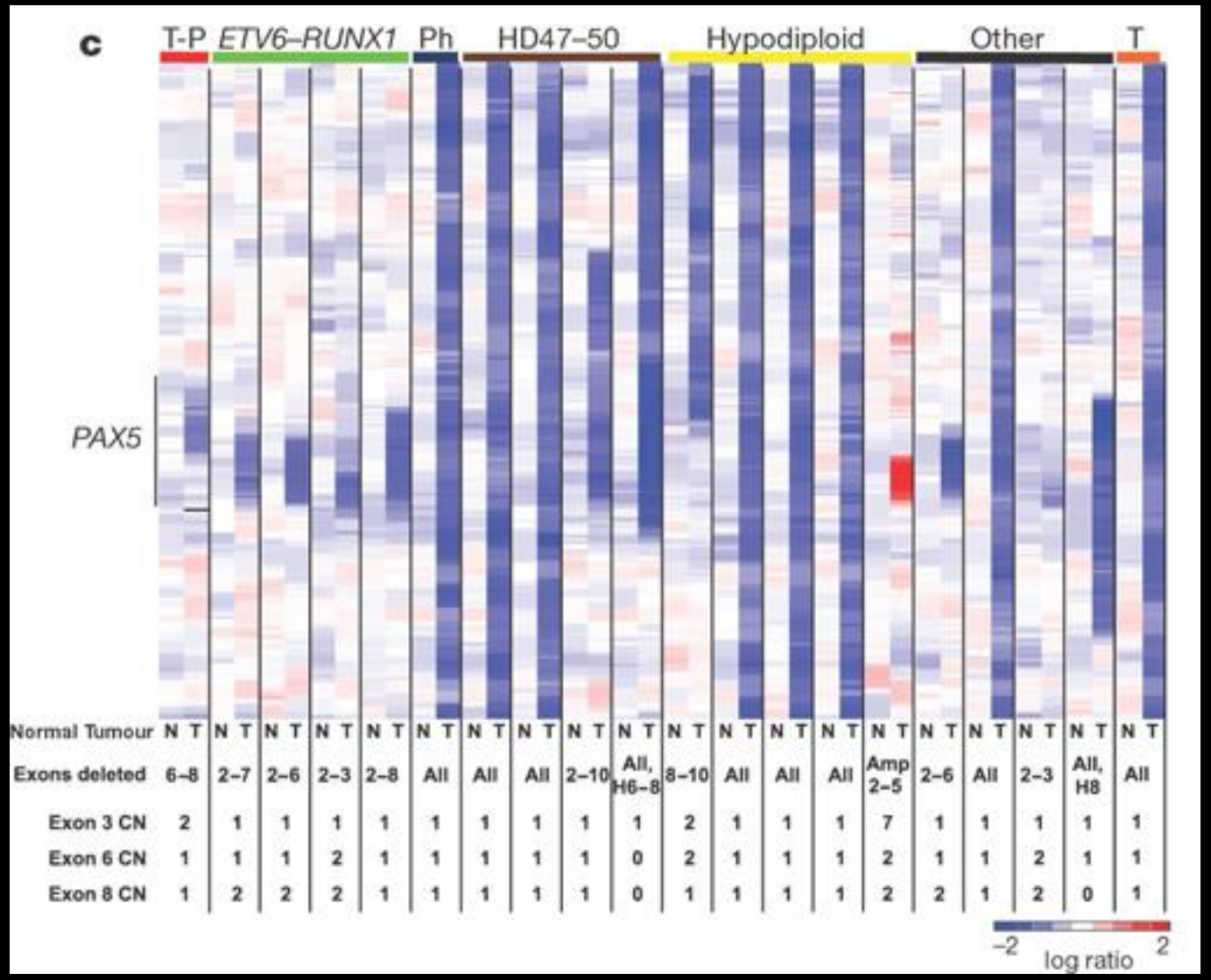
Discovery of Tumor Suppressor Genes in ALL: SNP Array Based Discovery



- Affymetrix SNP Array Platform
  - set of SNPs in human genome
  - allele call (G, T, G/T) and copy number assessment for each SNP
  - assumes equal amplification of different loci/alleles
- Study of 210 cases of B-ALL using SNP arrays
  - Also analyzed remission DNA as germline control

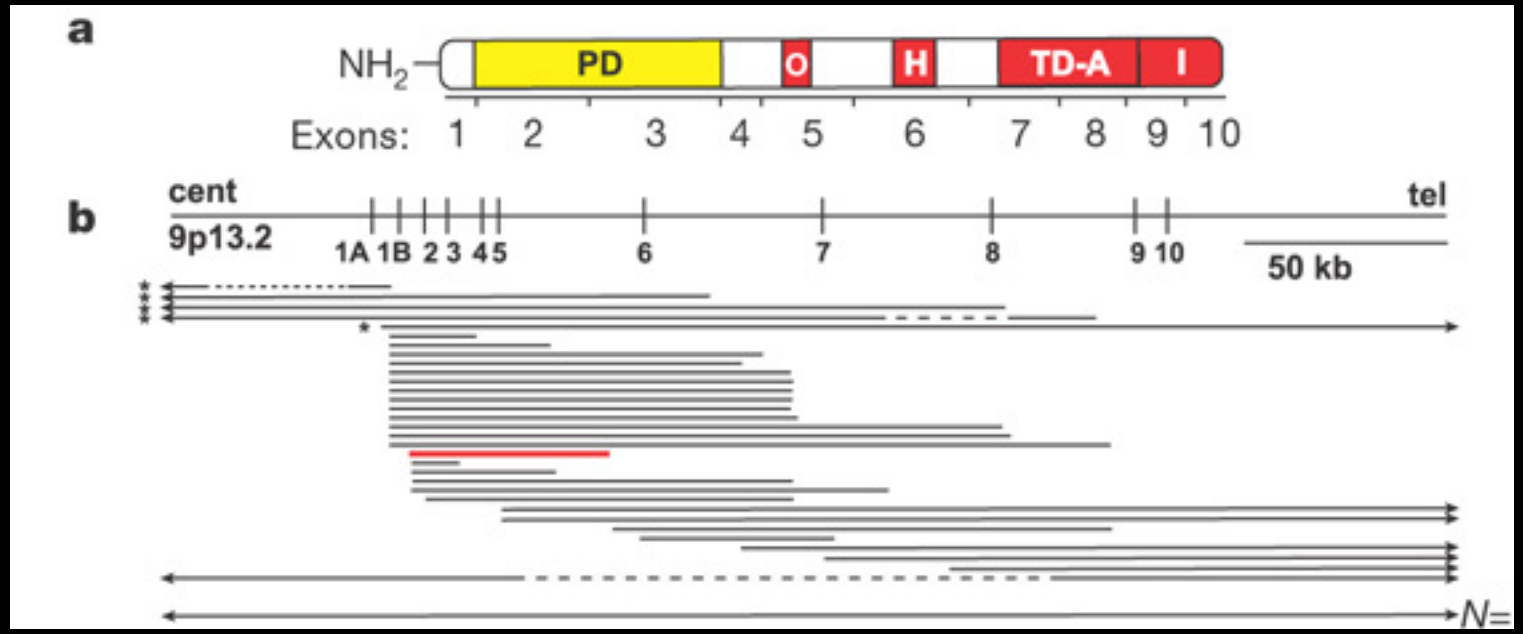
Mullighan et al. Nature 2009

PAX5 deletions in ALL



- copy number changes involving PAX5 in 57/192 B-ALL cases

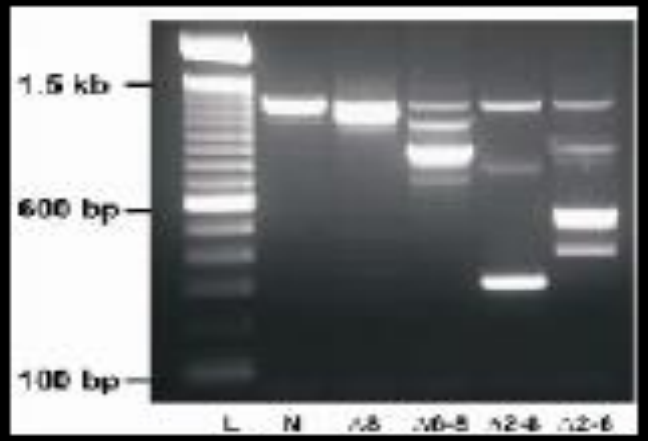
PAX5 deletions in ALL

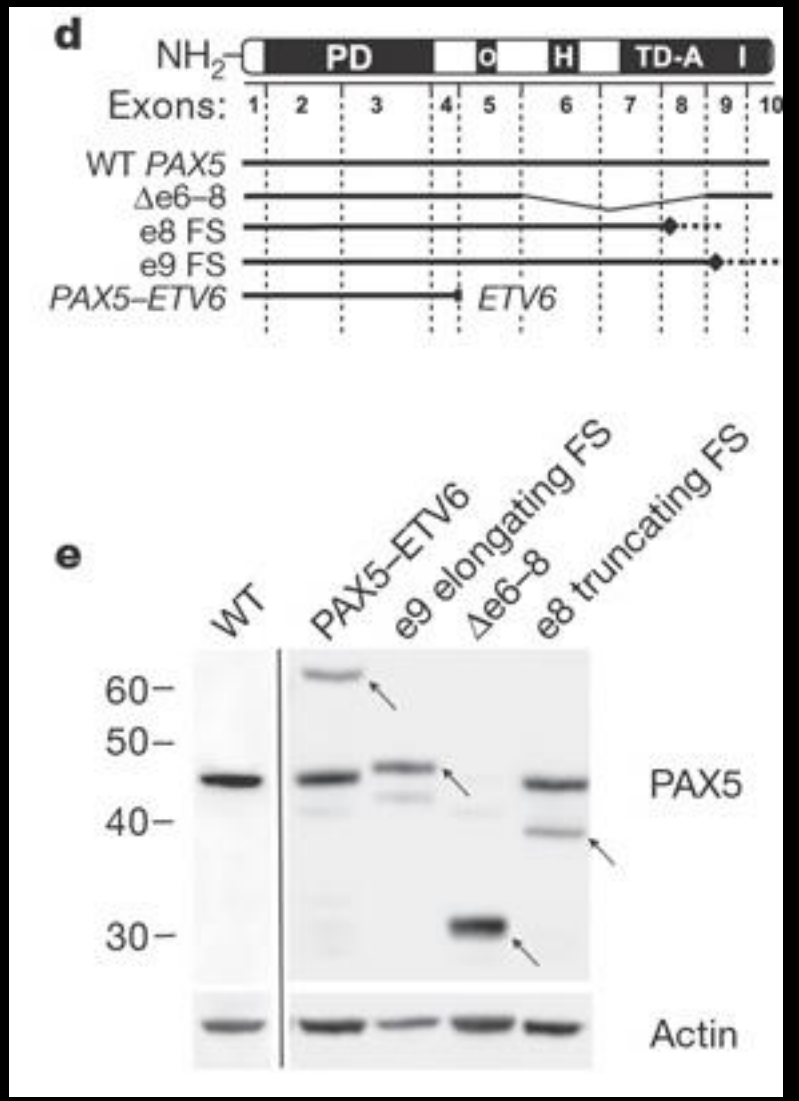


- different types of deletions
  - 7 cases with deletions of PAX5 and adjacent genes
  - 19 cases with deletion of chromosome 9 or 9p and all of PAX5
  - 5 cases with deletion of large portion of 9p including 3 ‘end of PAX5
  - 25 cases with deletions restricted to PAX5
  - only 3 cases with bi-allelic loss->acts in a HAPLOINSUFFICIENT STATE

PAX5 deletions in ALL

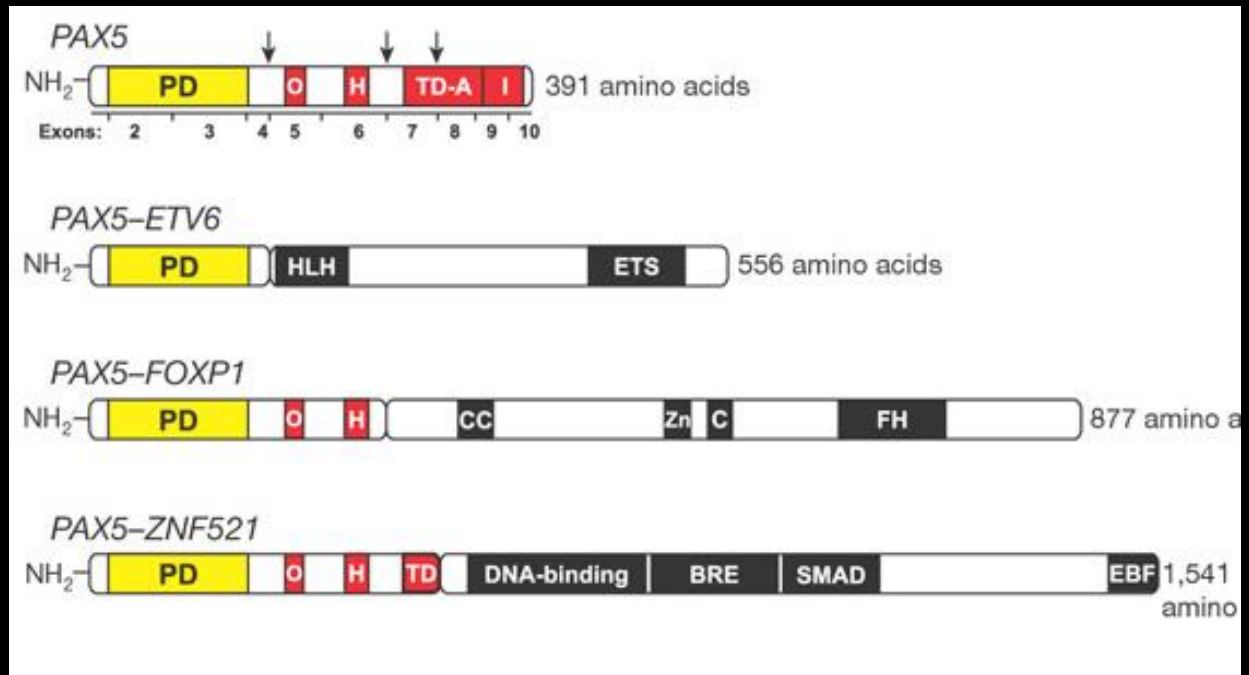
- 25 cases with deletions restricted to PAX5
  - 23 had intragenic deletions
  - internally deleted transcripts that lack DNA binding domain
  - confirmed by RT-PCT/Western blot of leukemic cells





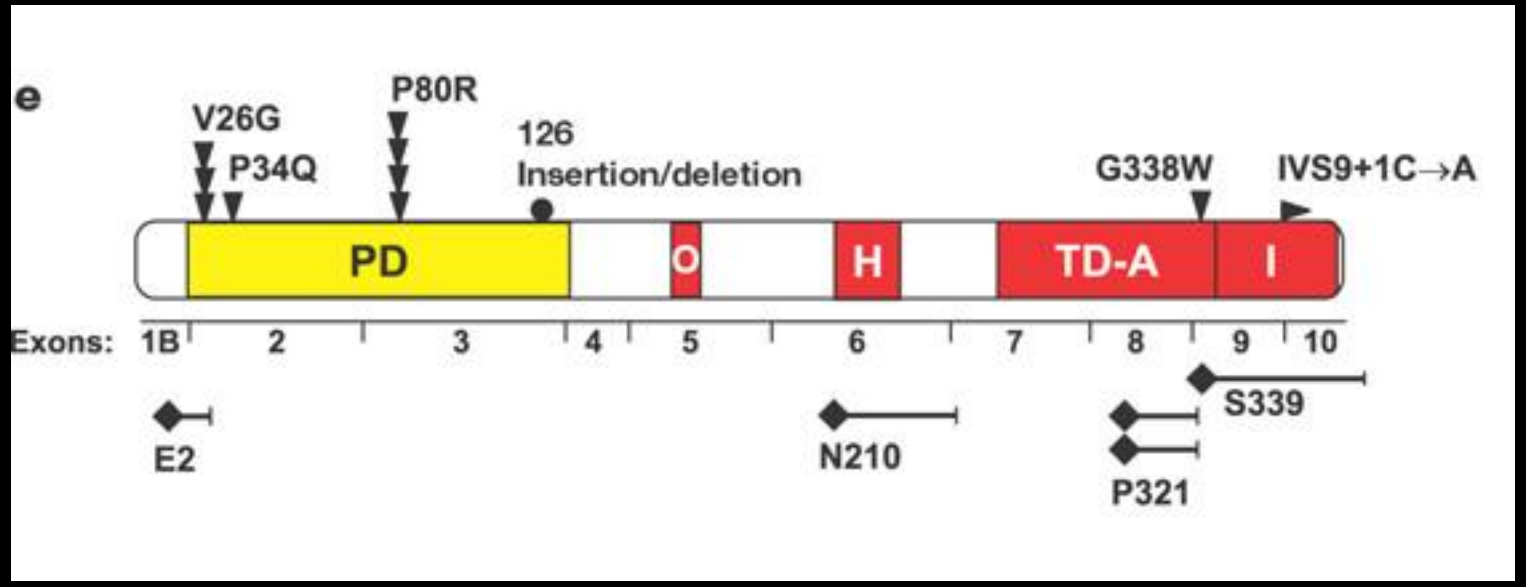
PAX5, continued…

- 5 cases with 9p deletions including 3’ end of PAX5
  - 2 had t9:12->ETV6-PAX5 fusions
  - 2 of remaining 3 cases->RACE identified novel translocations
    - PAX5-FOXP1
    - PAX5-ZNF521
  - confirmed by RT-PCR, FISH, sequencing



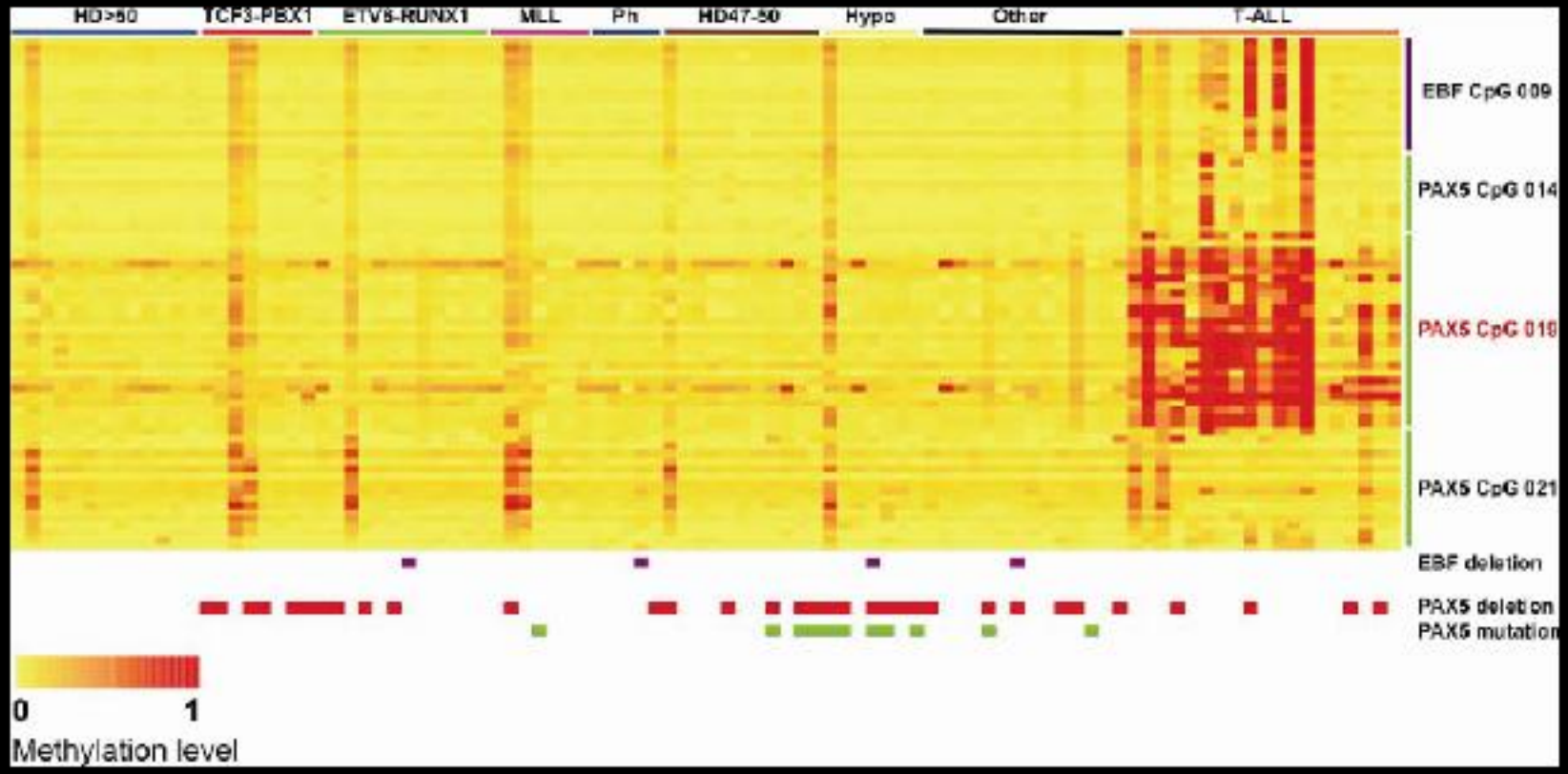
PAX5, continued…

- Sequence analysis of PAX5 (11 exons 242 samples)
  - 14 cases with point mutations: somatic in 13 cases with remission material
  - some mutations in cases with PAX5/9p deletions, in some cases
  - misssense, frameshift, splice site mutations



PAX5, continued…

- Methylation analysis of PAX5 (11 exons 242 samples)
  - detected high level methylation of PAX5, in T-ALL, not B-ALL



Other B-cell genes are deleted in B-ALL

- IKZF1 - 17 cases with deletions (no mutations)
  - IKZF1 -/- mice have early B-cell arrest
  - dnIKZF1 predisposes to T cell neoplasms

- IKZF3 - 3 cases with deletions

- LEF1 - 3 cases with deletions

- TCF3 - 3 cases with deletions

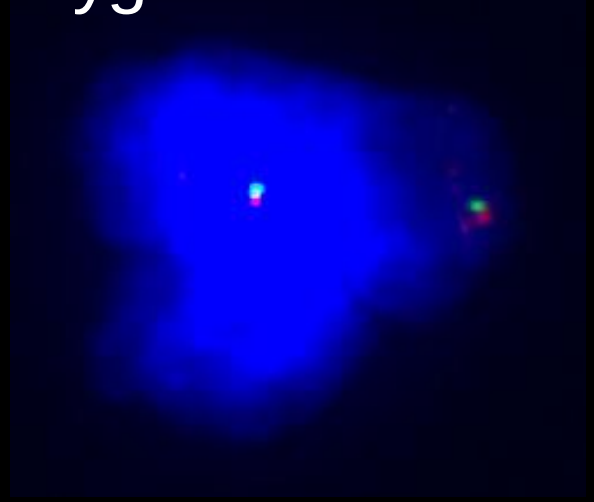
- BLNK - 2 cases with deletions

- These mutations were not mutually exclusive of PAX5 mutations consistent with multiple somatic hits in SAME pathway

- Rare in T-ALL

JAK2V617F homozygosity: uniparental disomy or copy neutral LOH

Pt with homozygous *JAK2V617F* mutation

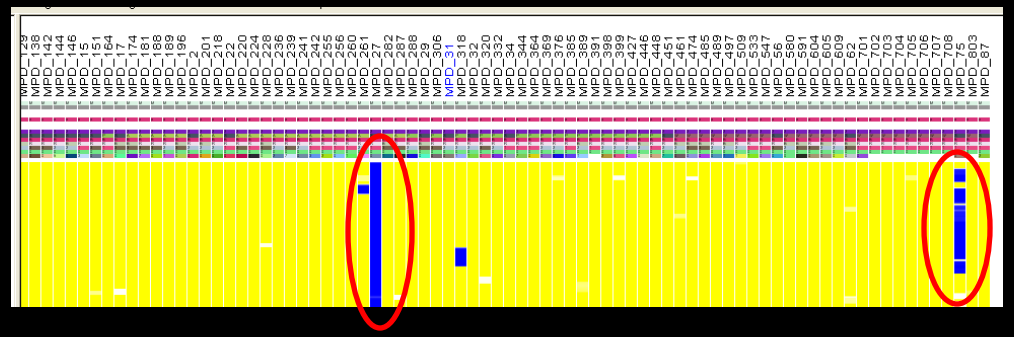


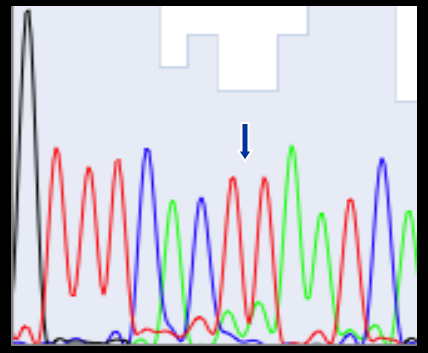
FISH: 2 copies of *JAK2* locus

- Homozygosity of *JAK2V617F* is due to duplication of mutant allele, and not deletion of wild-type allele

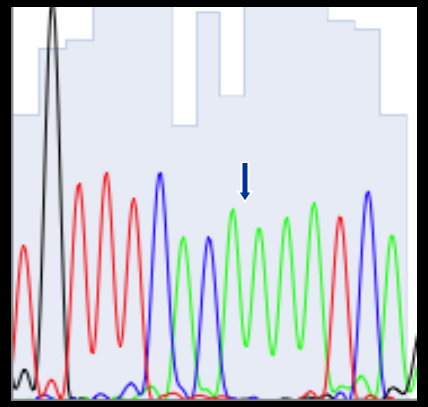
- This is seen with other activating alleles in myeloid malignancies (*FLT3*, *MPL*)

SNP Array Based Identification of 9p UPD





Granulocyte

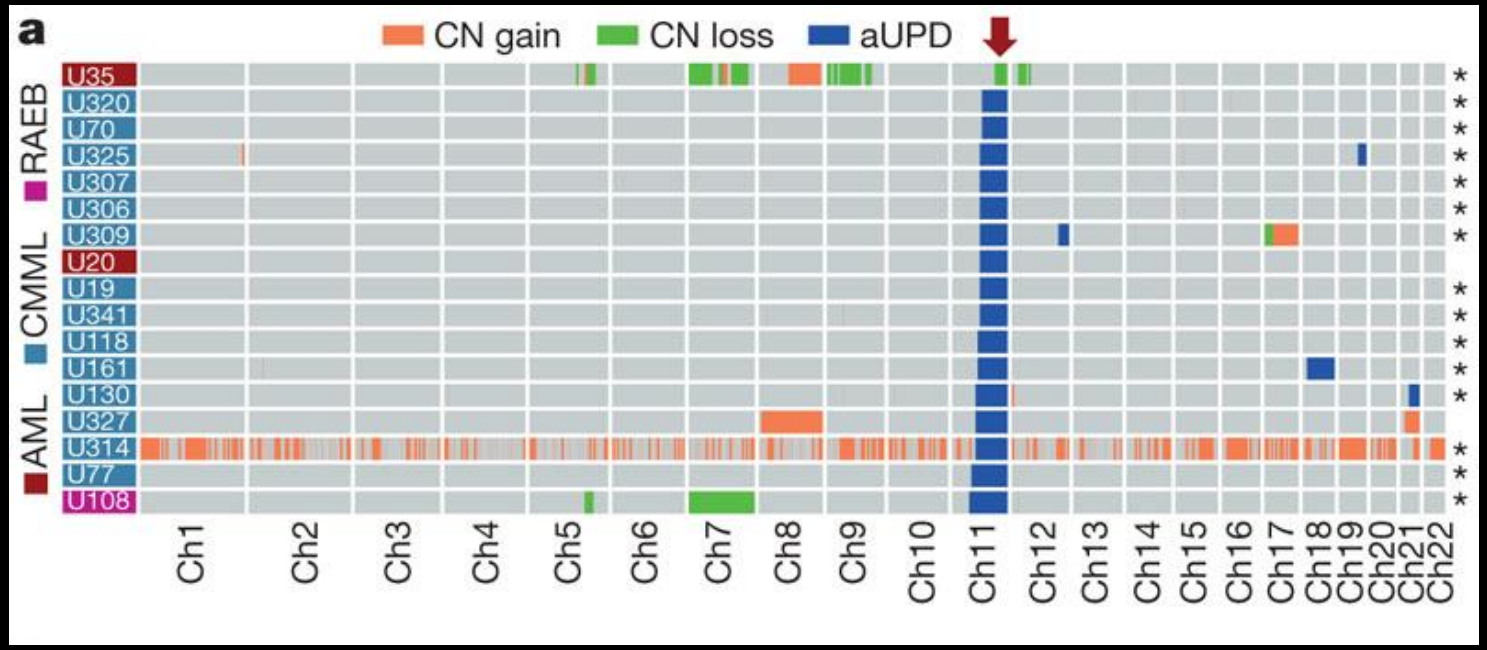


Normal

- 2 *JAK2V617F*-negative PV cases with 9p24 UPD
  - -Sequence analysis-> homozygous JAK2 exon 12 mutations

Can this approach be used to identify novel disease alleles?

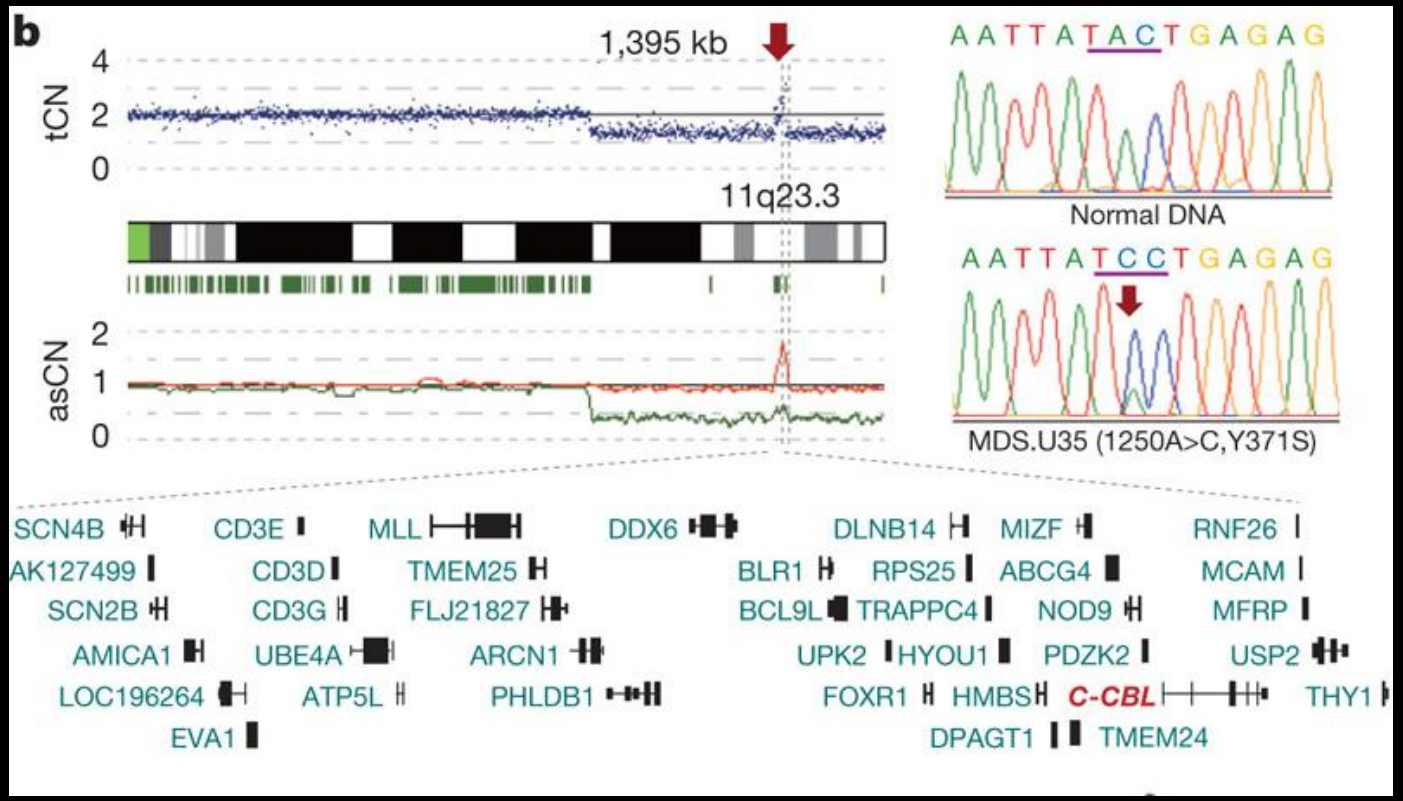
Identification of 11q UPD and C-CBL mutations



Identified large region of CN-LOH on 11q - followed with high throughput sequencing

Sanada et al. Nature 2009

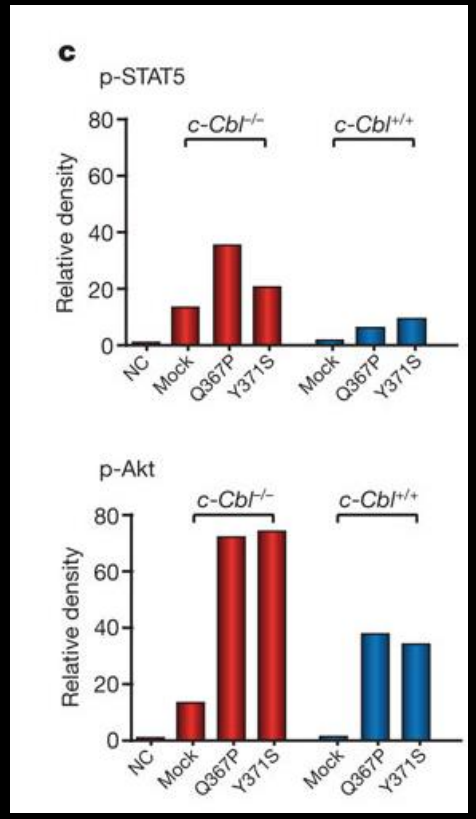
Identification of 11q UPD and C-CBL mutations



Somatic c-CBL mutations in MDS, MPN patients

Sanada et al. Nature 2009

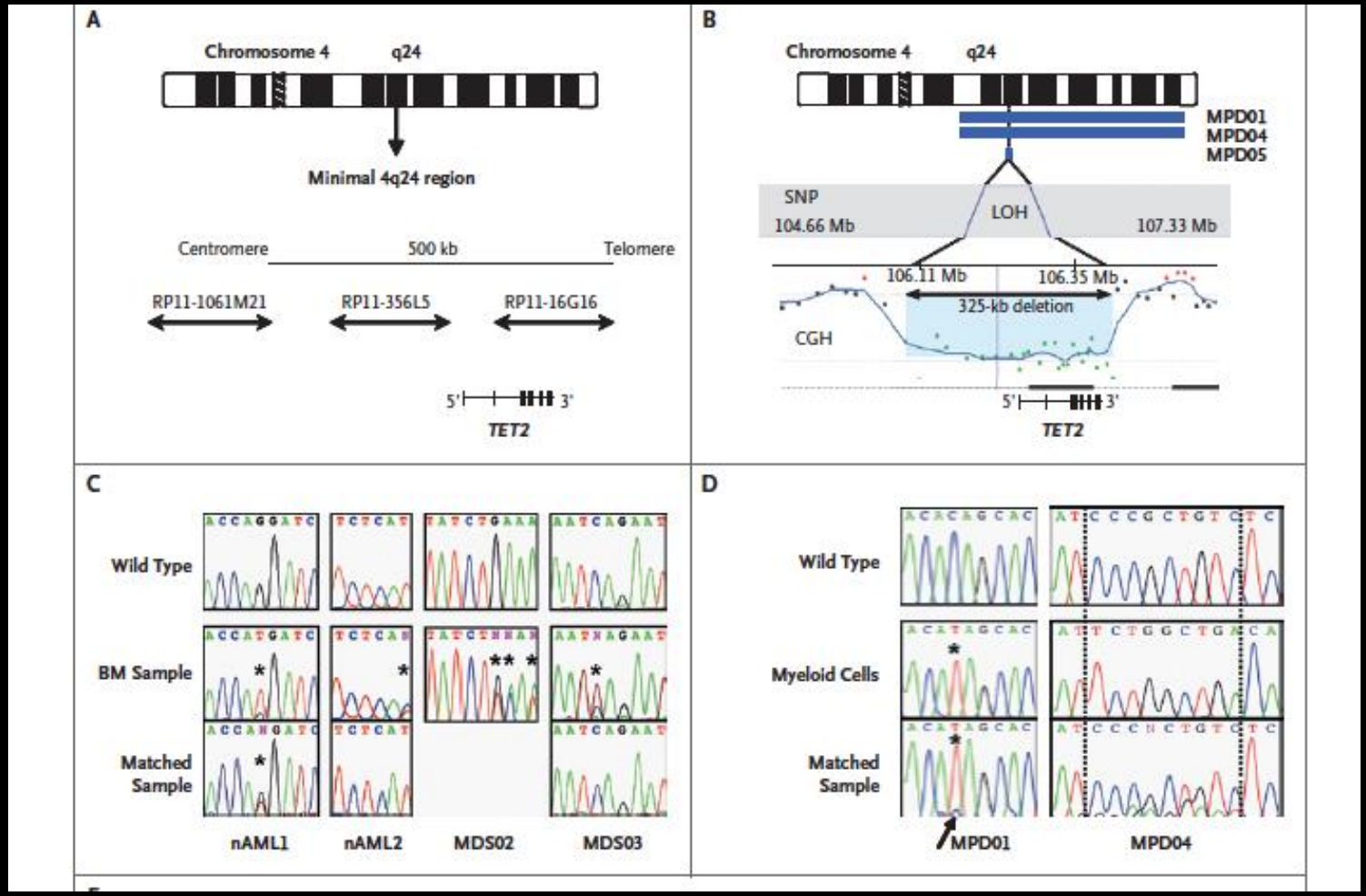
Functional studies with C-CBL mutations



- Mutant CBL scores in signaling and transformation assays
- Markedly enhanced by lack of WT allele
- So UPD not only selects for higher dose of oncogene, but also to remove WT allele and facilitate mutant mediated transformation
- Will inform murine model development

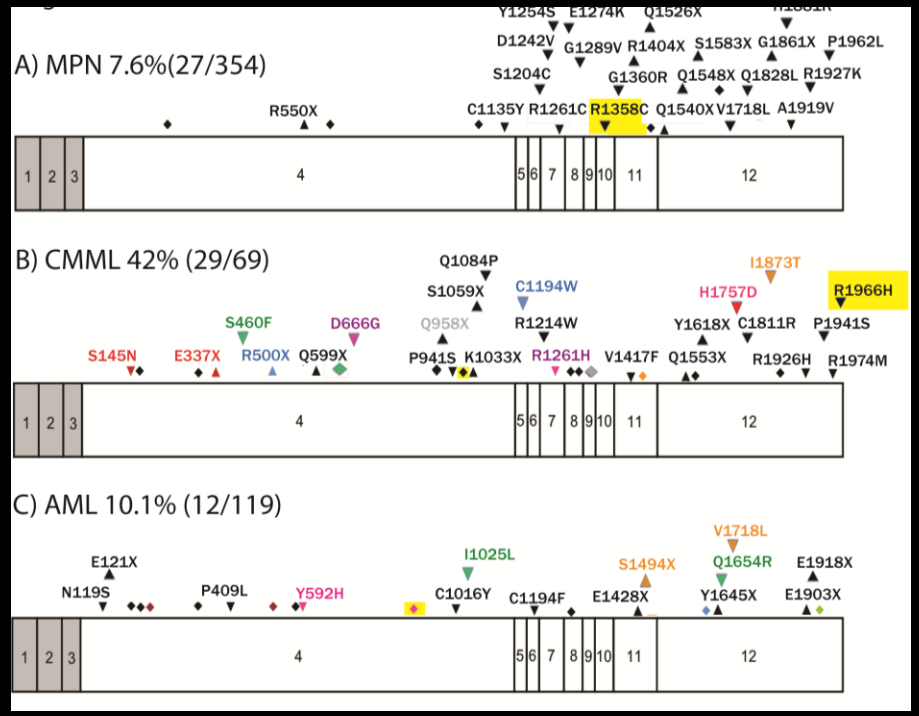
Sanada et al. Nature 2009

LOH/deletions can identify novel tumor suppressors



Delhommeau et al. NEJM 2009

TET2 mutations in myeloid malignancies

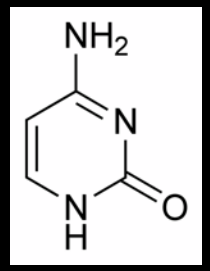


- Nonsense/frameshift>missense mutations, consistent with tumor suppressor function
- However most mutations are heterozygous – consistent with potential role as a haploinsufficient tumor suppressor
- No clear role for TET2 mutations or even function of TET2 protein at time of discovery
  - The genetics alone strongly suggested this was a driver mutation

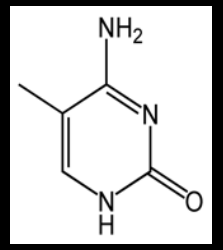
Abdel-Wahab et al. Blood 2009

Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1

Mamta Tahiliani,[1] Kian Peng Koh,[1] Yinghua Shen,[2] William A. Pastor,[1] Hozefa Bandukwala,[1] Yevgeny Brudno,[2] Suneet Agarwal,[3] Lakshminarayan M. Iyer,[4] David R. Liu,[2]* L. Aravind,[4]* Anjana Rao[1]*

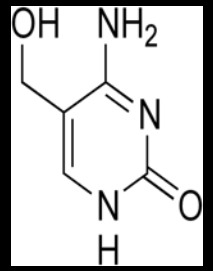


Cytosine (unmethylated)



Methyl-Cytosine
(hemimethylated)

α-KG ↓ **TET 1-3**



Hydroxy-methyl-Cytosine

TET family enzymatic function is α-KG dependent

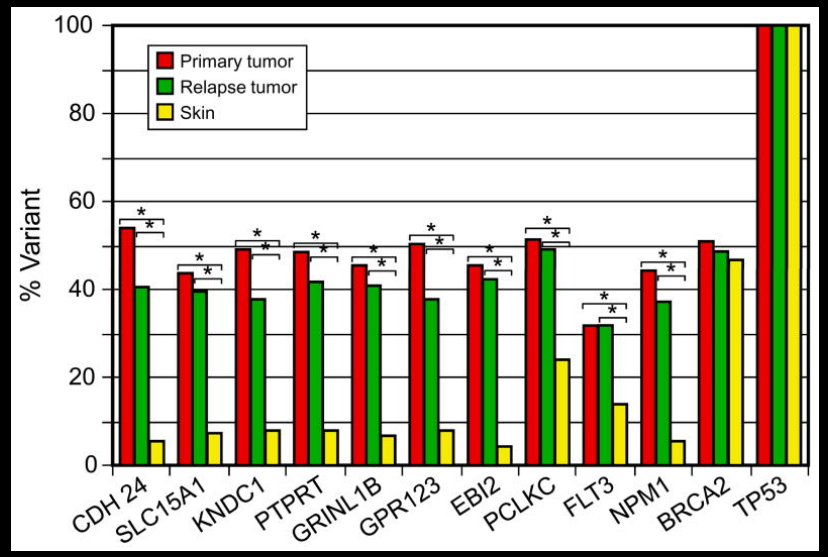
Inactivating mutations of TET proteins result in loss of DNA hydroxymethylation

Advent of whole exome/genome sequencing as a discovery tool to find new cancer genes

- 2007-9: Increase in throughput and reduction in cost made Sanger sequencing of large gene sets, even the entire set of known genes, a reality
- 2008-9: Next generation sequencing and advanced mapping techniques allowed for shotgun sequencing of whole genome
- 2010: Development of exome capture methods brought sequence analysis of coding exome down in cost – allow for analysis of much larger sample sets
- Paired end sequencing maps translocations
- RNA-sequencing – allows for mutation and translocation detection

Whole genome sequencing of an AML genome

- Ley et al. Nature 2008
  - 33 fold coverage of tumor and normal from single AML patient
  - Is that sufficent or saturating?
- Identified 10 somatic, nonsynonymous mutations in the AML genome
- 3 known mutations (FLT3, NPM1), 7 novel mutations
- None of novel mutations recurred in 187 additional patients



IDH1 Mutations in AML*

CDC42	Missense	S30L	Tolerated	597	1	1.03	49.27	46.3	27,990
NRAS	Missense	G12D	Deleterious	616	1	0.66	43.00	42.0	7,468
IDH1	Missense	R132C	Deleterious	445	1	0.81	46.06	63.9	11,400
IMPG2	Missense	G834D	Deleterious	472	0.018	0.67	46.22	0.4	NA
ANKRD26	Missense	K1300N	Deleterious	444	1	0.70	51.73	33.1	514
LTA4H	Missense	F107S	Tolerated	539	0.946	0.68	45.28	47.9	12,138
FREM2	Missense	Q2077E	Tolerated	464	1	0.37	48.92	0‡	NA
C19orf62	Splice-site	Exon 5-1	NA	444	1	0.27	38.71	38.8	5,021
SRRM1	Silent	P691	NA	553	0.988	0.97	46.61	ND	12,858
PCDHA6	Silent	A731	NA	NS	0.423	0.66	49.75	ND	Absent
CEP170	In-frame insertion	Codon 177 in-frame ins L	NA	513	1	0.28	28.57	52.0	15,298
NPM1	Frame-shift insertion	W288fs	NA	689	1	0	45.46	85.4	27,150

Whole genome sequencing of the same AML genomefrom before (!) identified somatic IDH1 mutation->seen in 8% of 187 additional samples

Prognostic/therapeutic relevance of these mutations not known at that time

Presence/Absence of IDH2 mutations in AML or other leukemias not known

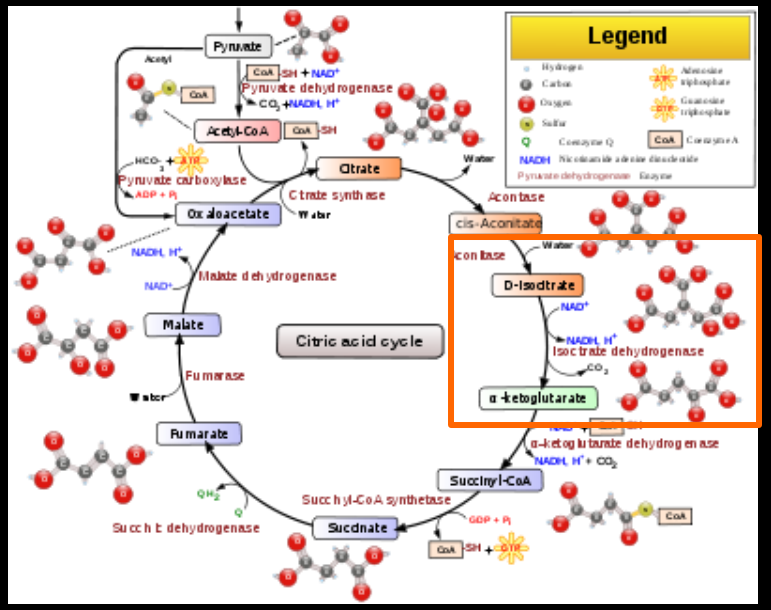
*Mardis *et al* NEJM 2009

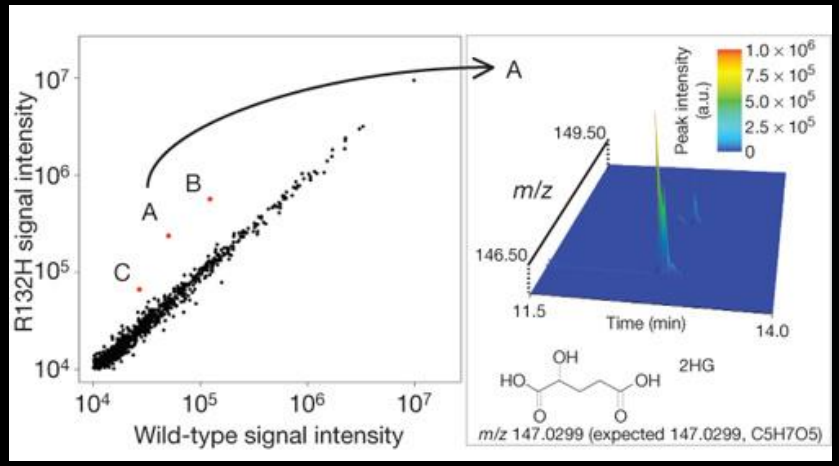
IDH Mutations in Malignant Brain Tumors

- Parsons et al. Science 2008 sequenced 22,000 genes in 22 patients with GBM
  - 12% had a point mutation in IDH1 at R132
- Subsequent studies identified a high proportion of IDH1 mutations in patients with lower grade gliomas>de novo glioblastoma multiforme
- All IDH1 mutations in glioblastoma occur at R132
- Yan et al. NEJM 2009 identified IDH2 mutations at R172 in patients with glioma: mutually exclusive with IDH1
- Initial studies suggested that thsse mutations were loss of function: mutant IDH enzymes lose the ability to convert isocitrate to alpha-ketoglutarate

IDH1 mutations acquire a novel enzymatic function

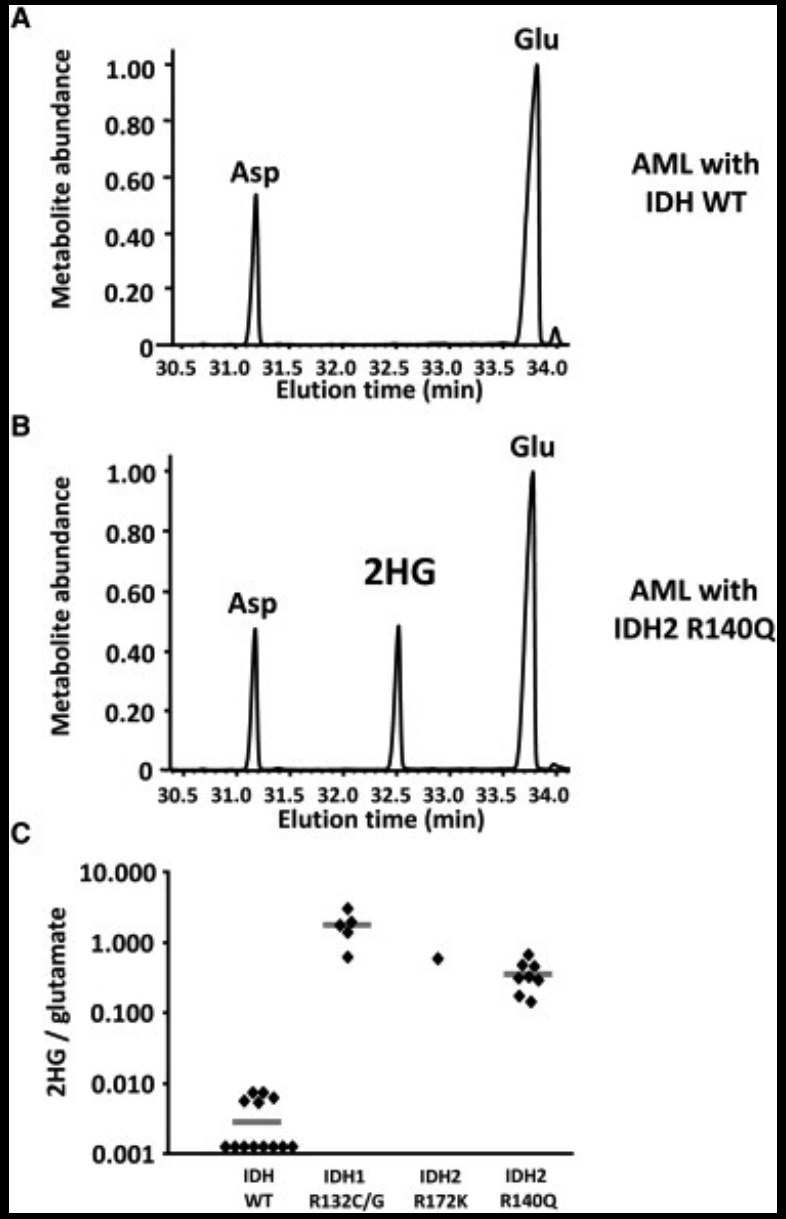
- Initial studies suggested that IDH1 mutations resulted in loss-of-function for the ability to convert isocitrate to alpha-ketoglutarate
- However unbiased metabolomic profiling found that IDH1 mutant allele expression resulted in production of 2-hydroxyglutarate, an aberrant metabolite normally in the serum at very low levels
- IDH1 mutant gliomas produce a vast excess of 2HG, such that it is readily detected using mass spec based approaches
- The mutant enzyme requires alpha-KG to make 2-HG – explains the retention of a wildtype IDH allele – both WT and mutant IDH represent potential therapeutic targets





Dang et al. Nature 2009

IDH2 mutations in AML



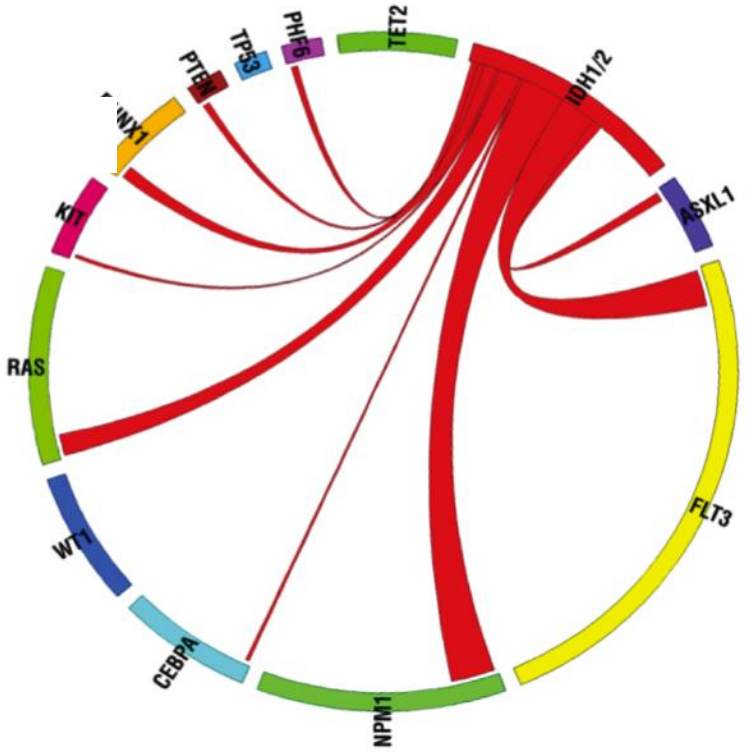
- Elevated 2-HG levels in IDH1-wildtype patients led to discovery of IDH2 mutations in AML
- Most common IDH mutation in AML is IDH R140Q – not seen in glioma
- The overall incidence of IDH1/2 mutations is 15-30%, most common in older patients, normal karyotype

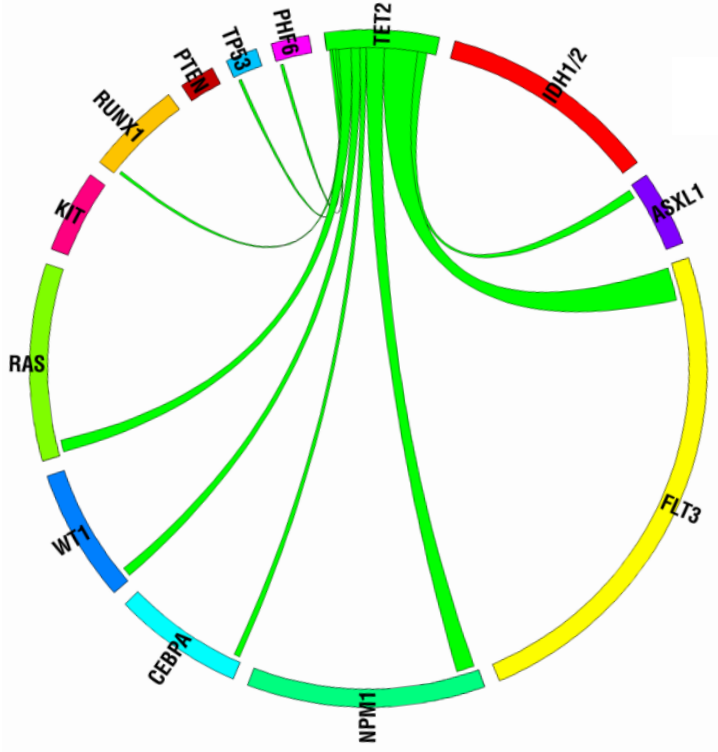
Ward *et al.* Cancer Cell 2010
Marcucci et al JCO 2010
Gross et al. J Ex Med 2010

Human genetics is always right: using mutational studies to elucidate AML pathogenesis

- By profiling primary patient samples we can improve our understanding of AML biology
- We can identify lesions that commonly occur together (NPM1/IDH) to guide development of new models, pathways to transformation…
- But…we can also identify mutations which NEVER occur together and define novel complementation groups/mutational classes
  - Would suggest that specific genes function in a pathway
  - Or that specific genes have a “synthetic lethal” interaction
- We hypothesized that we could elucidate the function of IDH mutations in AML by identifying mutations exclusive of IDH mutations of AML

ECOG 1900 Cohort: IDH1/2 mutations mutually exclusive of TET2 mutations





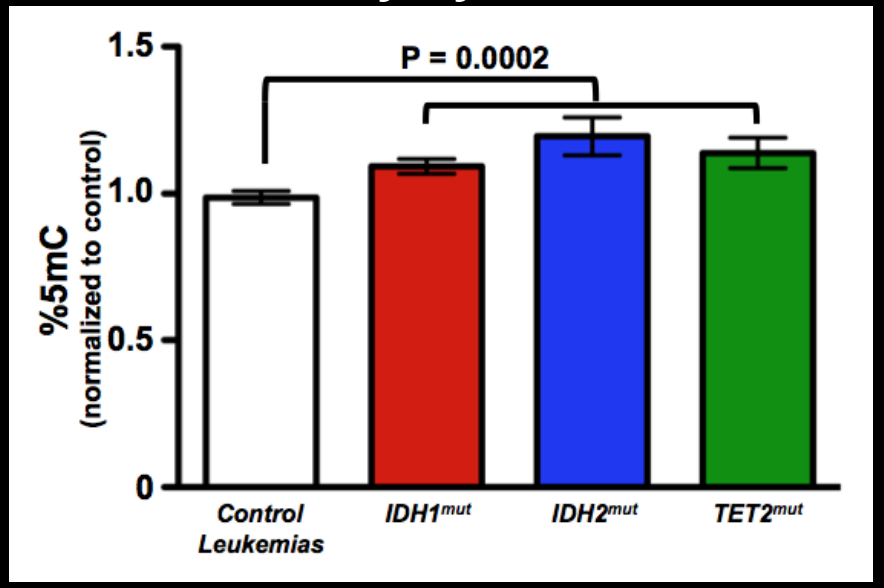
	TET2 Wildtype	*TET2* Mutant
IDH1/2 Wildtype	300	28
IDH1/2 Mutant	57	0

[a] P-value = 0.009 (Left-tailed Fisher's exact test)

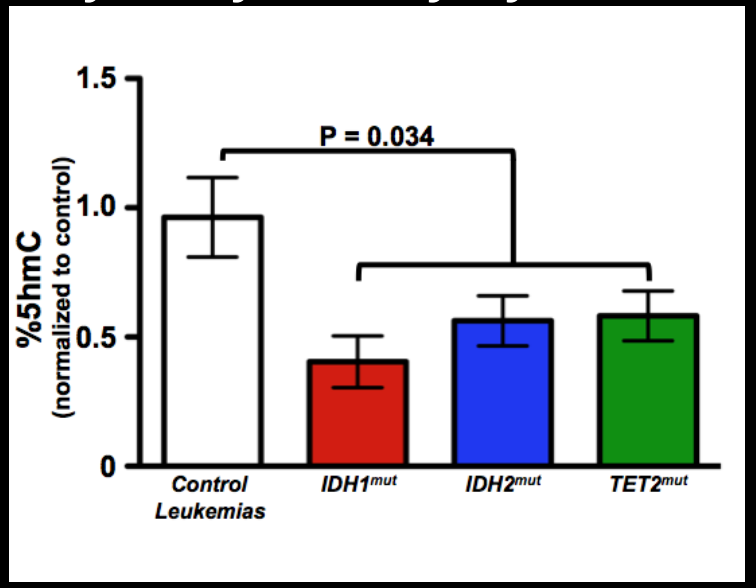
Figueroa, Abdel-Wahab, Lu *et al,* ***Cancer Cell*** 2010

AML patient samples->decreased 5-OH-methylcytosine and increased cytosine methylation with IDH/TET2 mutations*

Methylcytosine



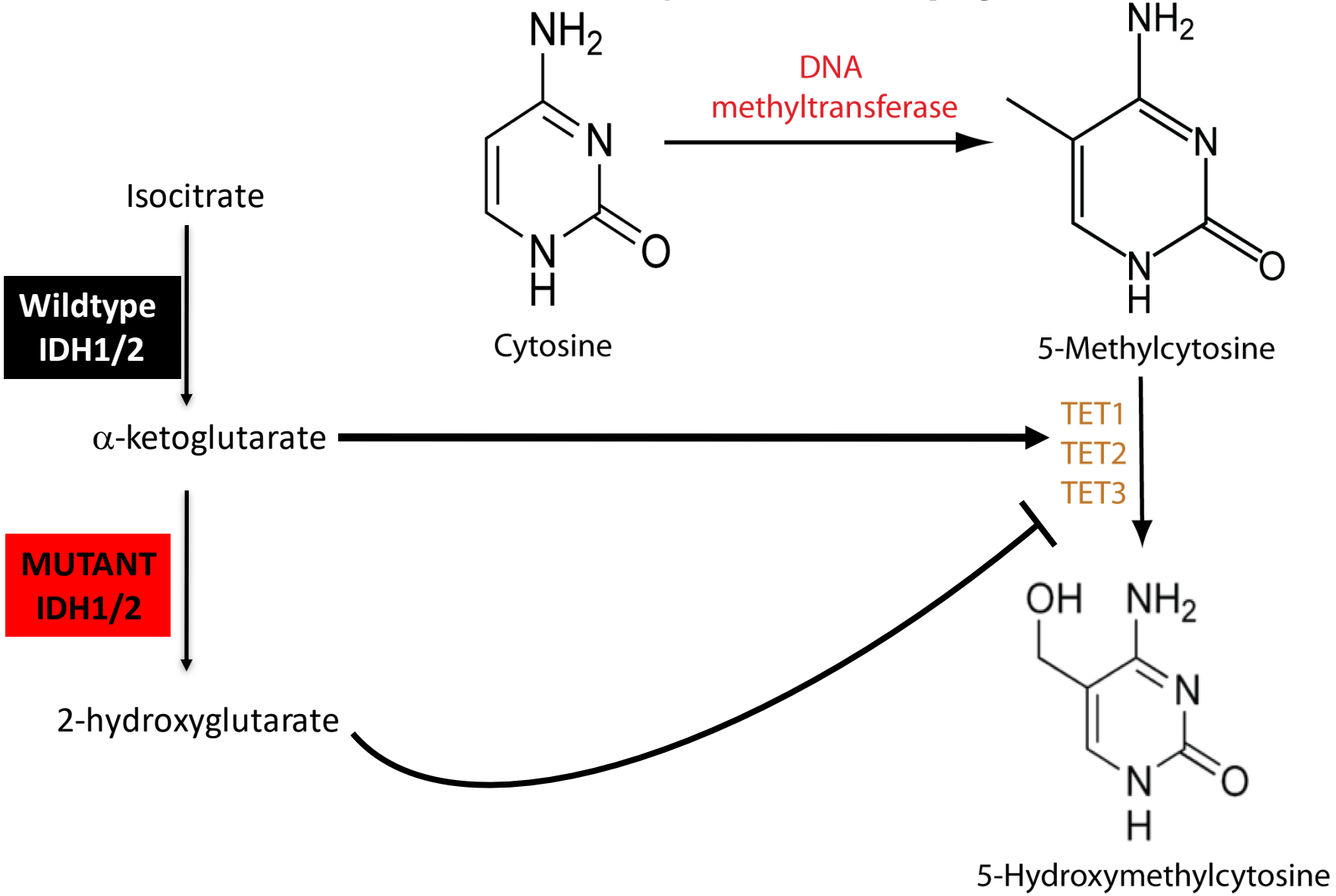
Hydroxy-Methylcytosine



Done using LC/MS – critical as not all methods distinguish mC from HmC

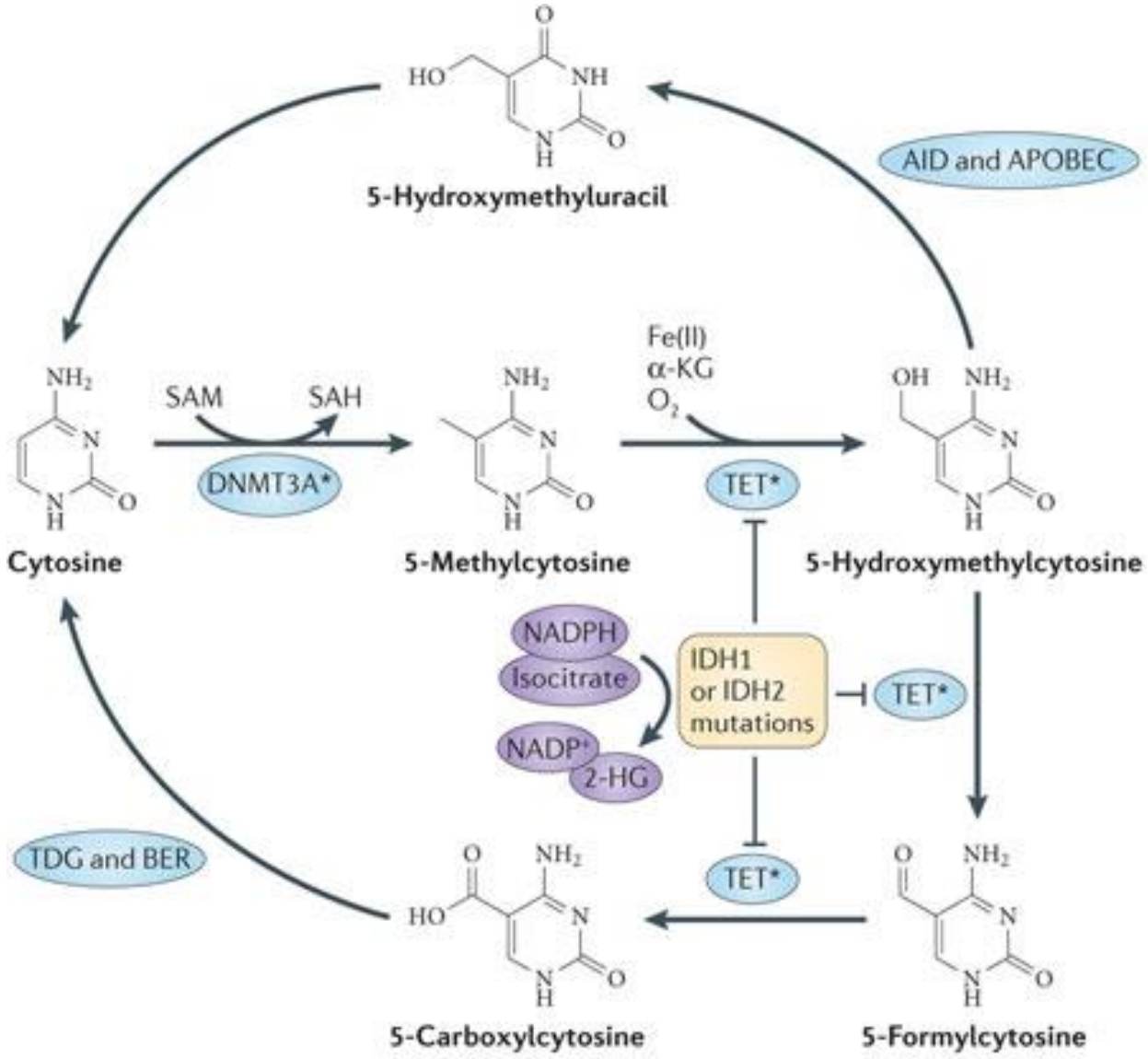
Ken Figueroa, Raajit Rampal, Lucy Godley

IDH1/2 and *TET2:* convergent mechanism of transformation by mutations in metabolic enzymes and epigenetic modifiers



How do these alleles contribute to hematopoietic transformation?

Mutations in Genes Which Regulate DNA Modifications in AML



- How do these alleles contribute to hematopoietic transformation?
- How do they affect the response to anti-leukemic therapies?

Clonal Hematopoiesis in Aging

- Nonrandom X-inactivation rations in blood cells of female elderly subjectd consistent with clonal outgrowth (Busque, Gilliland)
- Increased myeloid bias, risk of myeloid leukemias with increasing age
- Multiple Hypotheses for mechanism:
  - Caused by mutations conferring selective growth advantage in stem cells.
  - Stochastic clonal dominance secondary to stem cell depletion
  - Genetic trait
- Hypothesized clonal hematopoiesis due to somatic mutations-> exome sequencing of granulocyte/normal DNA on elderly subjects with clonal hematopoiesis

*Busque, Patel, *et al. Nature Genetics* 2012

Somatic TET2 mutations in clonal hematopoiesis

Nucleotide substitution[a]	Amino-acid substitution	Chromosome	Position
c.286_298delCGCAC AGTTAGTG	p.Arg96Asnfs*12	4	106155385
c.1330delA	p.Thr444Hisfs*6	4	106156429
c.1348delA	p.Lys450Lysfs*2	4	106156447
c.1547delC	p.Pro516Hisfs*16	4	106156646
c.1630C>T	p.Arg544*	4	106156729
c.3311_3312insAT	p.Phe1104Leufs*3	4	106158411
c.3991A>C	p.Thr1331Pro	4	106182952
c.5200delA	p.Met1734Leufs*11	4	106196867
c.5575insT	p.Ile1859tyrfs*16	4	106197239
c.5725G>T	p.Glu1909*	4	106197392

Clonal Hematopoiesis and Somatic Mutations

- Premalignant clonal state induced by somatic mutations in known leukemia genes
  - Mutations in TET2 in patients with clonal hematopoiesis (Busque et al. Nature Genetics 2012)
  - Mutations in DNMT3A, TET2, IDH1/2 in preleukemic stem cells (Jan *et al.* STM 2012, Shlush *et al.* Nature 2014)
  - Somatic mutations in hematopoietic cells of patients with solid tumors (Xie *et al.* Nature Medicine 2014) and in population based cohorts (Jaiswal/Ebert *et al.*, *McCarroll et al* NEJM)

- We now know that most adult, dividing tissues show evidence of somatic mutations and clonal expansion->most commonly in cancer initiating genes

- May represent the first frontier for effective malignancy prevention studies
  - Identify and intervene on patients with somatic mutations to prevent hematopoietic malignancies and other sequelae

Somatic Clonal Expansion is Common Across Different Tissues

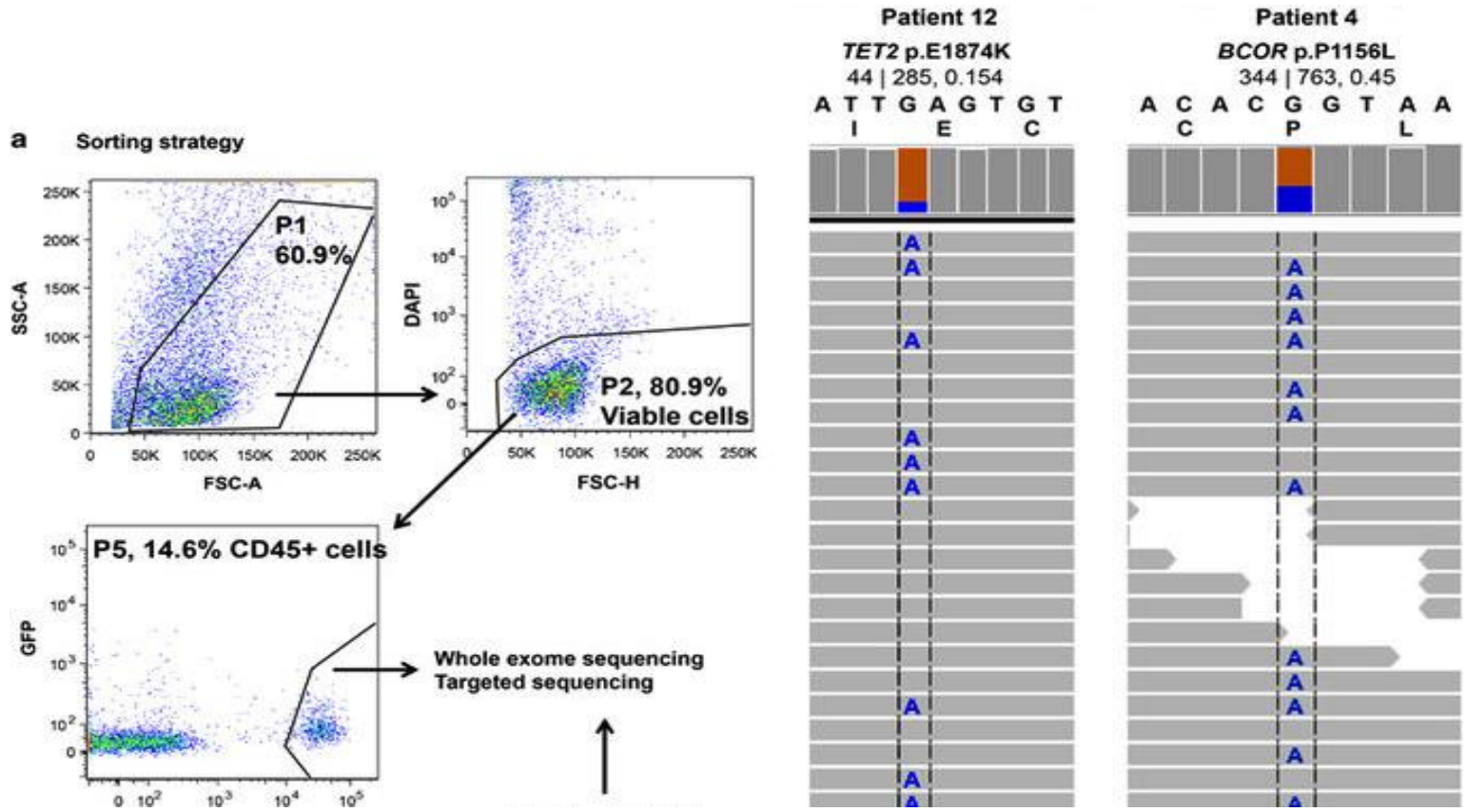
- Age dependent acquisition of somatic mutations has been identified in many dividing tissues
  - P53/other mutations in sun exposed skin (Jonason et al. PNAS 1996, Martincorena et al. Science 2015)
  - Notch mutations in normal esophagus (Yokoyama et al. Nature 2019)
  - Somatic mutations in normal liver->increased in cirrohosis (Brunner et al. Nature 2019)

- It is likely that all dividing tissues have a rate of somatic mutations, and that some of these will be in known/putative cancer driver/initiator genes->increase clonal fitness

- This has significant potential clinical implications
  - Diagnostics: mutations in “normal” tissue may not be informative to make a cancer diagnosis->may indicate cancer risk>diagnosis, whereas other mutations may be more linked to overt transformation
  - Circulating DNA tests may pick up pre-malignant clonal expansion->need properly designed studies to delineate potential clinical implications
  - May impact normal physiology in different organs independent of malignancy

- Clonal hematopoiesis
  - Circulating cells: easier to detect and study
  - Greater capacity to infiltrate different organs->implications for pathology in extra-hematopoietic sites
  - A “vanguard” for discovery science and translation in the field of clonal expansion/cancer risk

Another Twist in CH: Clonal Hematopoiesis and Epithelial Malignancies

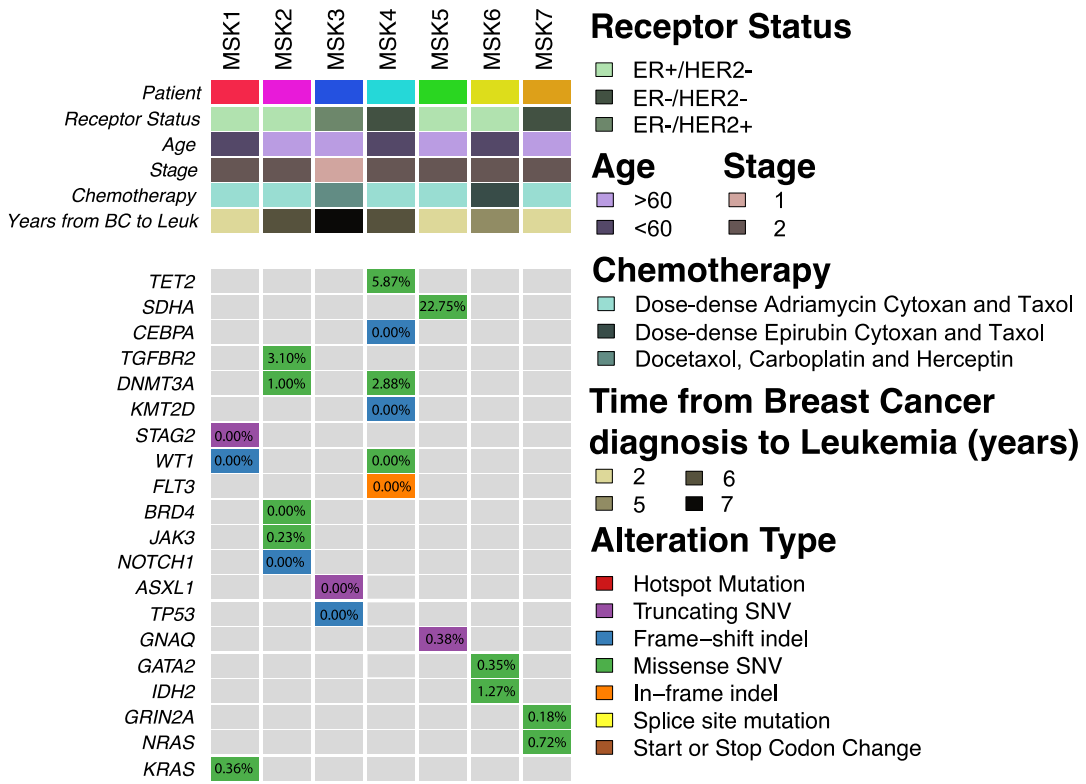


- Flow sorted infiltrating WBC from 15 patients undergoing surgical resection
- Identified somatic mutations in known cancer (leukemia genes) in 10/15 patients
- Associated with increased pathologic leukocyte infiltrates

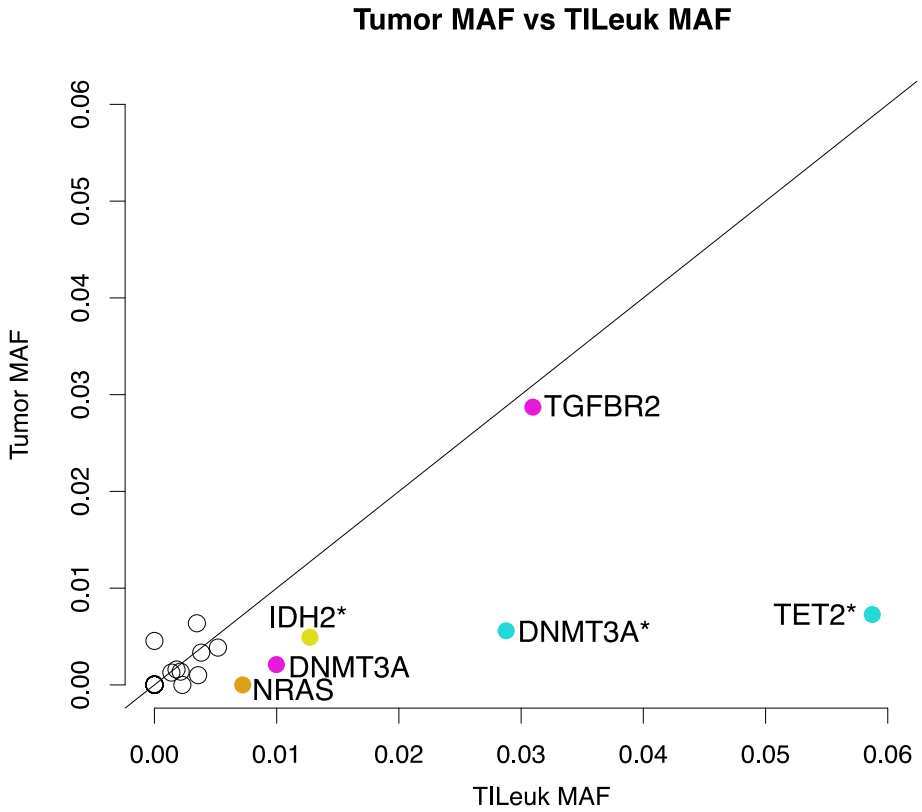
Kleppe, Comen, Norton et al, Nature Breast Cancer Journal 2015

Clonal Hematopoiesis and Epithelial Malignancies

A



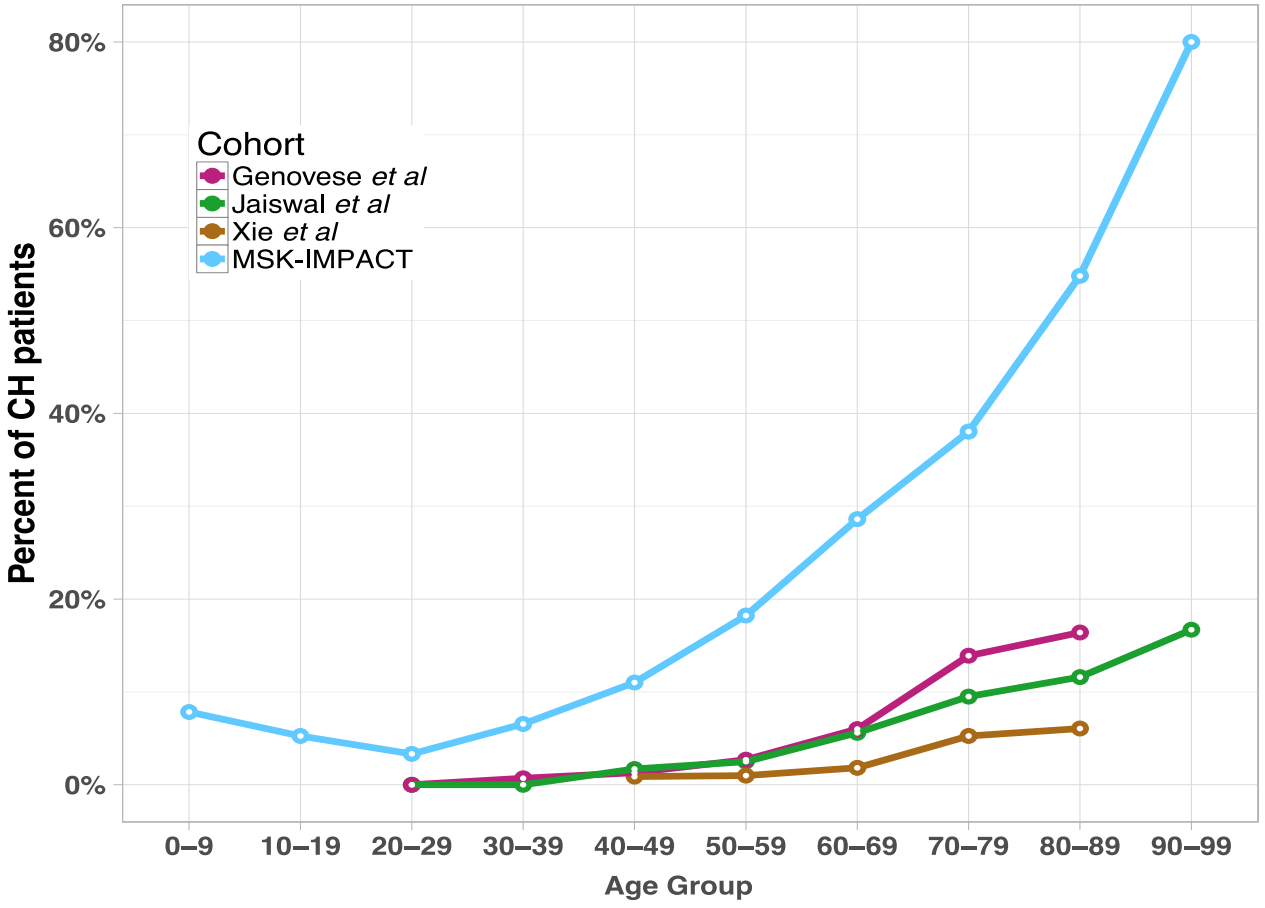
B

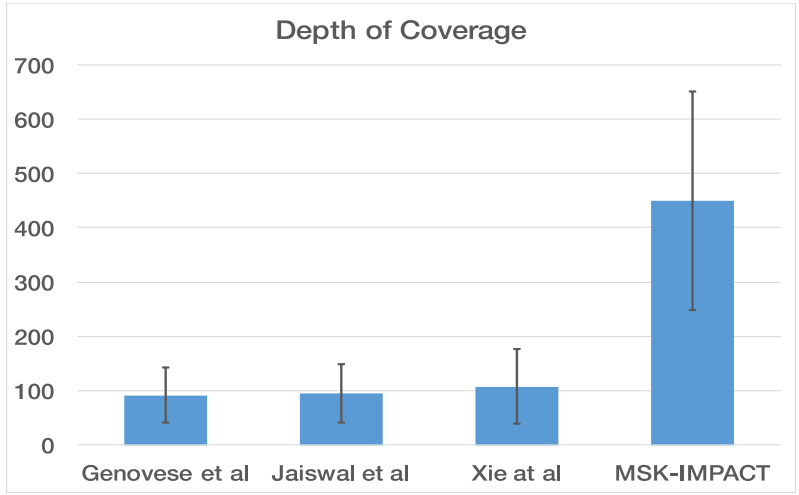


- Analysis of 7 breast cancer patients who subsequently developed MDS/AML
- 4 of 7 patients had leukemic mutations detectable in tumor-infiltrating leukocytes
- Were detectable as lower VAF alleles in tumor sample->early harbinger of secondary leukemia?

Comen, Bowman et al JNCI 2020

CH Prevalence in Solid Tumor Patients



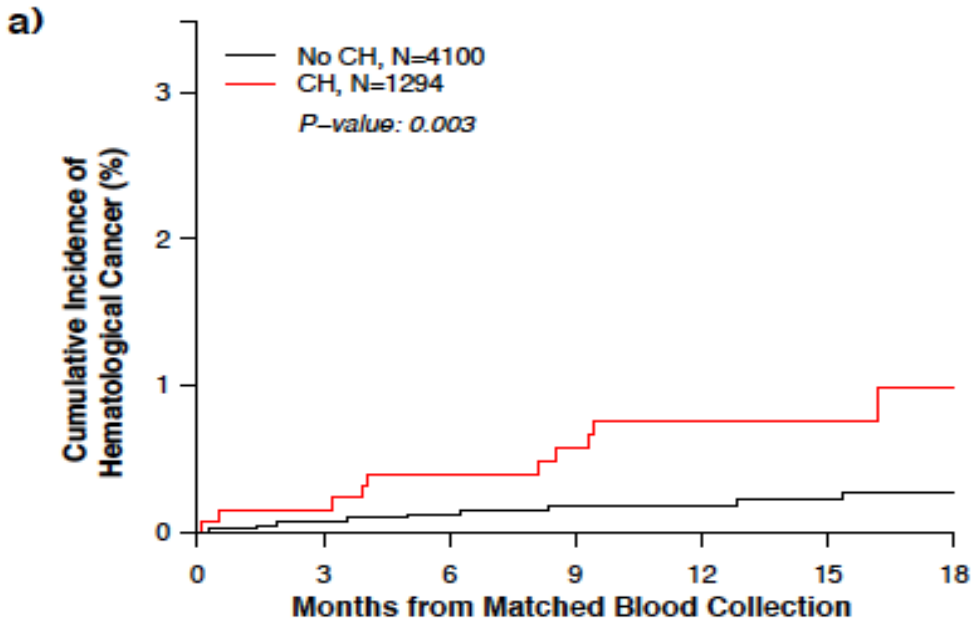


- Not clear if this is due to
  - increased coverage of our assay
  - ability to compare to matched tumor
  - true difference in CH incidence in solid tumor patients vs. healthy controls

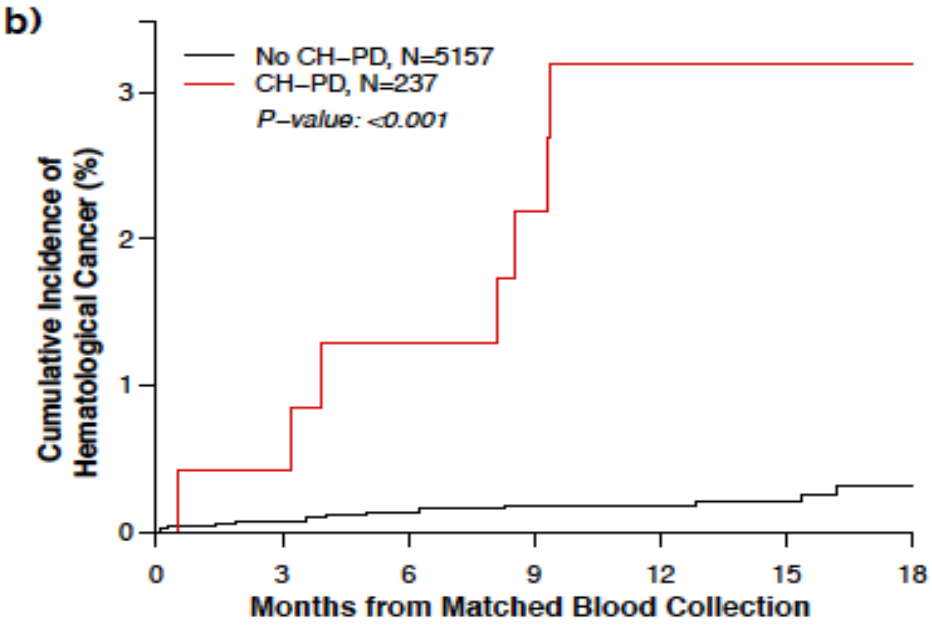
- Currently comparing incidence in cancer patients vs. controls using similar analysis

CH is associated with Decreased Overall Survival and Increased Risk of Blood Cancers

Patients with CH



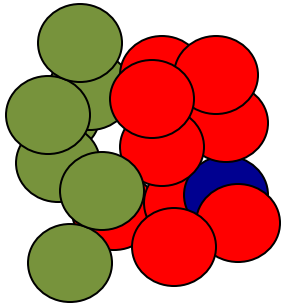
Patients with CH/PD



- Cause of death determined to be related to primary solid tumor in 98% of deaths
- Seen both for CH-PD (>10%) and CH overall

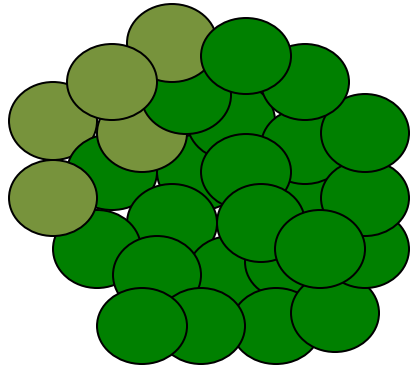
Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies

Clonal Hematopoiesis w/ Somatic Mutations DNMT3A, TET2, ASXL1, JAK2…



Acquisition of Additional mutations

Myeloid Malignancy

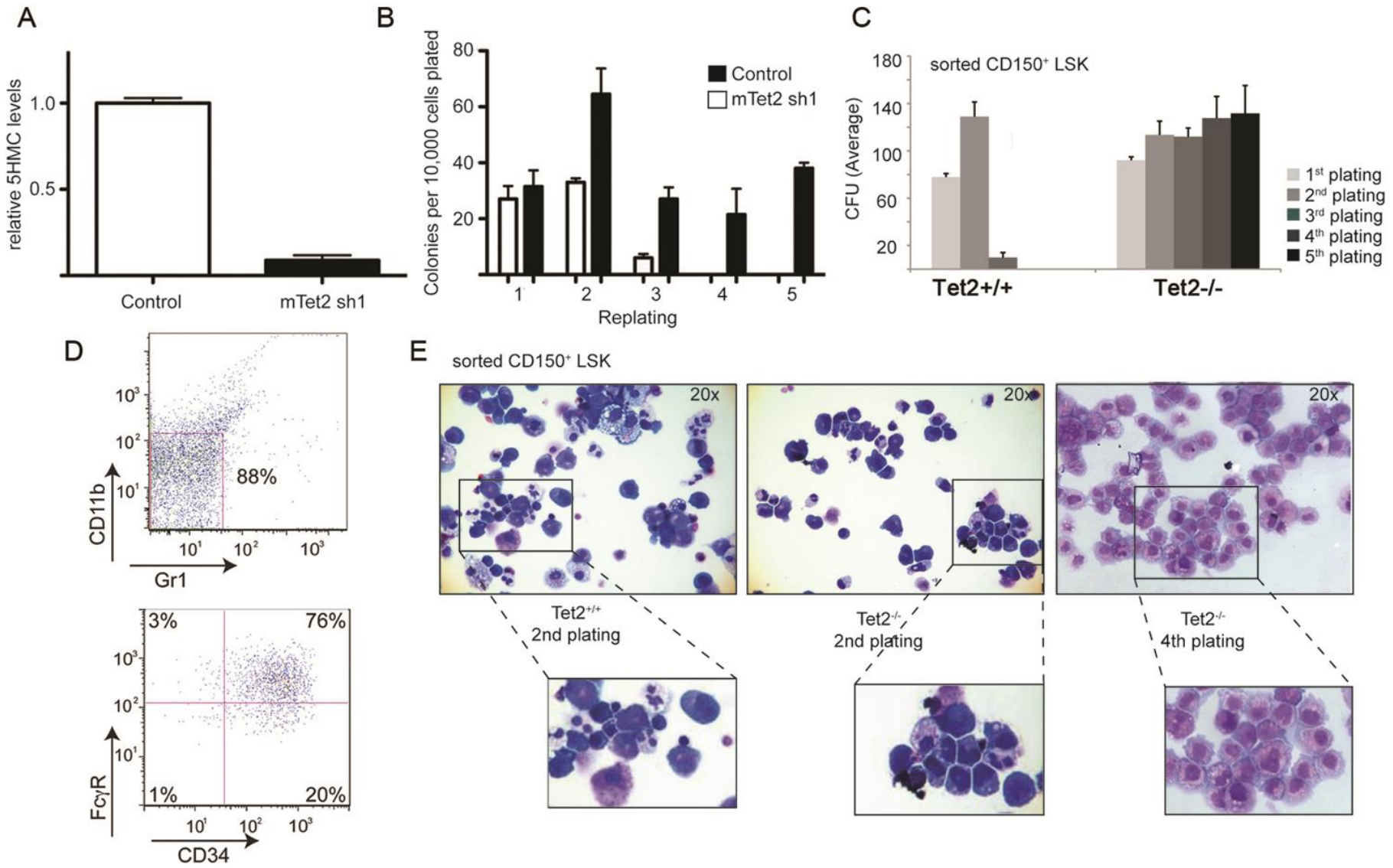


- What are the events which drive CH
- What is the implication of CH on clinical outcome, including cardiovascular disease and epithelial tumors
- How do we manage/follow patients who are found with CH in the clinical context

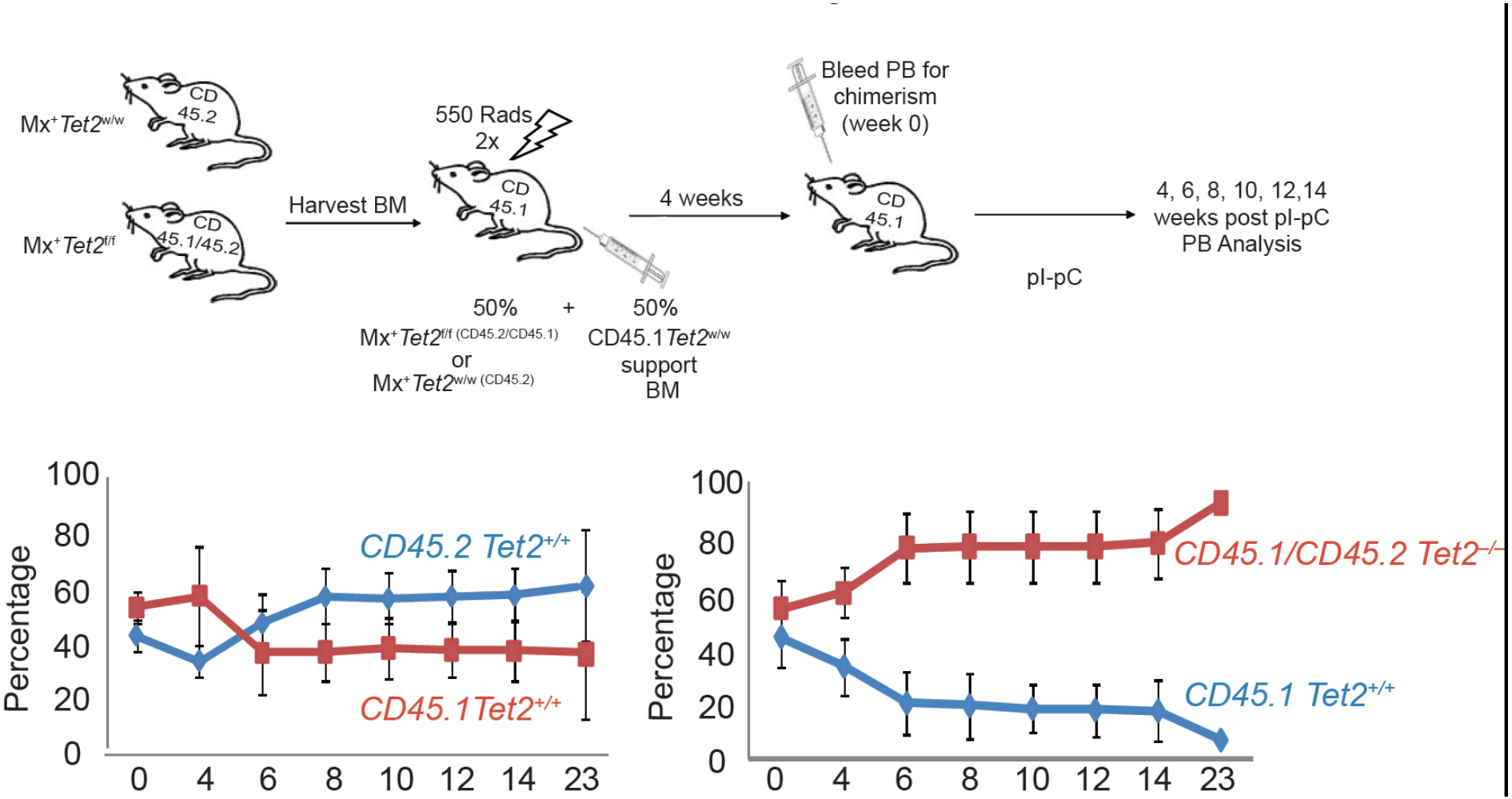
- What drives the acquisition of additional mutations (mutagenesis, selection)
- Does the stem/progenitor compartment which acquires additional mutations dictate risk of transformation

- Are CH mutations required for maintenance of the leukemic clone
- How do subsequent mutations cooperate with CH alleles to drive disease phenotype
- Are there therapeutic implications of targeting different mutant alleles including CH mutations

TET2 Deletion Leads to Serial Replating of Cells with a Progenitor Phenotype



TET2 Deletion Leads to Increased Competitive Transplantation in Vivo



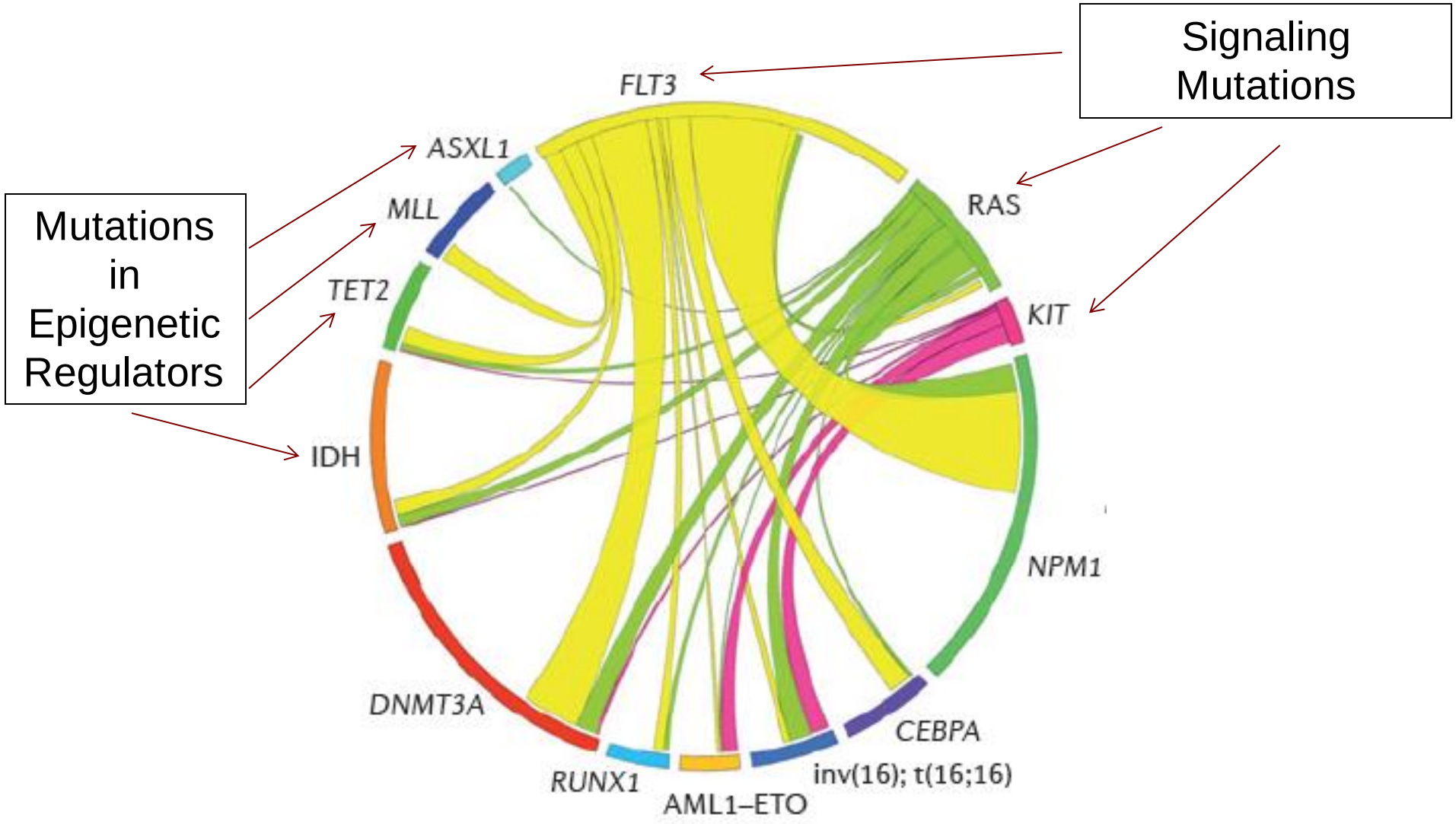
Kelly Moran/Linsey Reavie (Aifantis), Alan Shih/Omar Abdel-Wahab

Implications

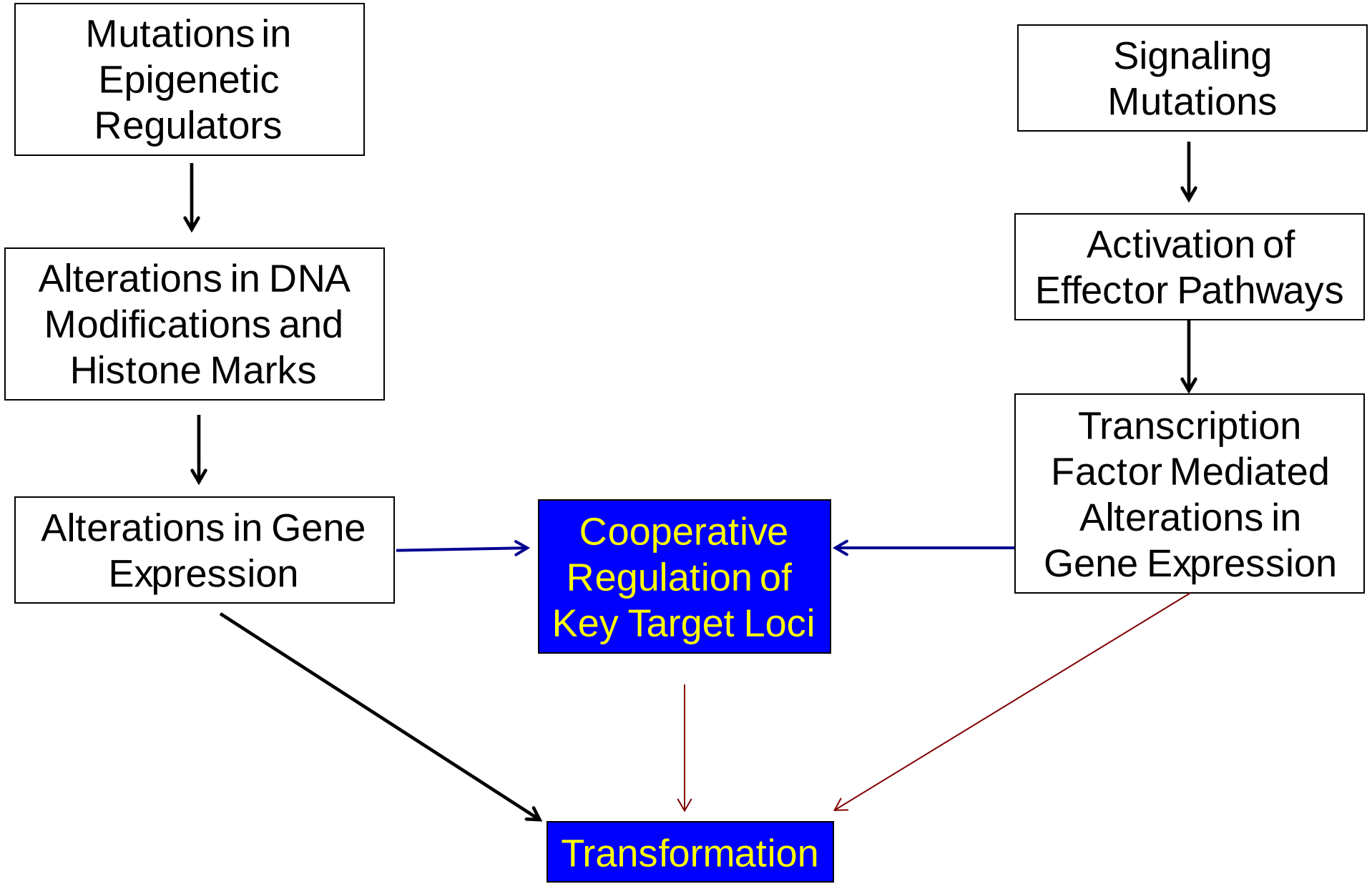
- Recurrent somatic mutations in TET2 are observed in patients with clonal hematopoiesis without overt disease
  - Consistent with a premalignant clonal state
  - Likely additional genes contribute to this process which may also contribute to pathogenesis of myeloid malignancies

- Prediction-> TET2 loss will increase competitive advantage of mutant stem cells over an extended period of time->predisposing cells to acquire additional alterations which result in MPN (JAK2), MDS (SF3B1), or AML (FLT3-ITD) based on specific genotype

Mutations in Epigenetic Regulators Commonly Co-Occur with Mutations in Signaling Effectors in AML



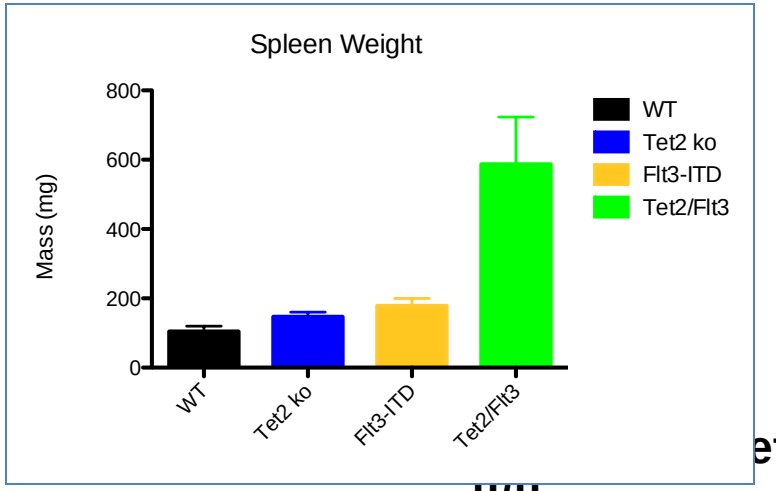
How do Mutations in Epigenetic Regulatory Proteins Cooperate with Mutations in Signaling Proteins

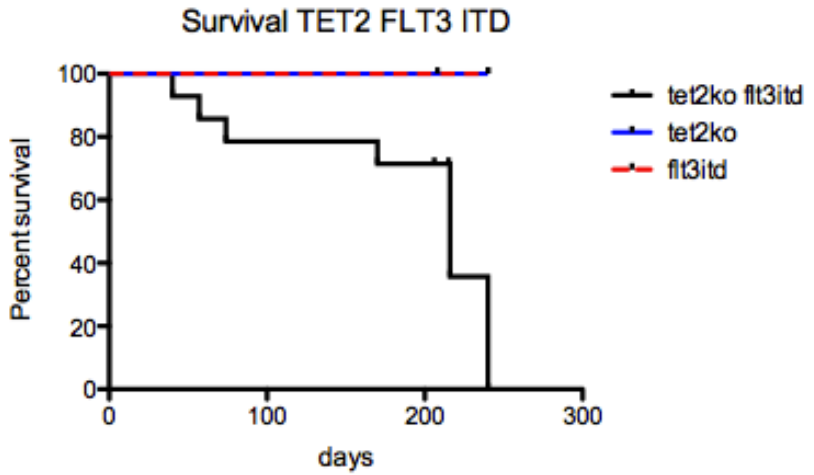


Mutational Cooperativity and Transformation

- How do mutations cooperate to induce AML?
  - Classical, additive model: mutations contribute distinct features to AML cells
  - Convergent model: mutations coordinately dysregulate specific, key target loci>result in cooperative effects on methylation/chromatin state and on gene expression
- Hypothesis: Epigenetic and signaling mutations have synergistic effects on epigenetic/transcriptional state which leads to leukemic transformation and is required for leukemia maintenance
- This has therapeutic importance: we now have drugs which target both classes of alleles
  - Need to understand how to combine (dose, schedule, context)

Modeling FLT3/TET2-induced AML

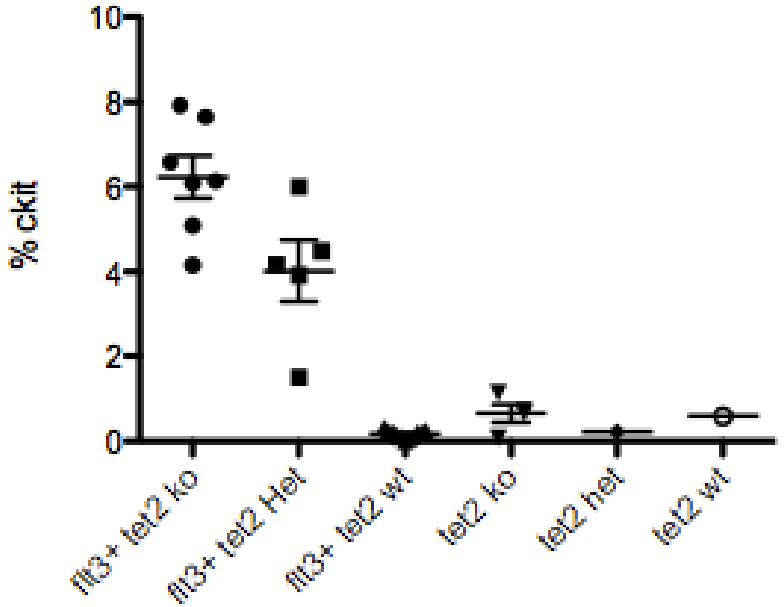


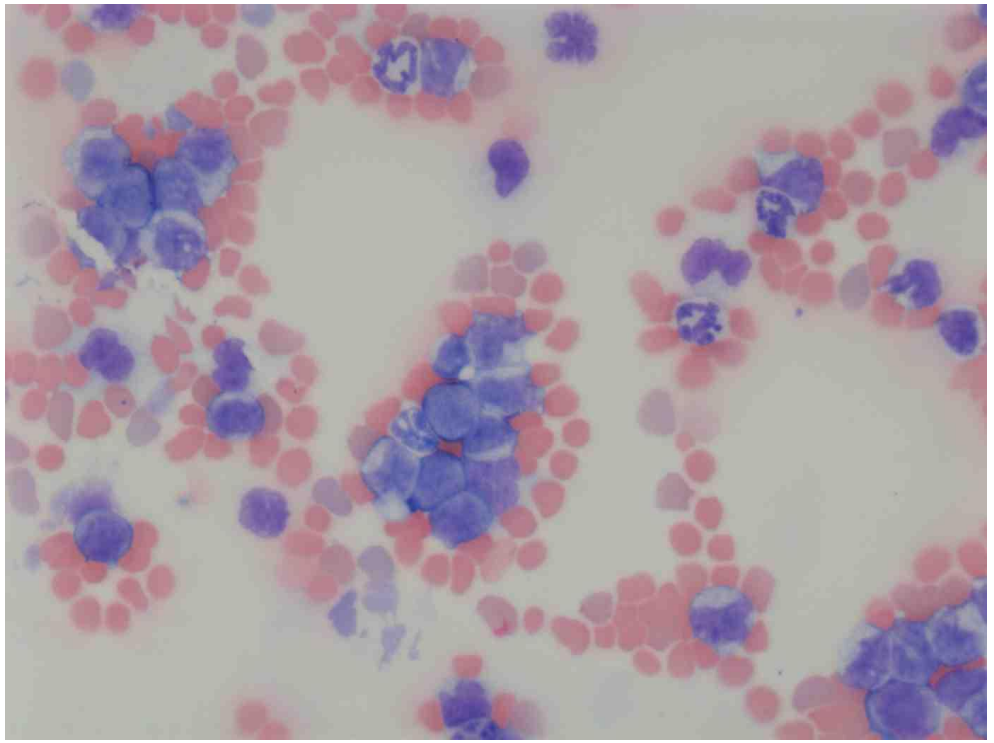


Vav cre Tet2 fl/fl + Flt3-ITD

*Shih *et al. Cancer Cell* 2015

AML in FLT3-ITD + TET2 Mice

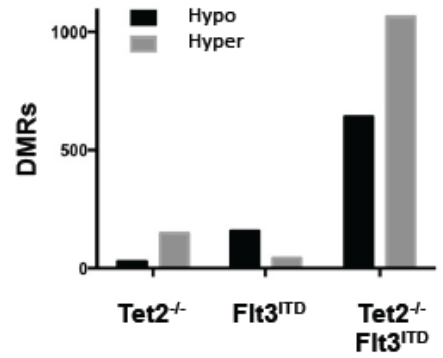


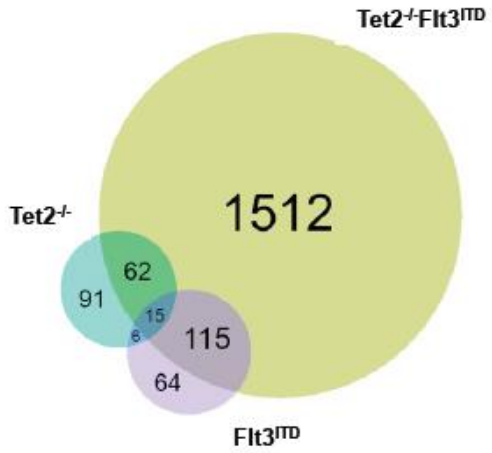


- Lethal, fully penetrant AML phenotype
- Resistant to chemotherapy, targeted therapies
- Defined leukemia propagating (stem) cell population: CD48+CD150-LSK

Shih *et al. Cancer Cell* 2015

FLT3/TET2 Mutant AML Stem Cells: Marked Alterations in DNA Methylation Not Seen with TET2 Loss Alone





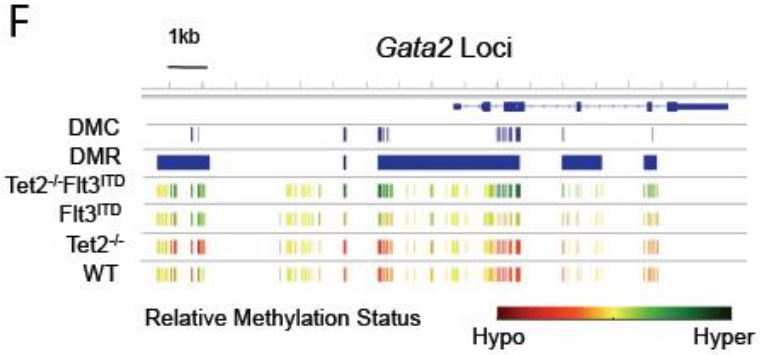
- 1789 loci wth differential methylation, much more than in TET2-deficient mice
- Majority of differentially methylated loci are hypermethylated and transcriptionally silenced

*Yanwen Jiang/Cem Maydan (Melnick), Alan Shih

TET2 loss/mutation and FLT3-ITD Have Synergistic Effects on DNA Methylation

GeneSymbol	Hyper			Hypo			log2FC
	T2F3	TET2	FLT3	T2F3	TET2	FLT3	
MN1	91	3	0	0	0	0	-2.24
HOXA3	51	0	1	0	3	1	-2.19
GATA2	39	0	12	0	2	1	-2.99
ZFPM1	27	0	2	0	0	0	-2.85
GM53	23	0	0	0	0	1	6.92
LTBP3	22	0	0	0	0	0	-1.68
GSE1	23	0	4	2	2	0	-1.42
FAM110A	17	0	0	0	0	0	-1.26
RAPGEF3	15	0	4	0	0	0	-2.88
TRIM47	14	0	3	0	1	0	-3.54
RUNX3	14	0	0	1	0	1	-1.05
GDF10	12	0	1	0	0	0	9.23
ADCY9	12	0	0	0	0	0	-1.64
HOXB3	12	0	0	0	0	0	-2.11

F

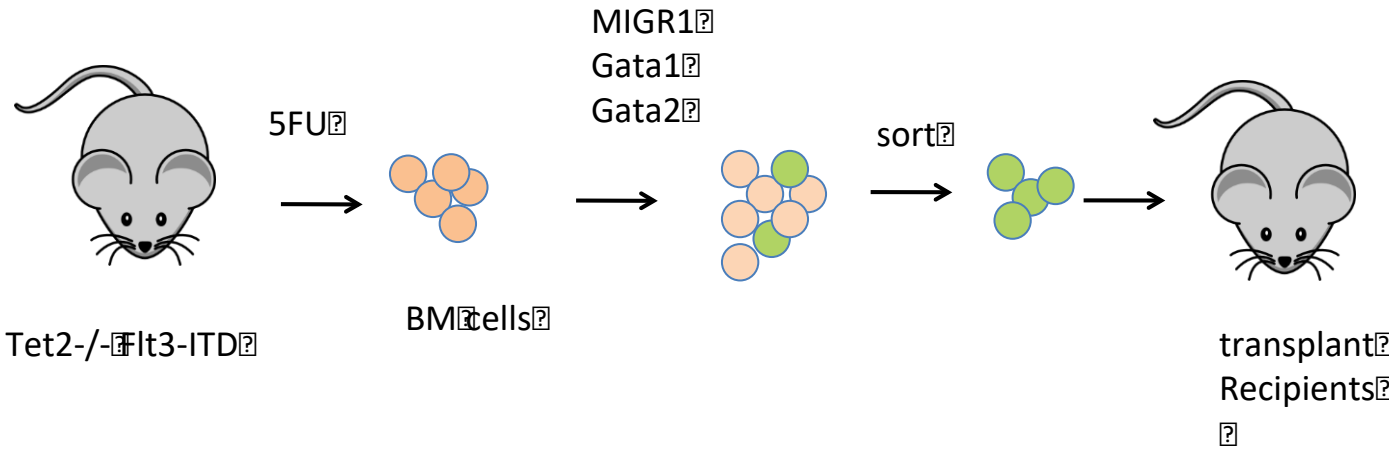


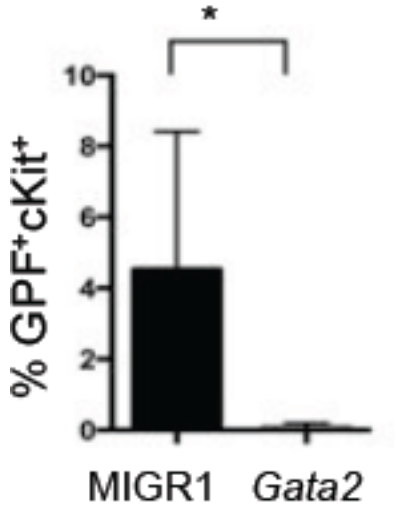
- Strong association between synergistic methylation changes and alterations in gene expression
- Most of these loci are also altered in TET2-mutant AML
- GATA2 mutations seen in AMLs->not in cases with TET2 mutations

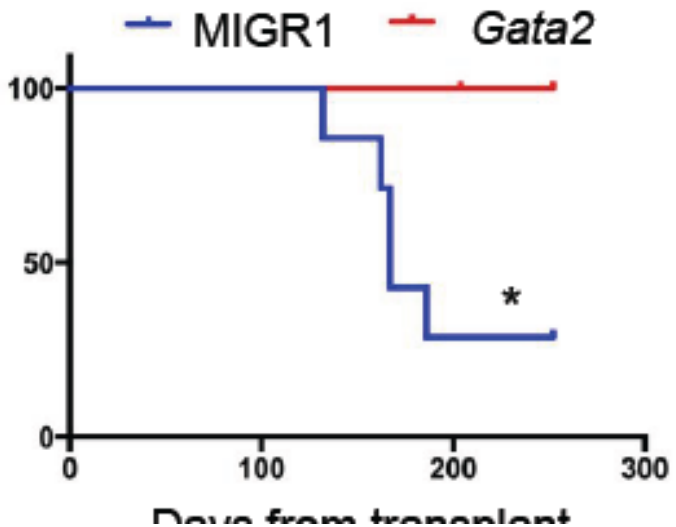
LSC-specific promoter methylation at GATA2 locus seen in TET2/FLT3 mutant cells (human and mouse)

*Yanwen Jiang/Cem Maydan (Melnick), Alan Shih

Rexpression of GATA2 abrogates in vivo transformation of FLT3/TET2-mutant AML cells

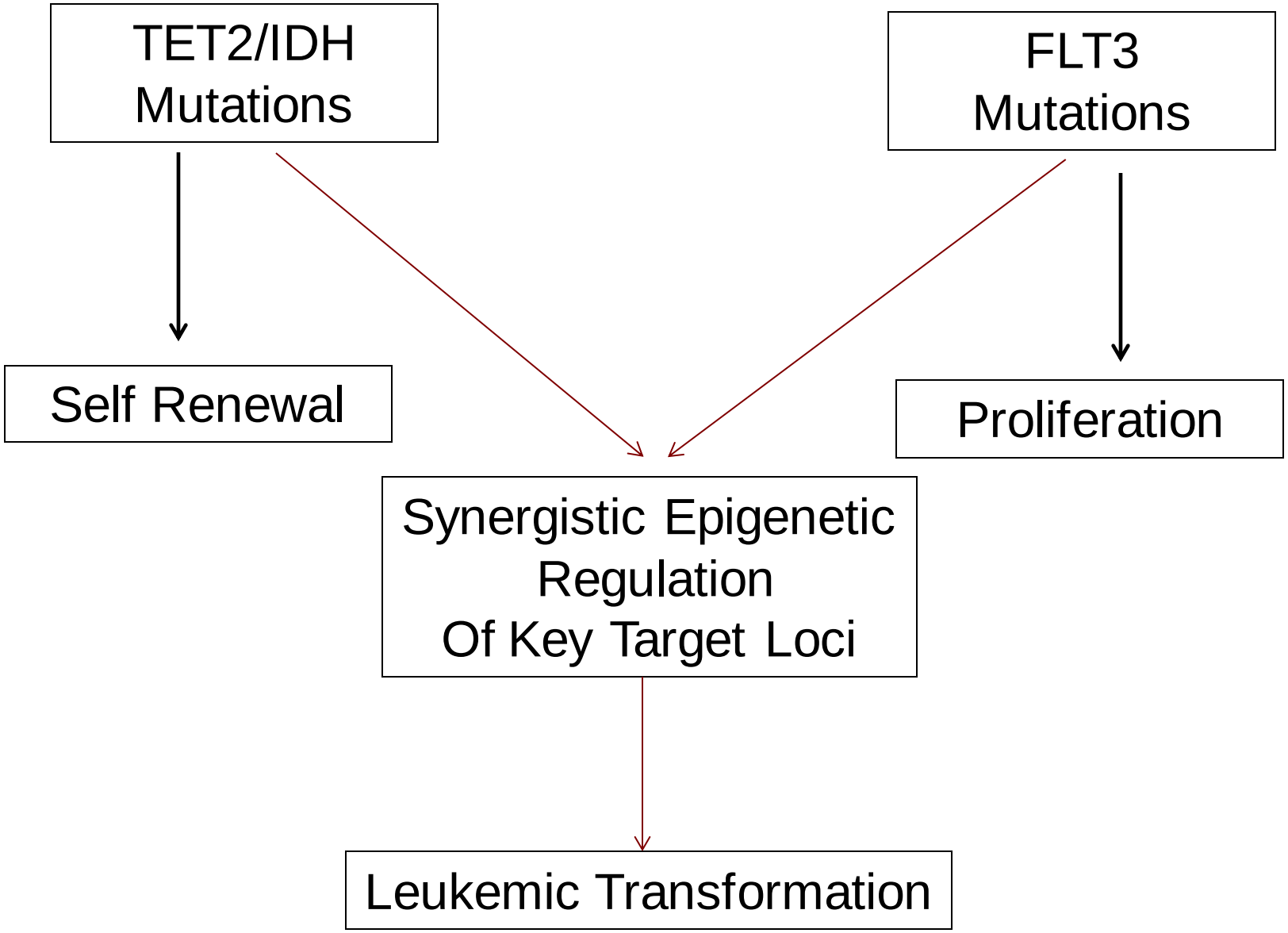






See in vivo differentiation of AML cells expressing GATA1/2 followed by disappearance of leukemic clone

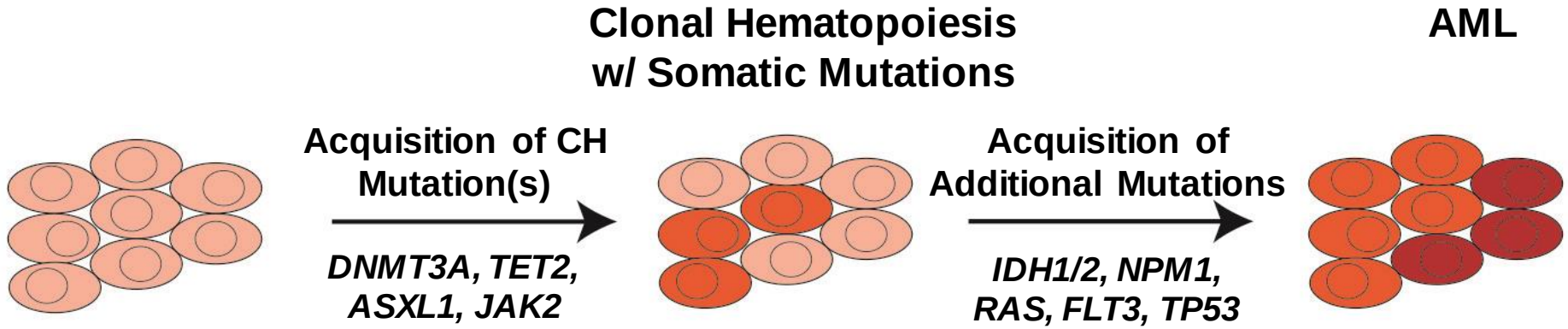
Convergent Transformation by Cooperating Disease Alleles in AML



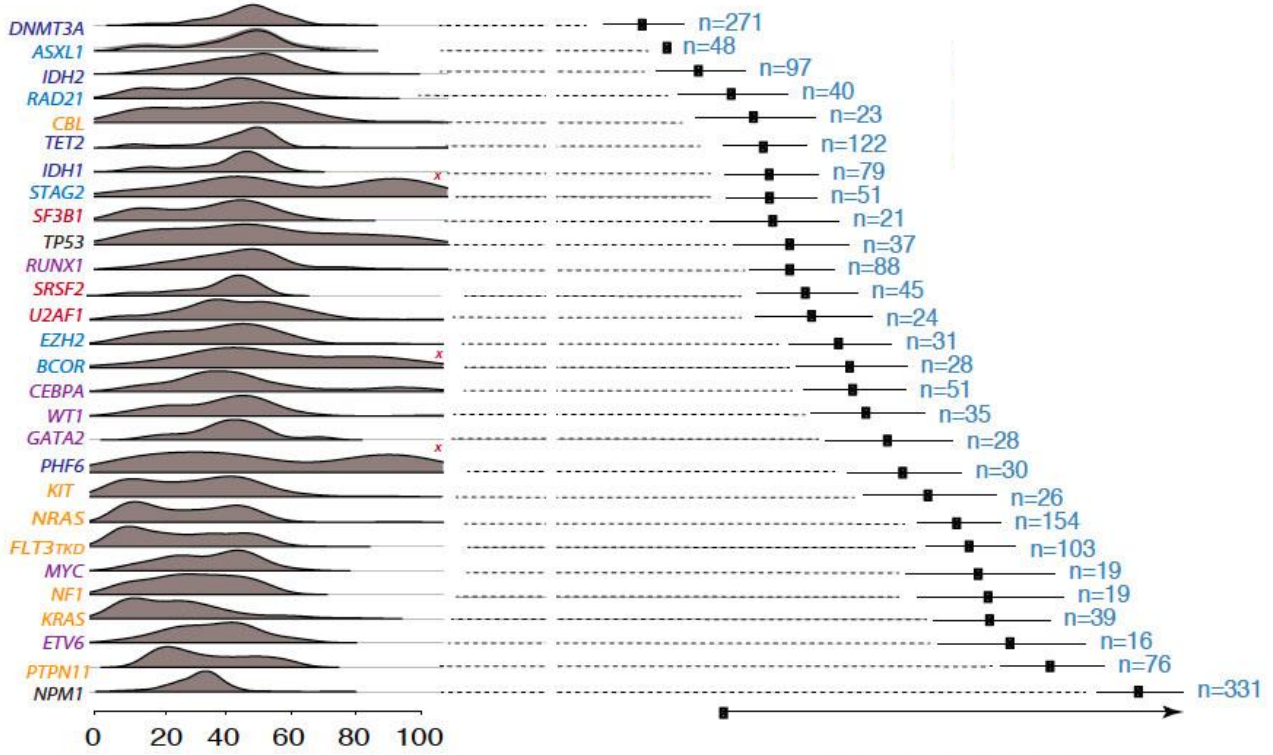
Classical model of carcinogenesis

- Candidate gene studies and mapping of LOH allowed Vogelstein and colleagues to identify a series of somatic events which occur during colorectal tumorigenesis
- But do all cancers follow such a carefully orchestrated model?
- In many cases the precursor/sequential lesions are not known, or easily accessible
- Alternative – catalog the set of genomic lesions in a full-flown cancer and “work back”

Bulk Sequencing suggests Stepwise Progression to AML but Cannot Elucidate Clonal Architecture or Clonal Evolution



- **Higher VAF= Earlier mutation**
- Order of Mutation inferred by variant allele frequency
- First mutations usually epigenetic modifiers (*TET2;DNMT3A;IDH1/2*)
- Later mutations (*NPM1;RAS;FLT3*) lead to progression to AML



Papaemmanuil, E. et al (2016) NEJM

Elucidating Clonal Evolution During Myeloid Transformation

- Can we better delineate the sequential acquisition of somatic mutations which induce progression from normal stem progenitors->CH->myeloid malignancies
- Can we understand what are the key genetic events, including co-occurring mutations, which promote clonal expansion and myeloid transformation
- Are there different genetic/evolutionary trajectories to AML based on initiating/cooperating mutations
- How does the milieu of clones change during leukemic transformation
- Can specific genetic alterations/mutational combinations be linked to genotype-specific phenotypes and therapeutic dependencies

Single Cell DNA Sequencing of CH/AML

(Miles, Bowman *et al Nature* Nov 2020)

- **Custom single cell DNA sequencing panel:**
  - 109 amplicons
  - 31 genes frequently mutated in MPN/MDS/AML
  - Tiles *DNMT3A* and *TET2*

- **17 Patient Samples** sequenced with **scDNA+Protein** platform:
  - CD3
  - CD11b
  - CD19
  - CD34
  - CD38
  - CD45RA
  - CD90

Characteristic	Value
Individual Patients	123
Individual Samples	146
Age at Sample Collection (yr)	66.4 ± 13.0
Sex	
Male	63 (51.2)
Female	60 (48.8)
Diagnosis at Sample Collection	
CH	14 (11.3)
MPN	14 (11.3)
AML	91 (74.0)
Newly Diagnosed	25 (20.3)
Newly Transformed	11 (8.9)
Relapsed/Refractory	56 (45.5)
tAML	4 (3.2)
sAML	18 (14.6)
Other (MRD+CR/MRD-CR)	3 (2.4)
Other (MDS, CMML)	4 (3.2)

Custom Platform

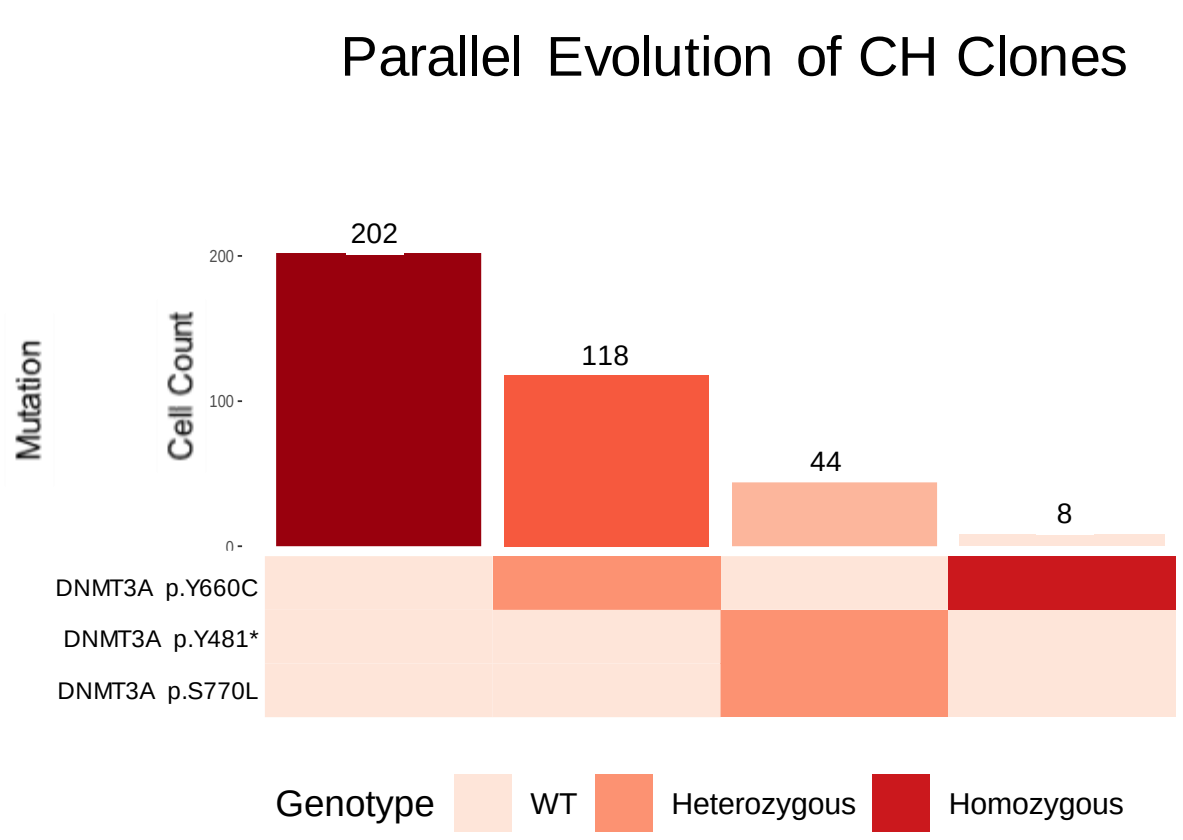
ASXL1	FLT3	NPM1	SF3B1
ATM	GATA2	NRAS	SRSF2
BRAF	IDH1	PHF6	STAG2
CALR	IDH2	PPM1D	TET2
CBL	JAK2	PTPN11	TP53
CHEK2	KIT	RAD21	U2AF1
DNMT3A	KRAS	RUNX1	WT1
EZH2	MPL	SETBP1	

Linde Miles

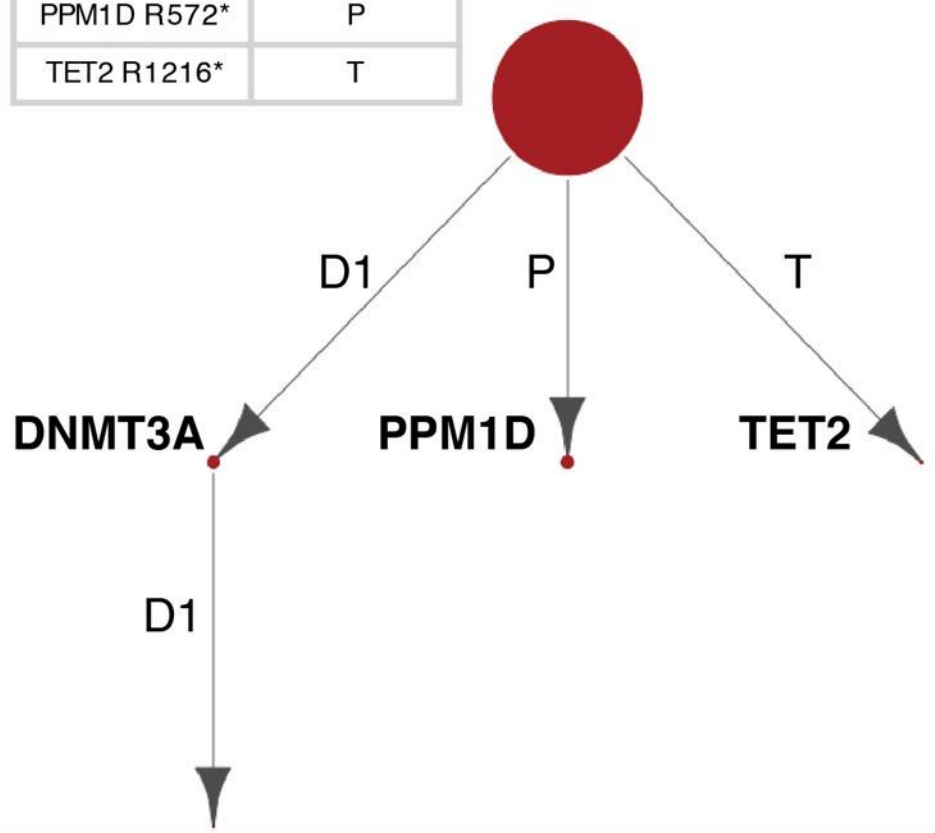
Bobby Bowman

Similar work from Koichi Takahashi (Nature Communications 2020)

Clonal Hematopoiesis: Parallel Evolution of Genetically Distinct Mutant Clones

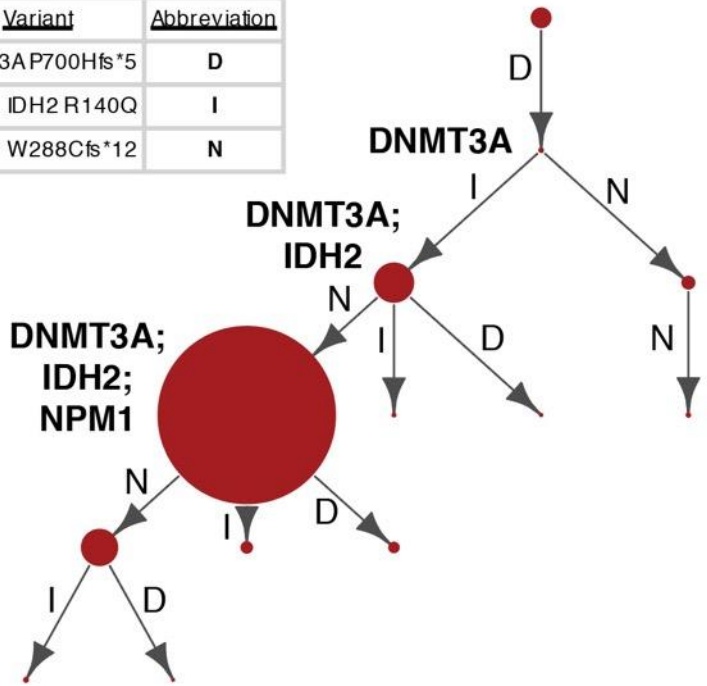


Variant	Abbreviation
DNMT3A Y359*	D
PPM1D R572*	P
TET2 R1216*	T

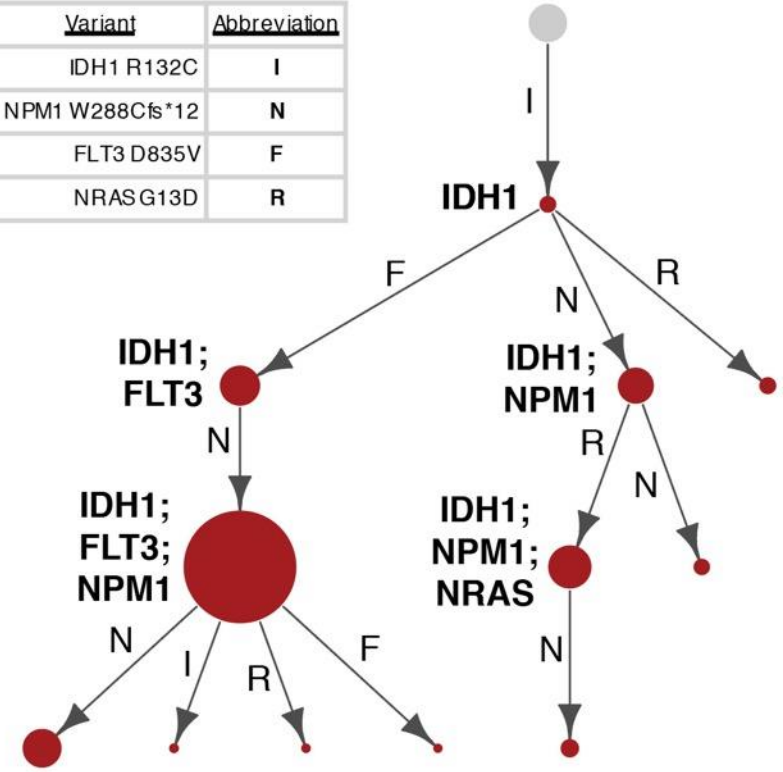


Defining Clonal Trajectories in AML

Variant	Abbreviation
DNMT3A P700Hfs*5	D
IDH2 R140Q	I
NPM1 W288Cfs*12	N

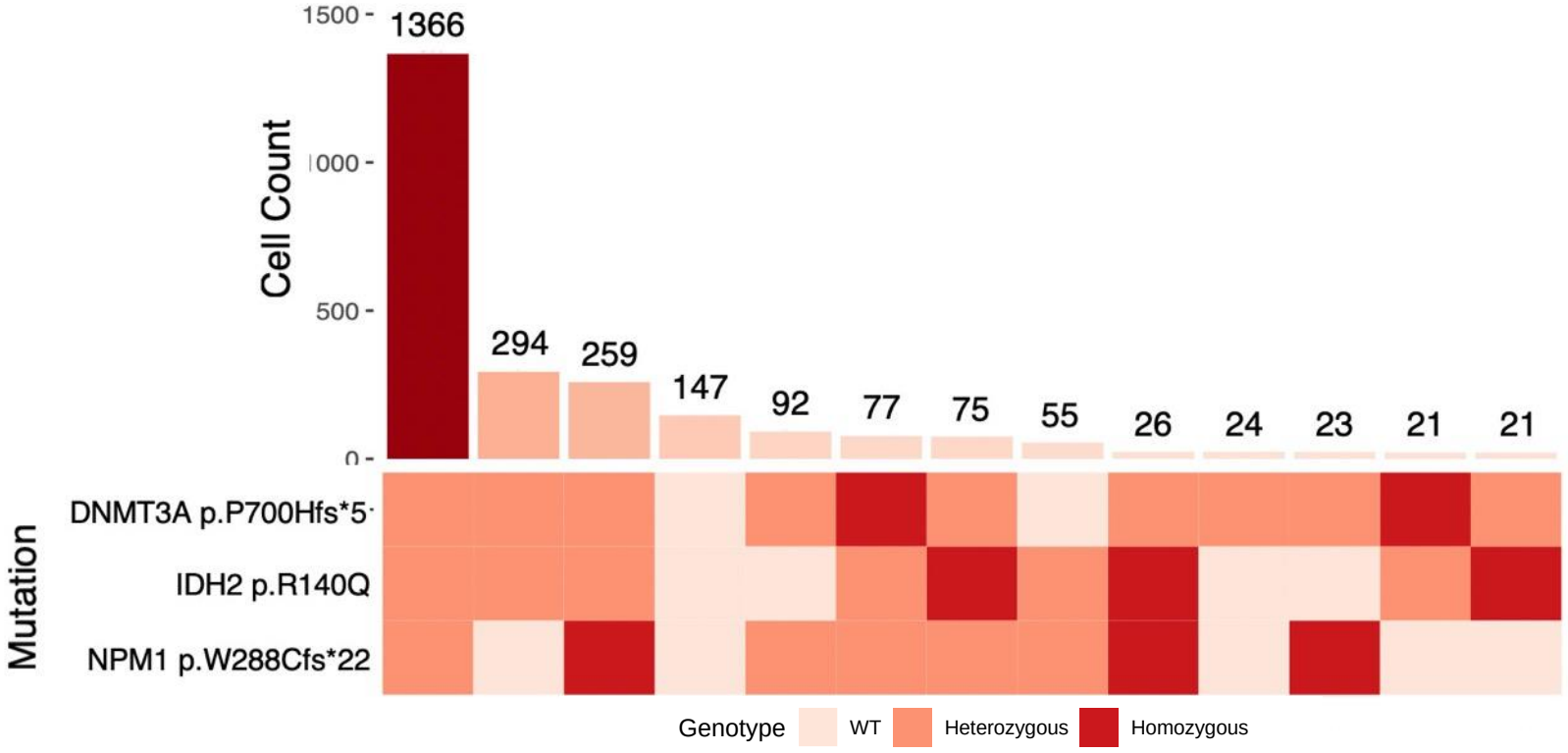


Variant	Abbreviation
IDH1 R132C	I
NPM1 W288Cfs*12	N
FLT3 D835V	F
NRAS G13D	R



- Used Markov decision process to generate genetic trajectories in each sample
- Determine the optimal/likely initiating mutation and subsequent trajectory

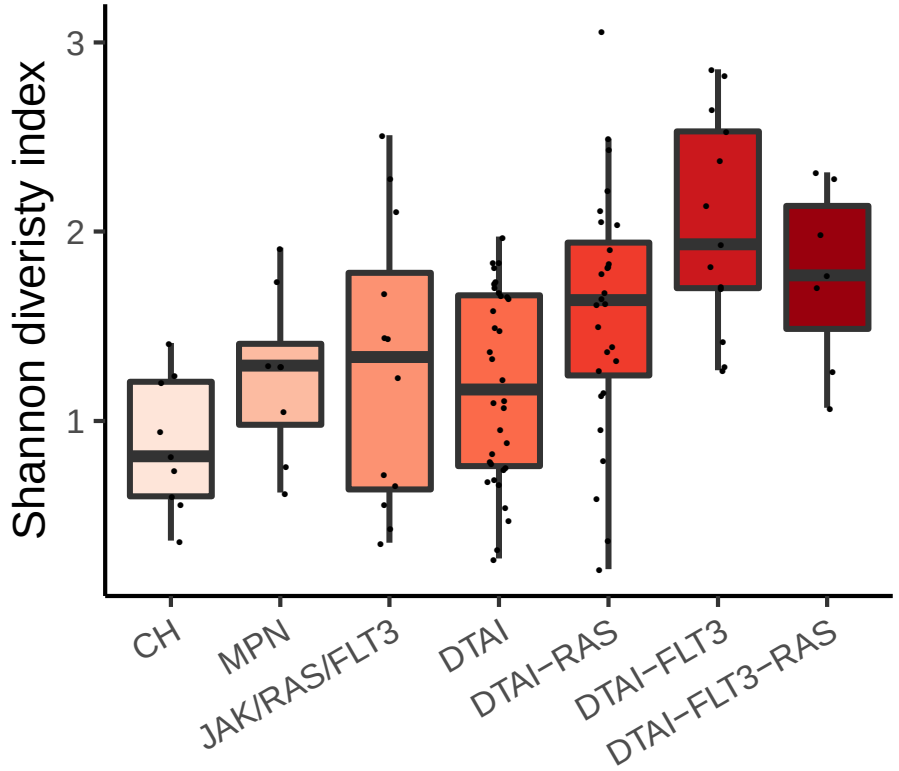
AML: Clonal Expansion/Dominance

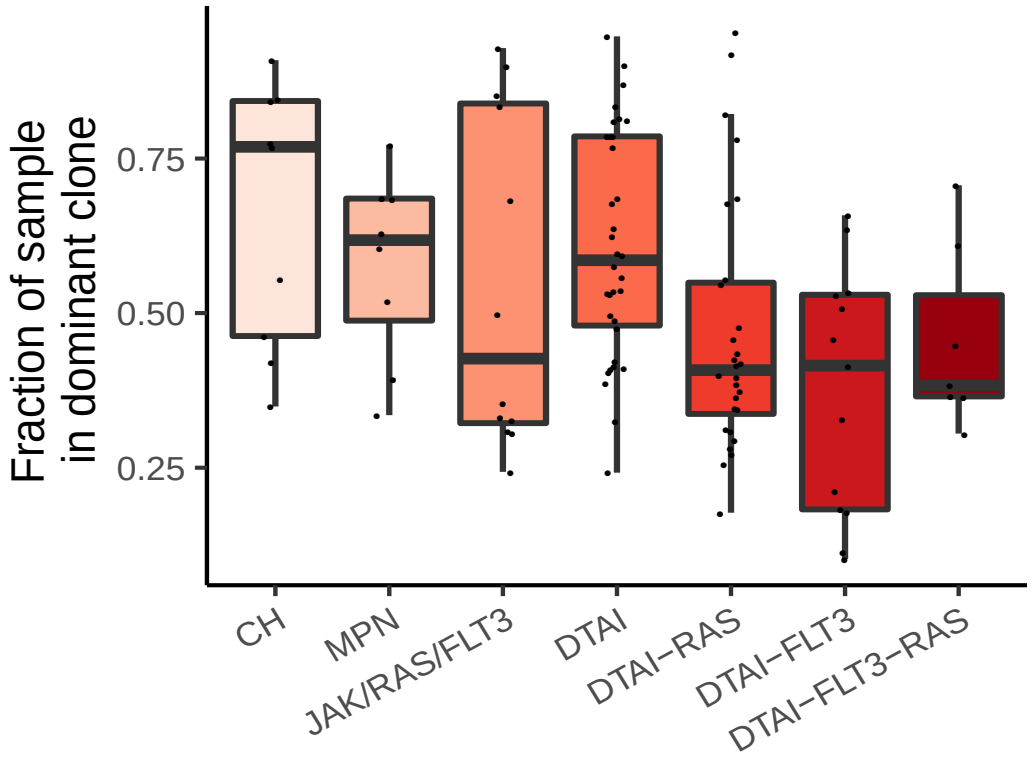


- 86% of the AML samples had 1 (75%) or 2 (11%) clones that accounted for more than 30% of the cells

- Suggests that there are specific mutational combinations which lead to competitive advantage/increased fitness
    - Enhanced proliferation/self-renewal compared to other clones
    - Cell non-autonomous suppression of other clones, including pre-leukemic clones

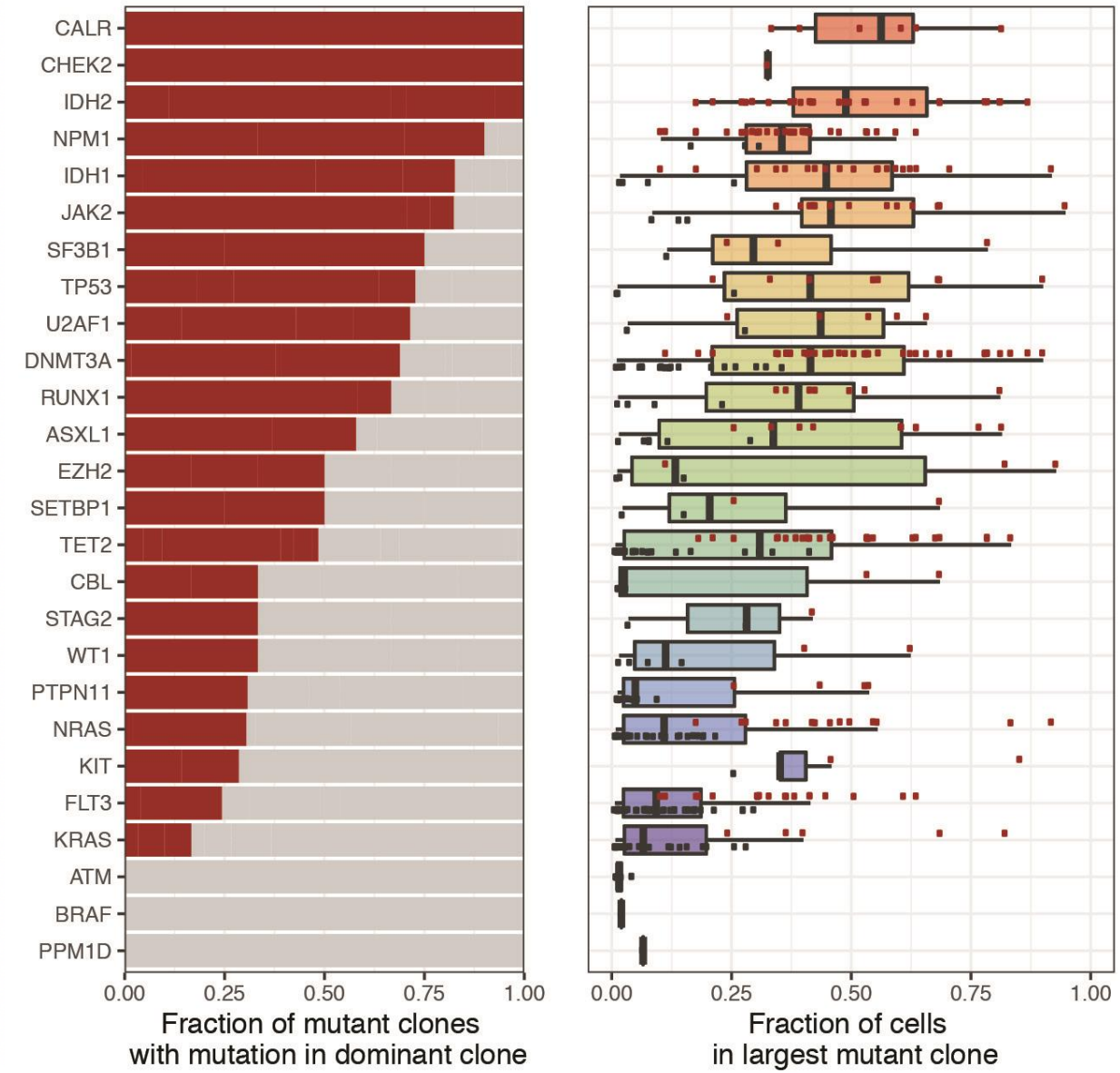
Disease Progression: Increased Clonal Diversity, Decreased Size of Dominant Clone





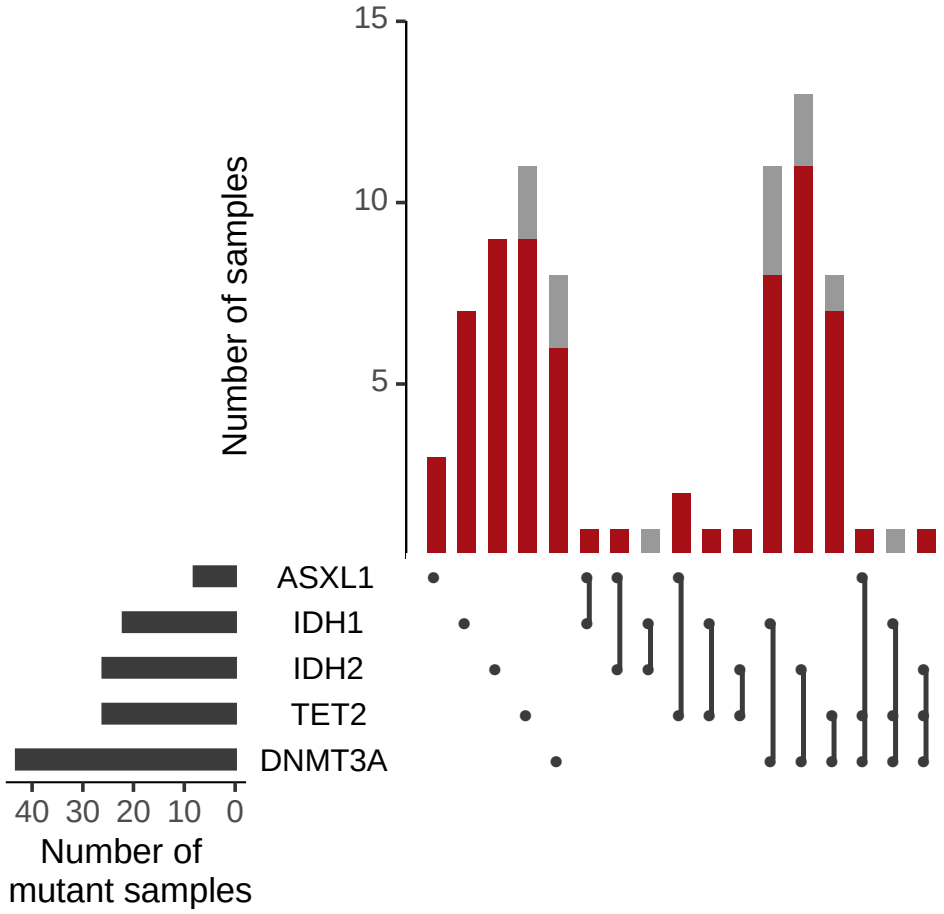
- Clonal diversity increases more substantively than mutational complexity
- Size of the dominant clone decreases with disease progression
- However, this is not associated with increased mutational burden in the largest/dominant clone
- Do specific mutations have distinct roles in establishing clonal dominance

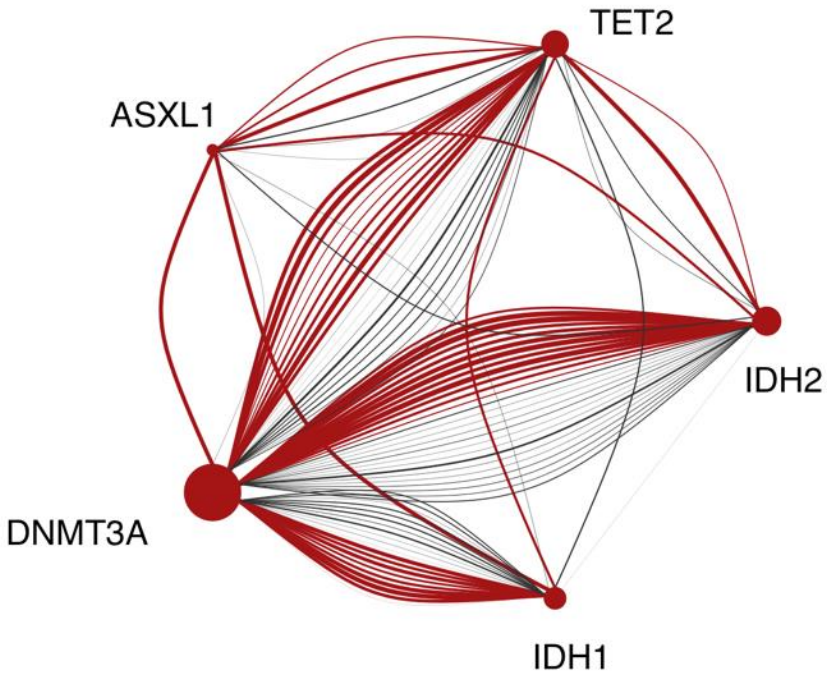
Mutations in Different Genes Are Differentially Represented in the Dominant Clone



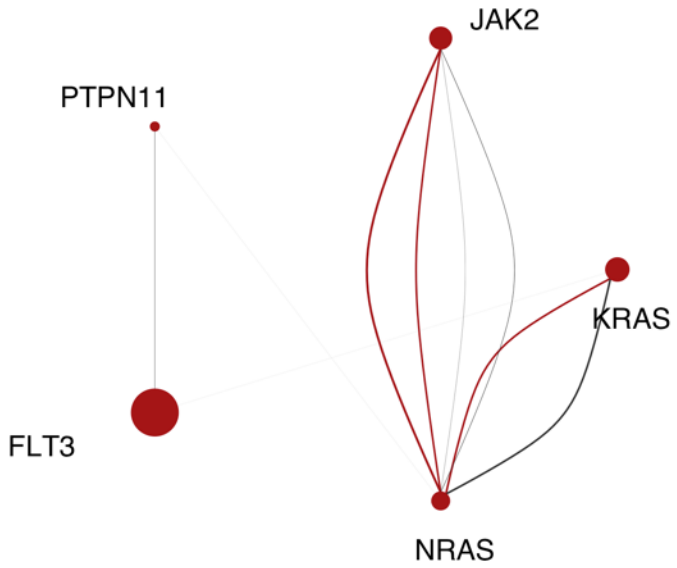
- Mutations in disease-defining genes (JAK2, CALR, NPM1c) are almost always in dominant clone
- Mutations in AML signaling effectors (FLT3, RAS) are rarely in the dominant clone
- Mutations in epigenetic regulators can be in dominant clone or only in subclones (TET2)
- What about mutational combinations?

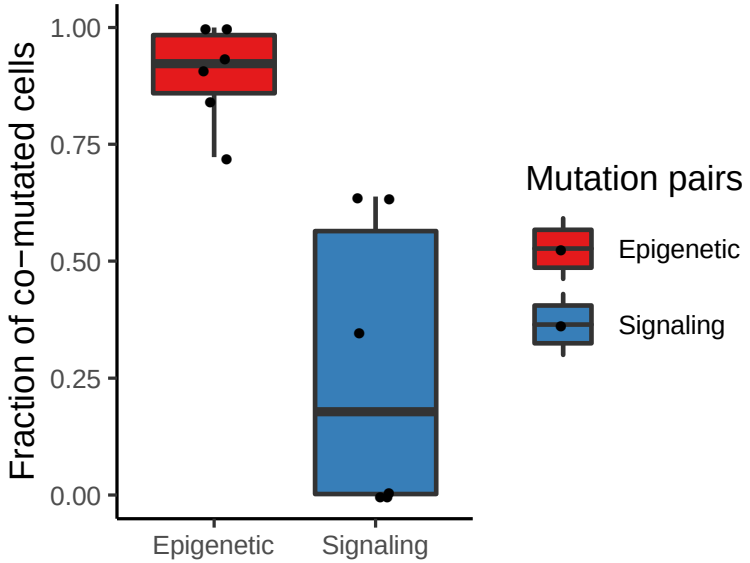
Epigenetic Modifier Mutations Co-Occur in the Dominant Leukemic Clone

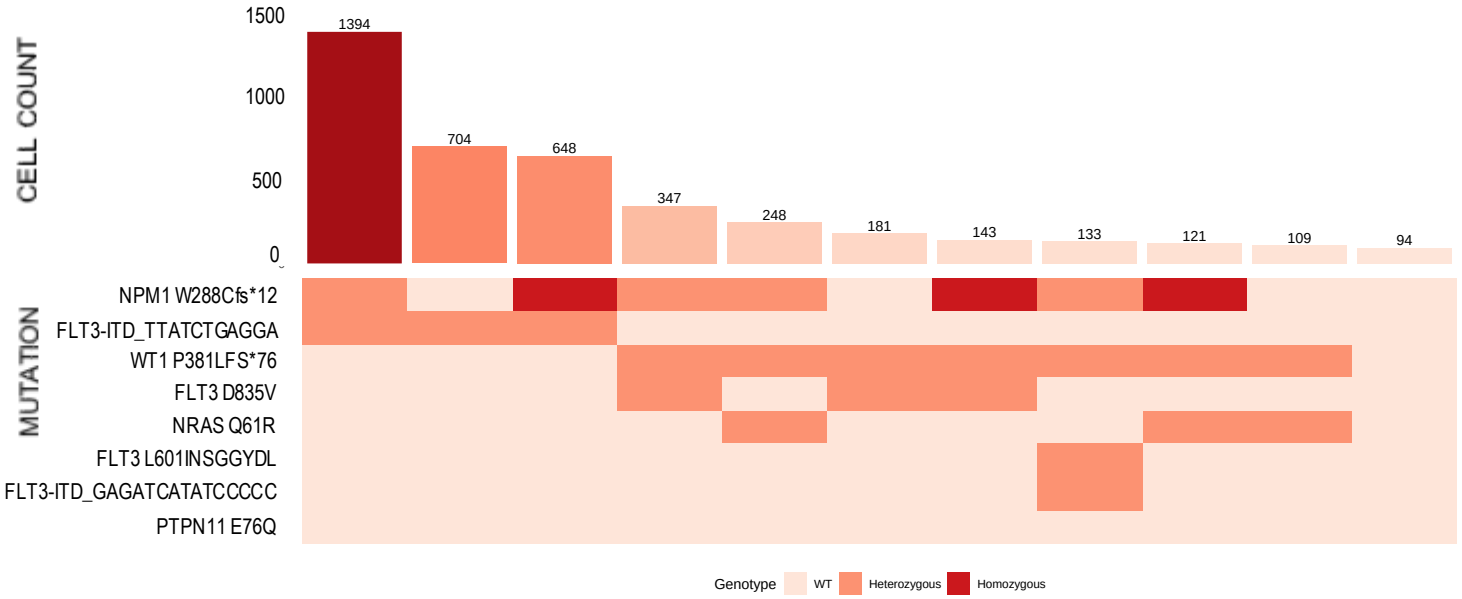




Signaling Mutations are Rarely Identified in the Same Clone or Same Cell

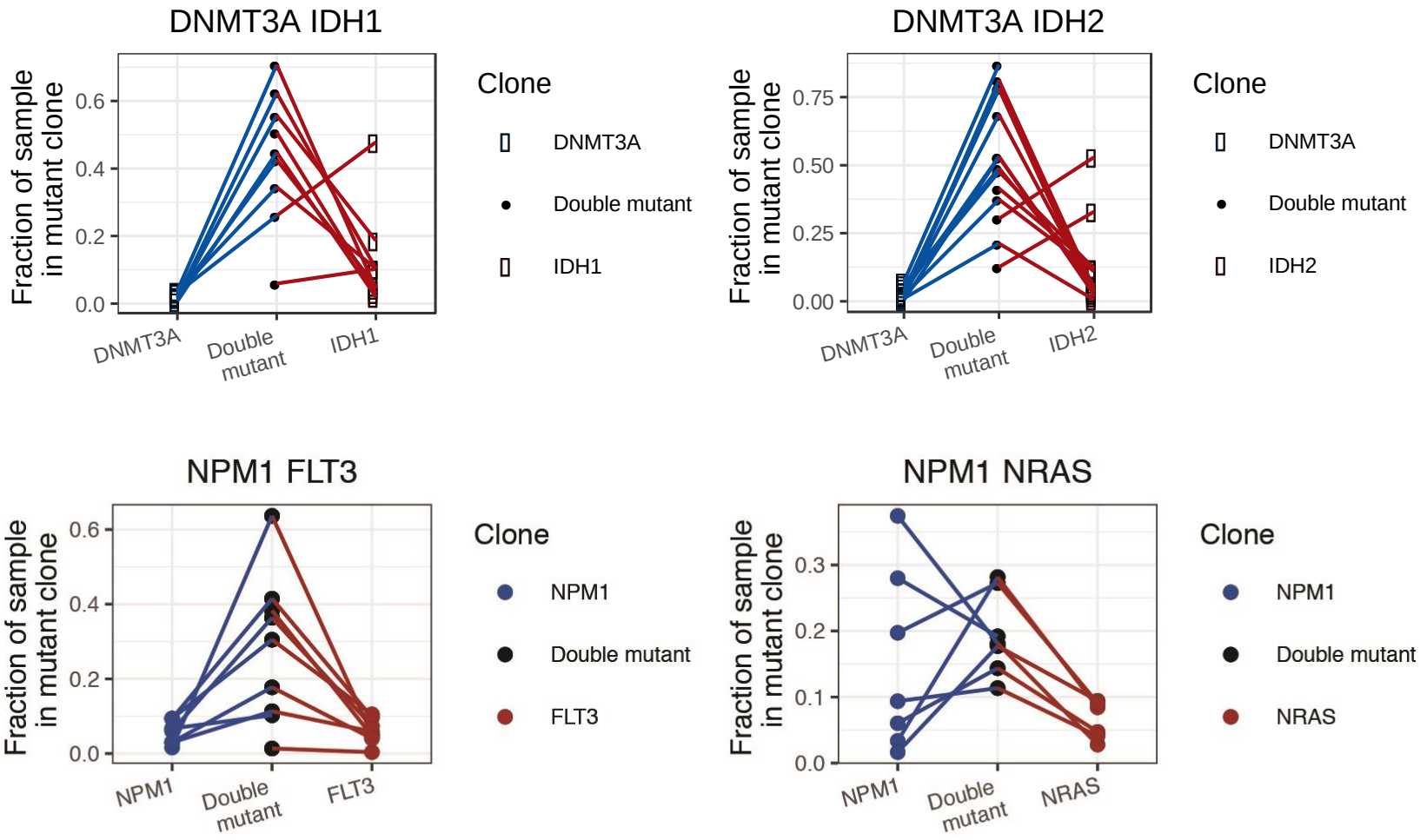






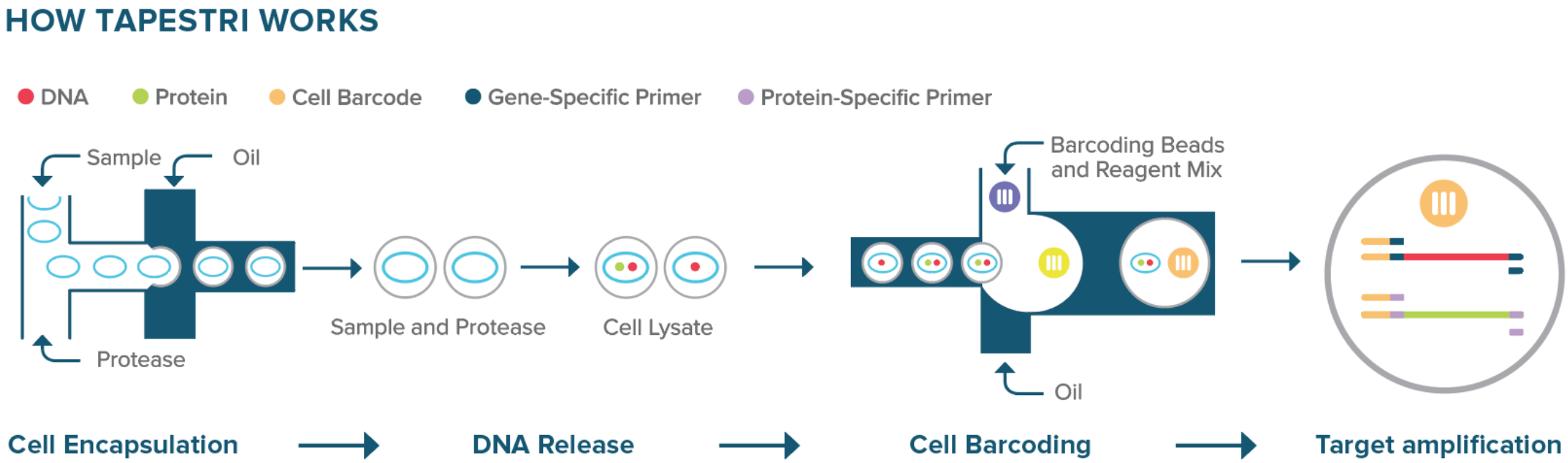
Exception: co-occurring JAK2/NRAS mutations

Mutational Cooperativity and Clonal Expansion

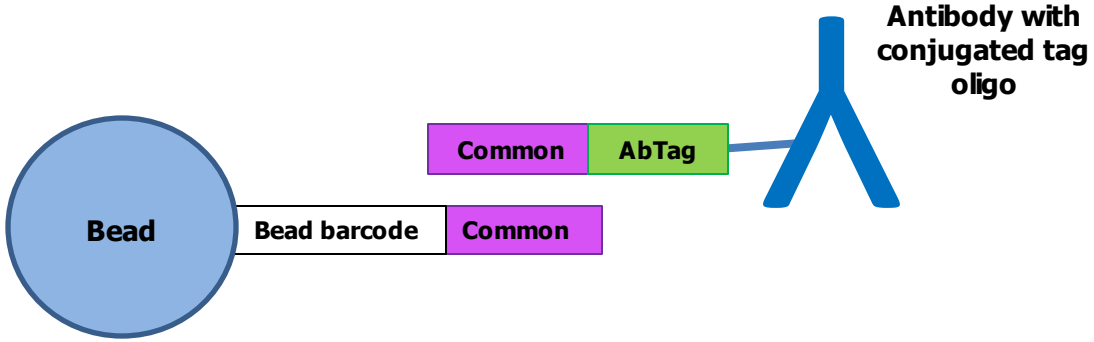


- Disease initiation is likely driven by single mutations or mutational combinations which modestly increase fitness of stem/progenitor cells (CH)
- We hypothesized that clonal expansion and dominance would be driven by specific mutational combinations
- See clear evidence of mutational cooperativity for some combinations (DNMT3A/IDH, FLT3/NPM1) but not others (NRAS/NPM1)
- Inform functional studies of mutational cooperativity on a clonal level

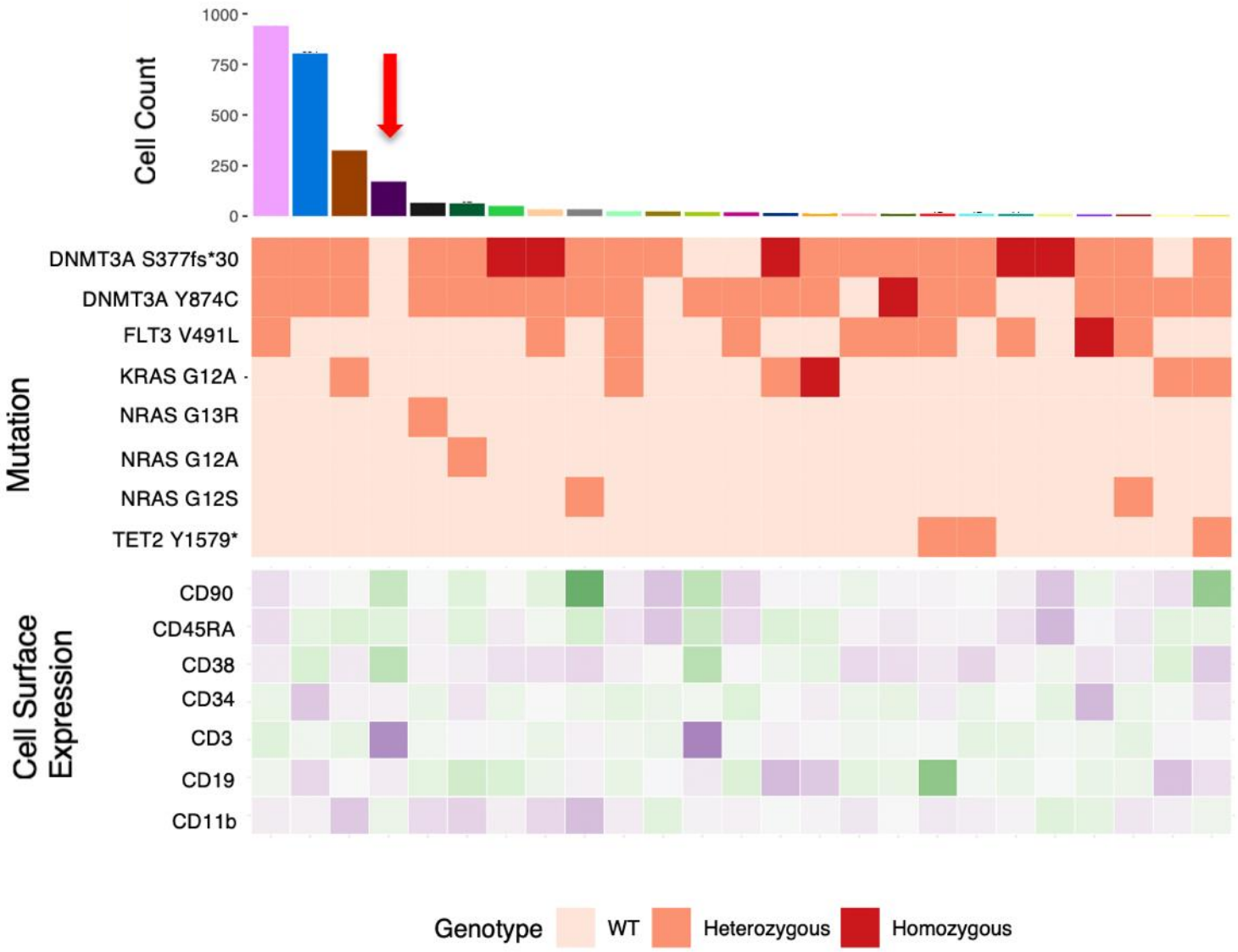
Linking Genotype to Phenotype at Single Cell Resolution

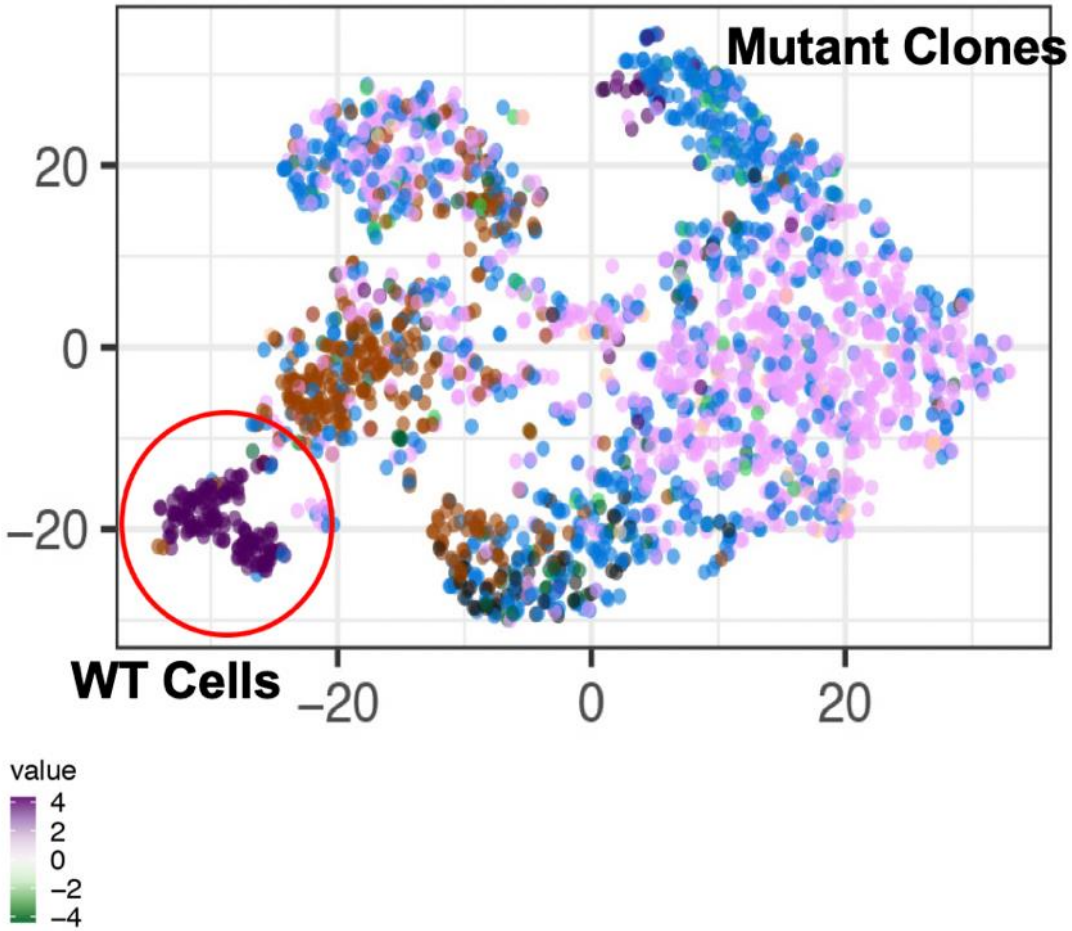


- Merge antibody staining protocol with Tapestri workflow
- Stain cells with oligo-conjugated antibodies
- Perform library prep on both Protein-derived library and DNA-derived library

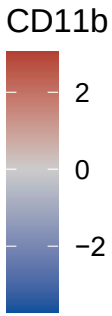


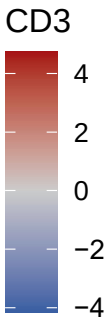
scDNA+Protein Sequencing Shows Immunophenotypic Differences between WT Cells and Different Mutant Clones

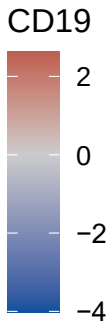




Mapping Clonal Output in Clonal Hematopoiesis





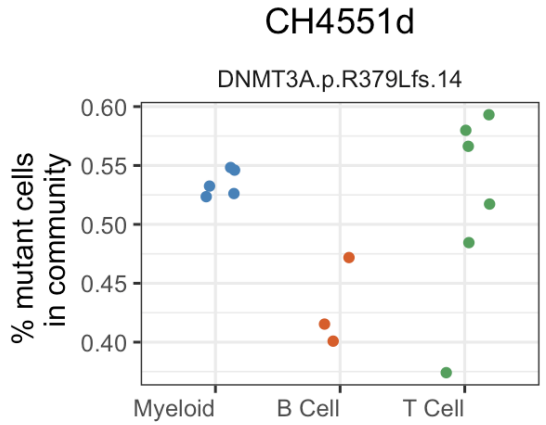


DNMT3A.p.R379Lfs.14

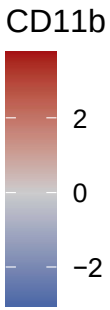
0

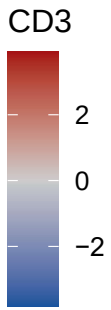
1

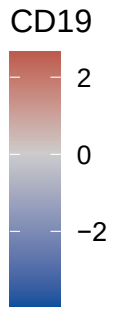
2

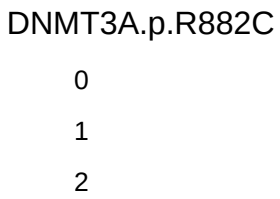


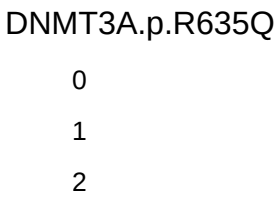
Mapping Clonal Output in Clonal Hematopoiesis

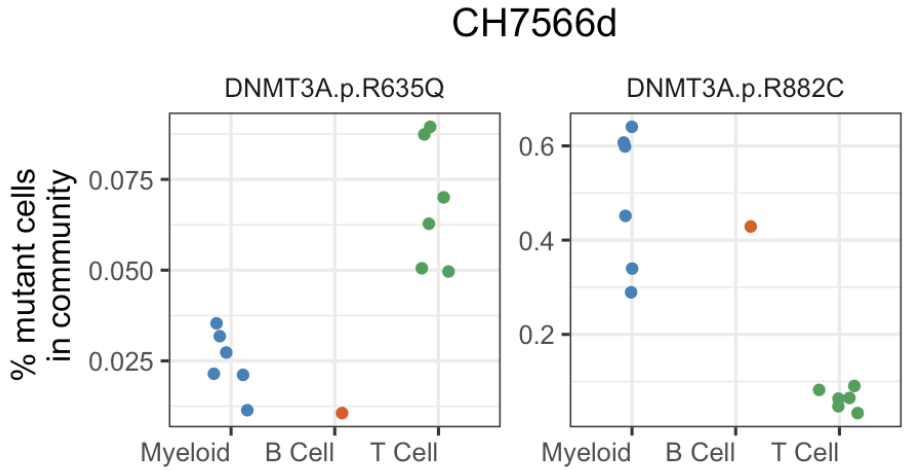




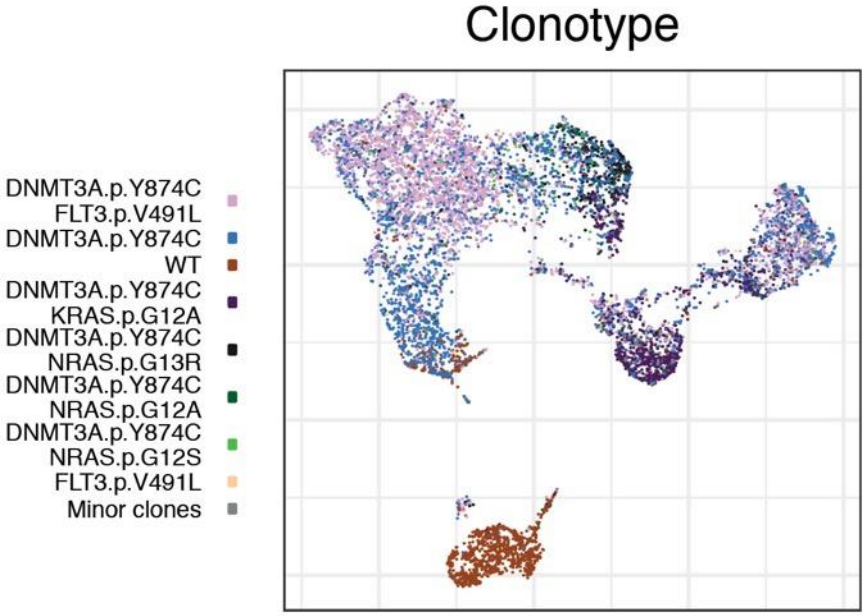


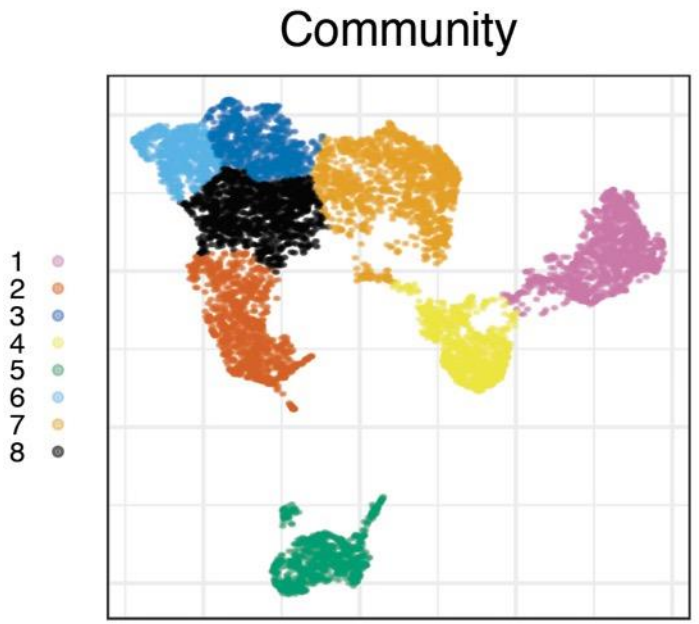




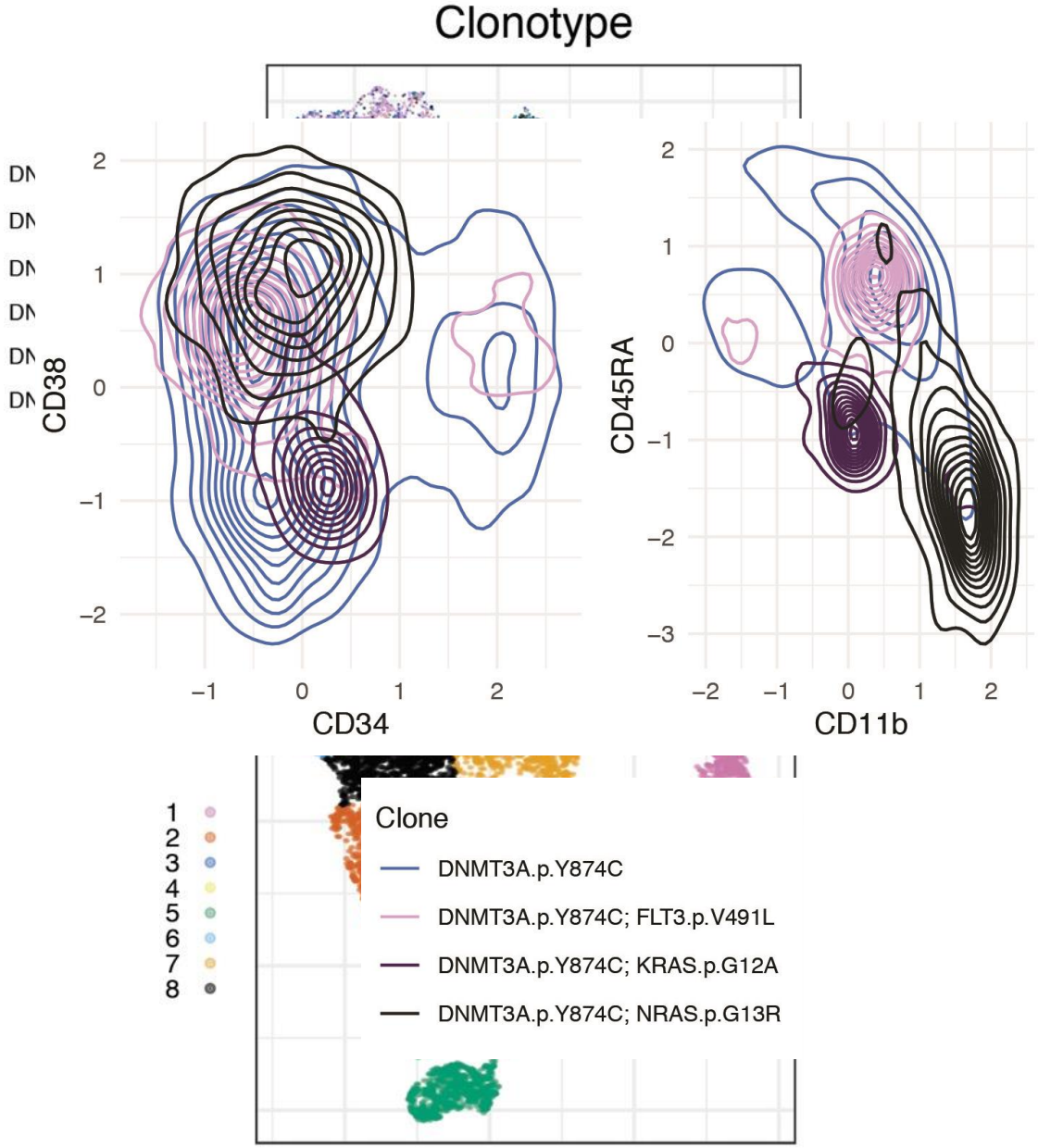


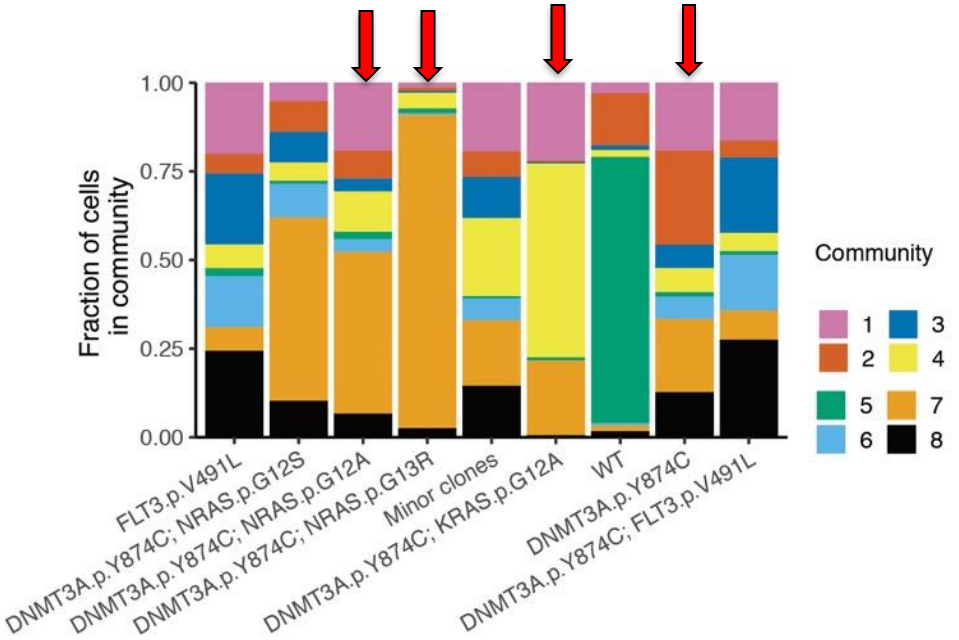
Cell Surface Protein Expression Differs based on Single Cell Genotype: Neighborhood Analysis

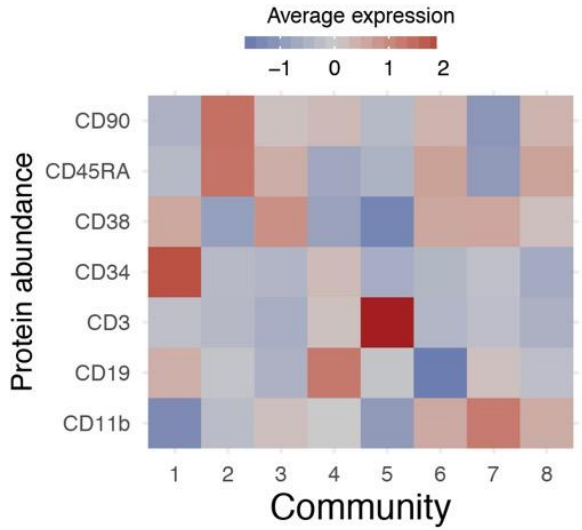




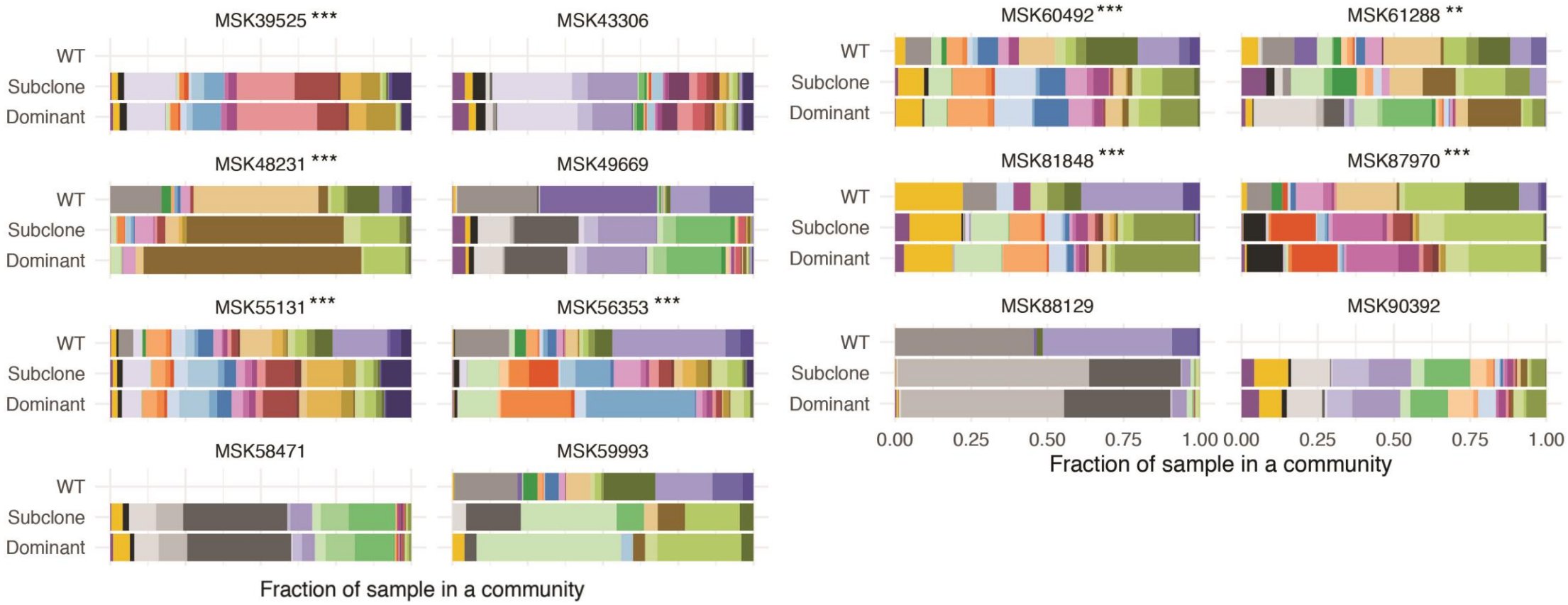
Cell Surface Protein Expression Differs based on Single Cell Genotype: Increased CD11b expression with RAS





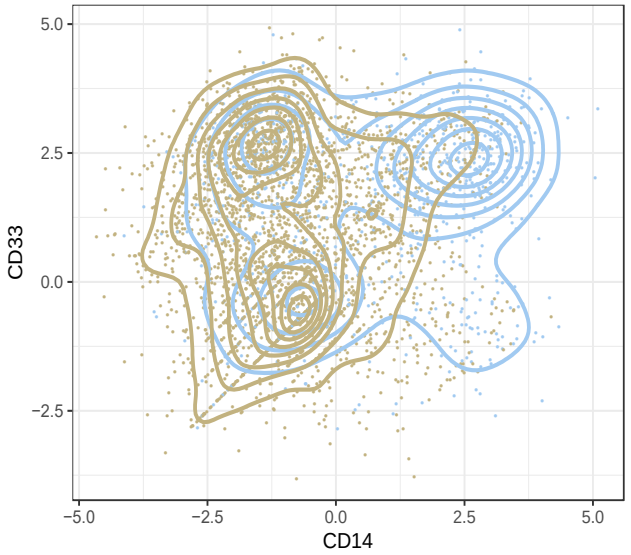


Changes in Communities/Marker Expression between Dominant Clone & Subclones



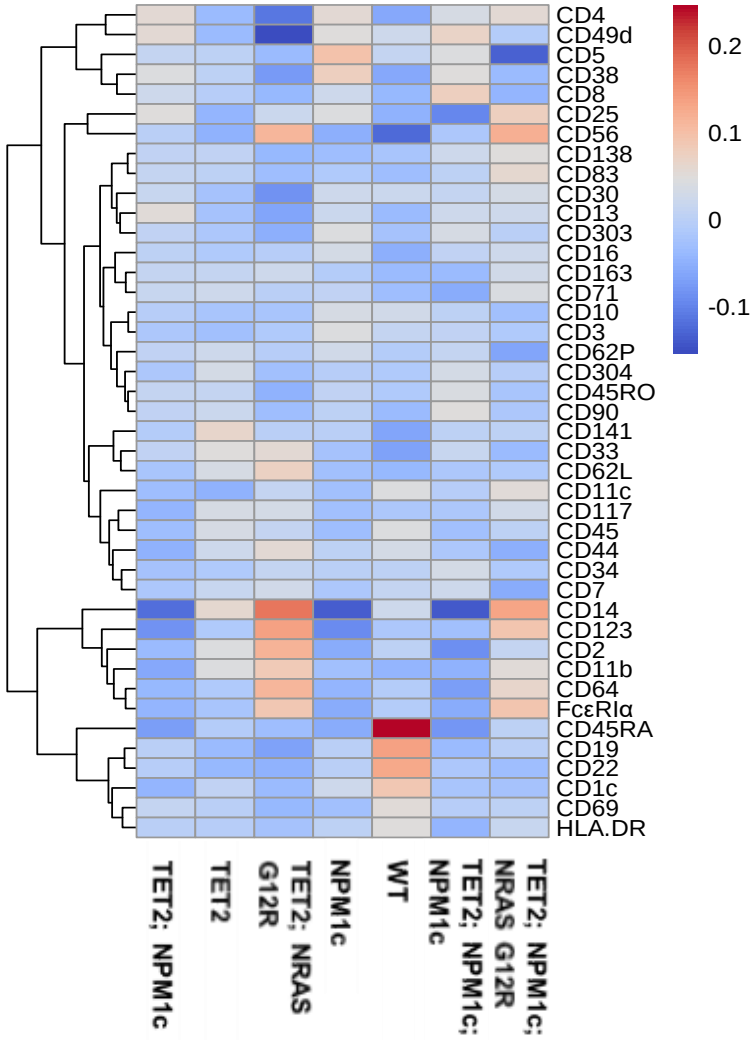
- 8/14 samples showed significant change in community representation between dominant clone and subclones

Dissecting Clone/Immunophenotypic Complexity



Clone

- TET2; NPM1c; NRAS G12R
- TET2; NPM1c; NPM1c
- WT
- NPM1c
- TET2; NRAS G12R
- TET2
- TET2; NPM1c



- **TET2/NRAS mutant clone shows highest expression of CD123, CD13, CD11b, CD2**
- **Can use this to delineate relationship between specific surface marker profiles and clonal composition; inform use of cell-targeting cellular/BITE-based therapies esp those targeting >1 antigen**

New Tools to Model Clonal Evolution

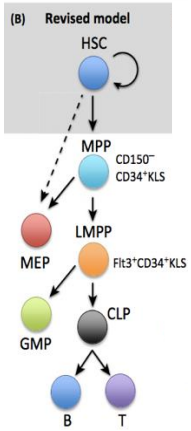
Genetic Control

Cre

Dre

Flp

Cell-type Control



Event Control

Nuclear localization

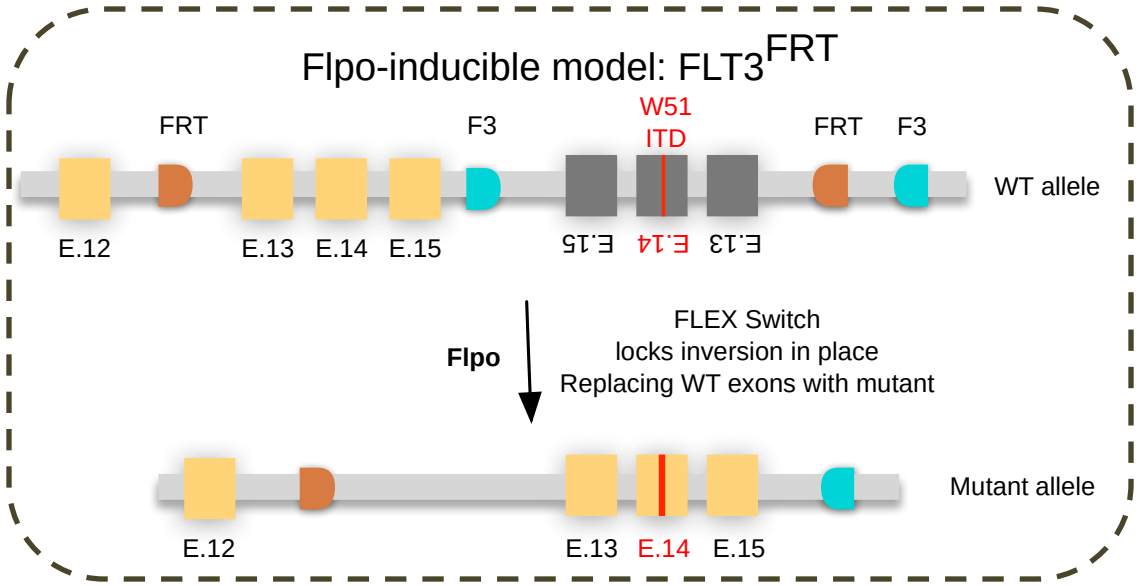
Protein Stability

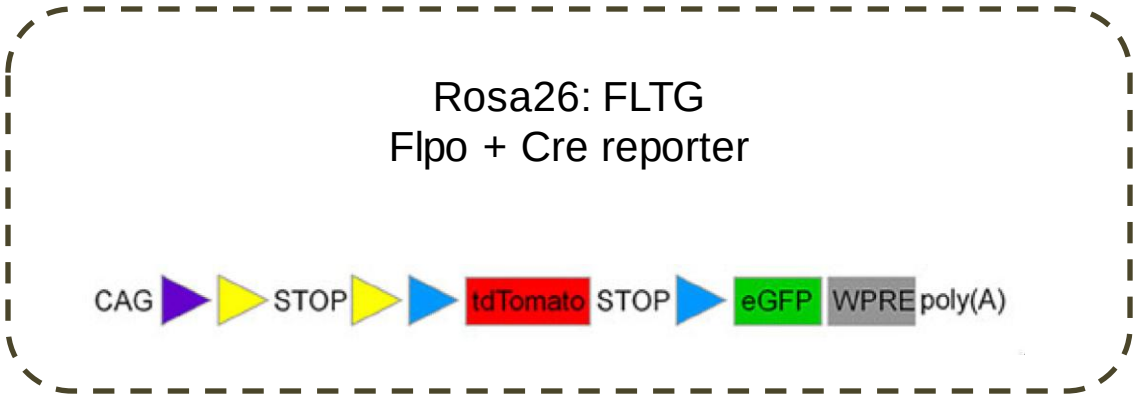
Long-term goals:

- Use human genetic studies to understand the order of events which lead to myeloid malignancies
- Develop genetic models to evaluate sequential mutagenesis
- Drive recombinase expression in different stem/progenitor compartments to allow for temporal and spatial control of mutational order in vivo

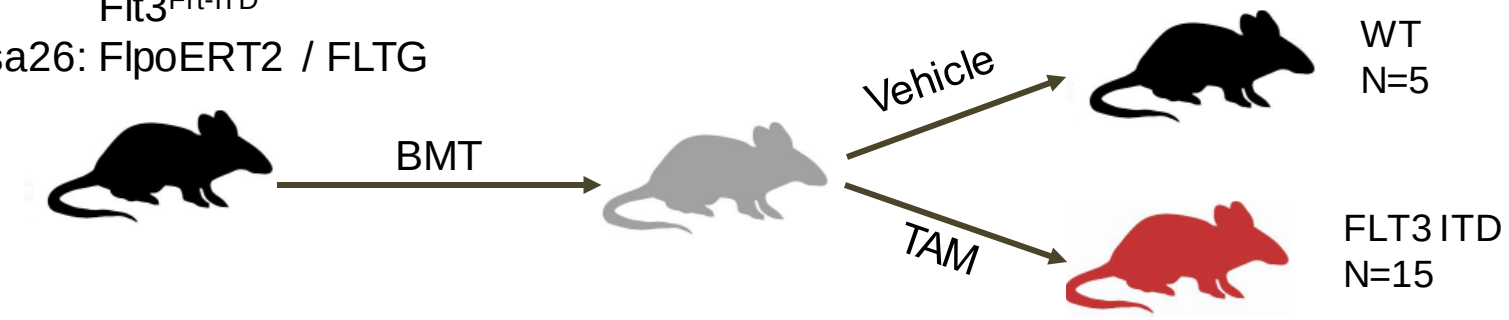
Bobby Bowman

Development of Flpo-inducible Flt3-ITD allele

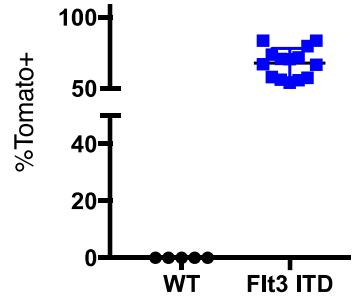




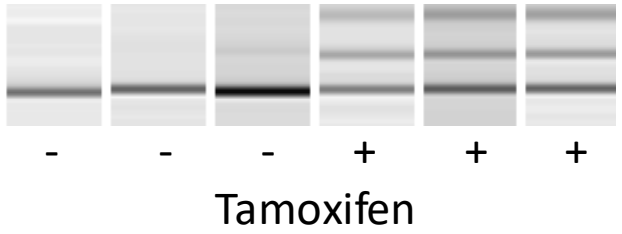
Flt3Frt-ITD
Rosa26: FlpoERT2 / FLTG



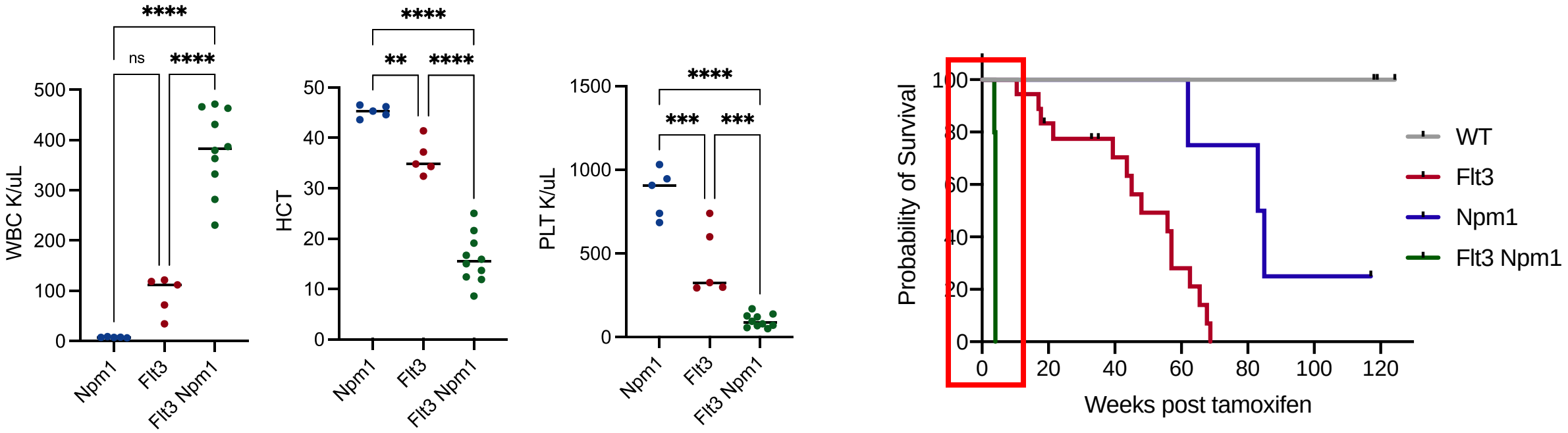
Recombination
4 weeks post TAM



Excision PCR for Flt3

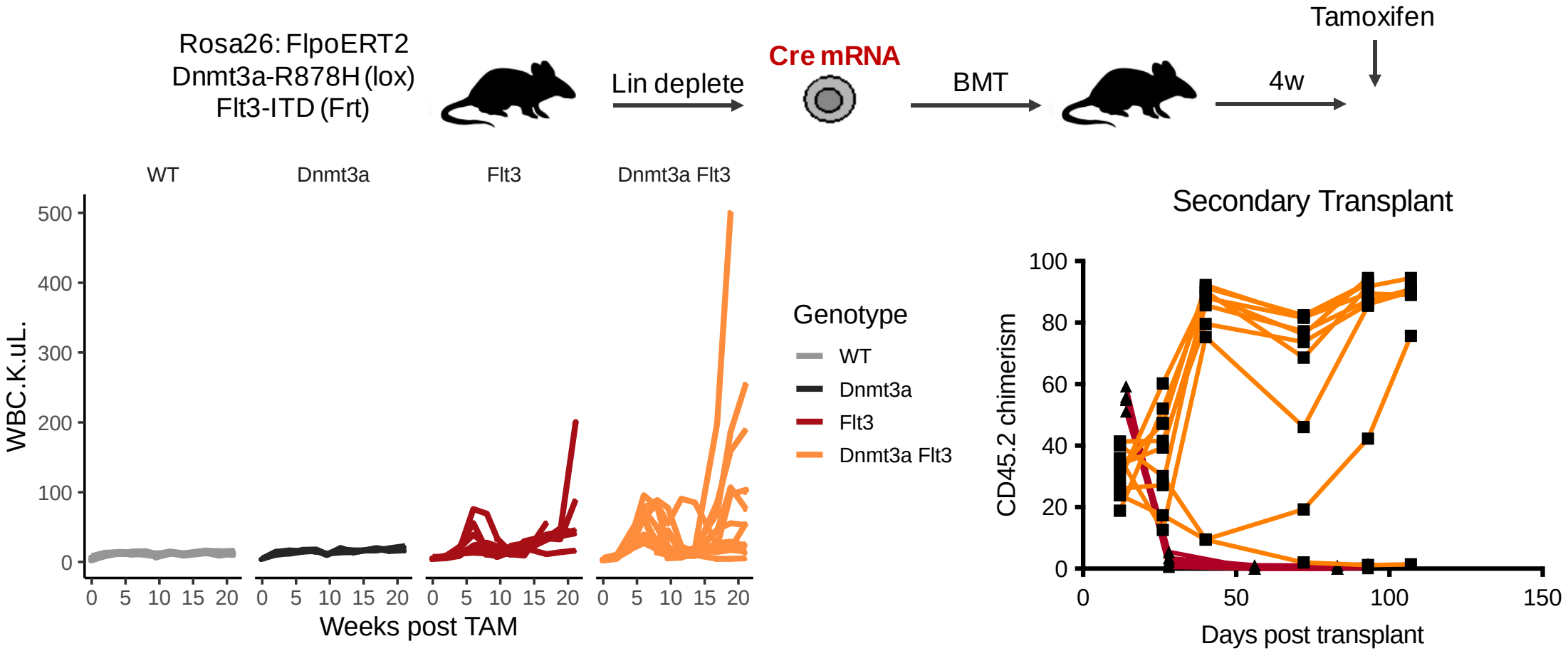


Simultaneous or sequential Npm1->FLT3-ITD: Fully Penetrant Lethal AML



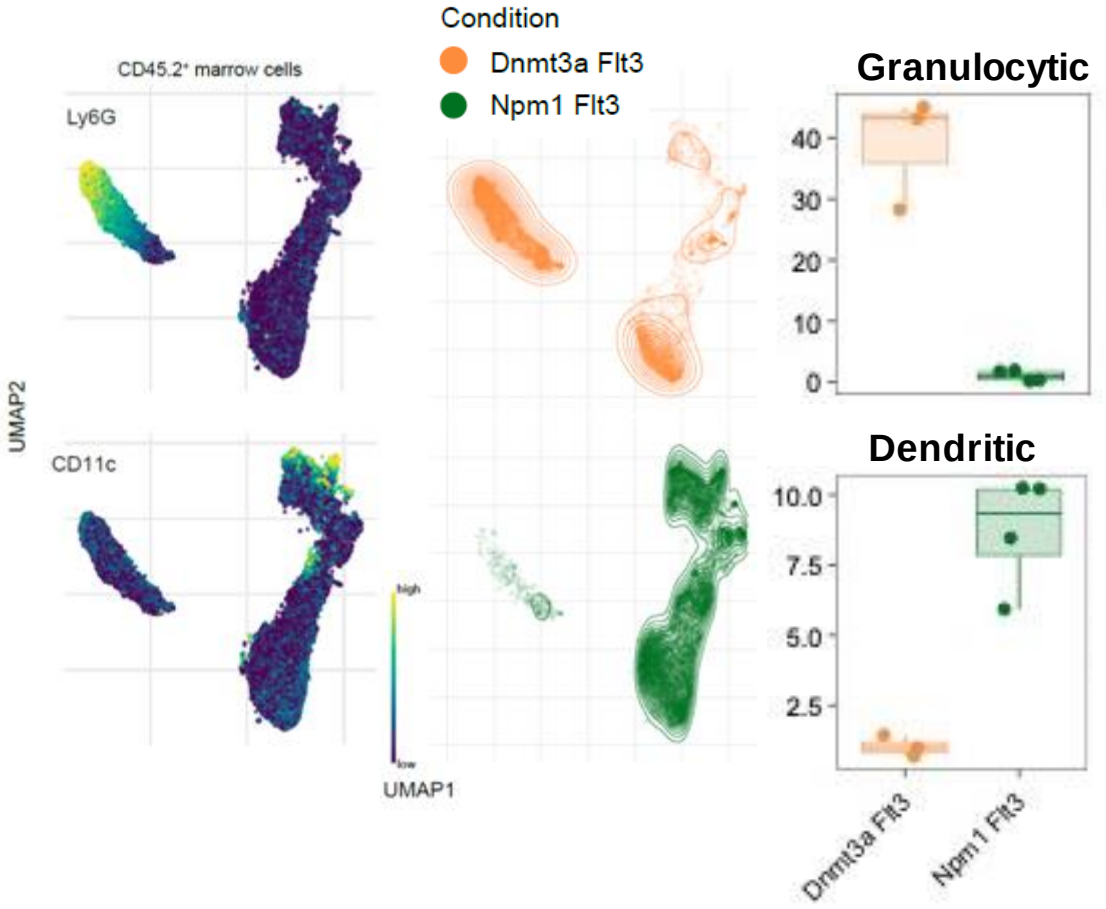
- No “feedback” after Flt3/Npm1 activation, whether simultaneous or NPM1c->FLT3
- Mice develop profound leukocytosis, anemia and thrombocytopenia.
- Secondary transplants give fully penetrant disease (20,000 splenocytes or whole marrow)

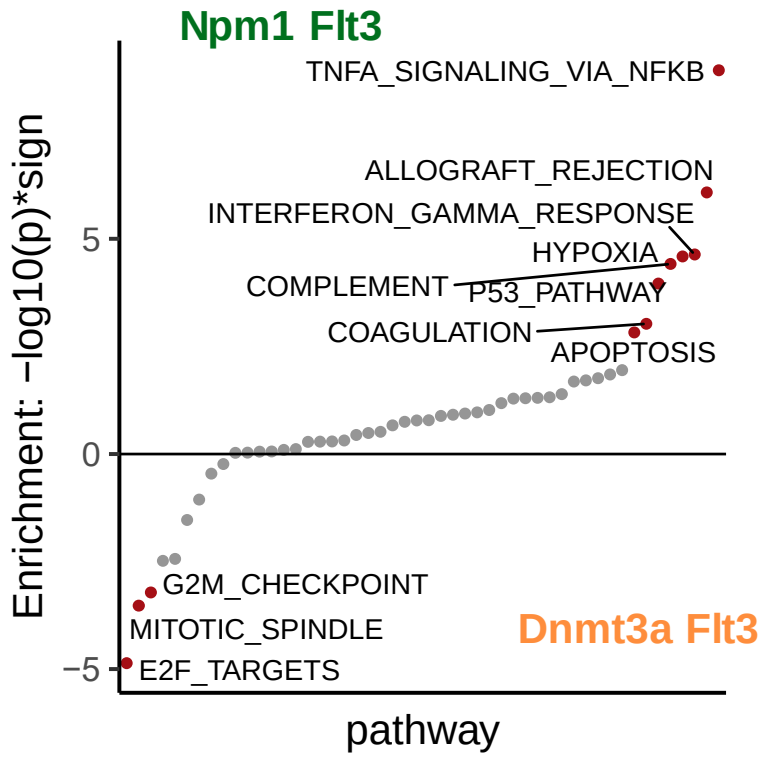
Sequential Dnmt3a R878H -> Flt3 leads to enhanced self-renewal->AML



- AML penetrance by histopathology ~40%, mild anemia and thrombocytopenia
- Secondary transplants propagate disease with ~70% penetrance ($1x10^5$ splenocytes), see enhanced competitive capacity of DNMT3A/FLT3-mutant cells

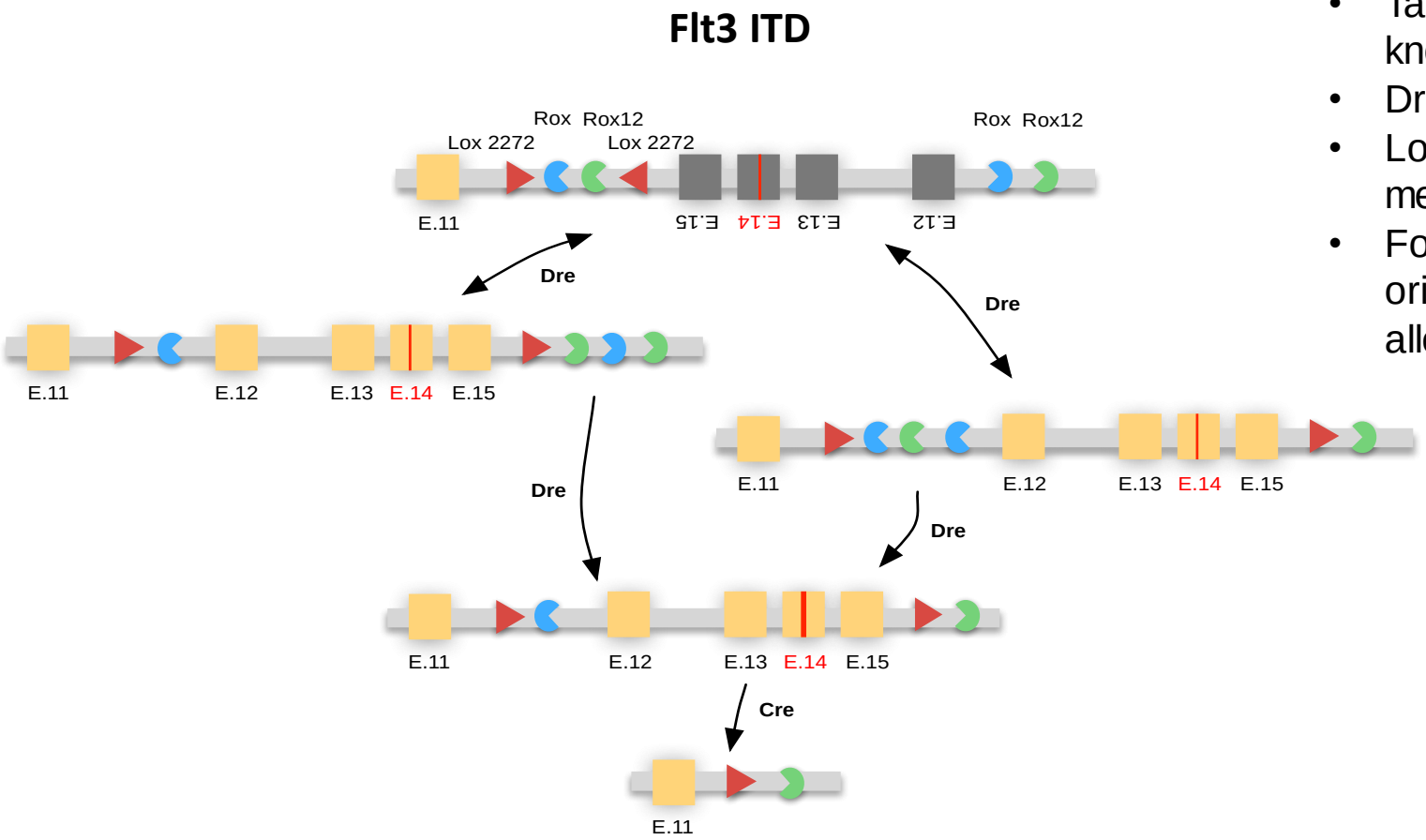
Different leukemic models possess divergent immunophenotypes and transcriptional outputs



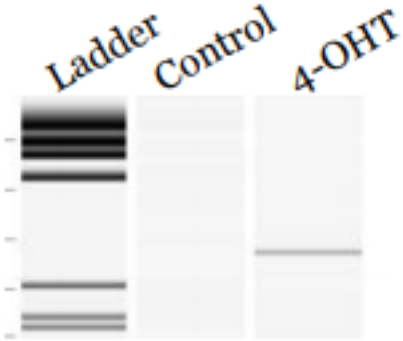


- Correlates with differential lineage marker expression in murine leukemia models and in primary AML samples

Dual recombinase models for reversible oncogenic mutations: GOLDI-Lox



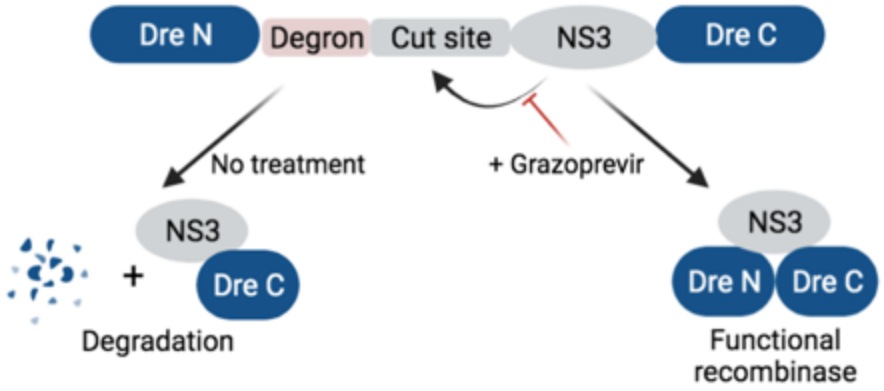
- Targeted to endogenous locus as an initial knockout allele
- Dre on (FLEx Switch); Cre off
- Lox2272 sites are too close to recombine, until Dre mediated recombination moves them further apart
- Following Dre; Lox2272 sites now point in the same orientation, and a pulse of Cre will delete the ITD allele leading to nonsense mediated decay.

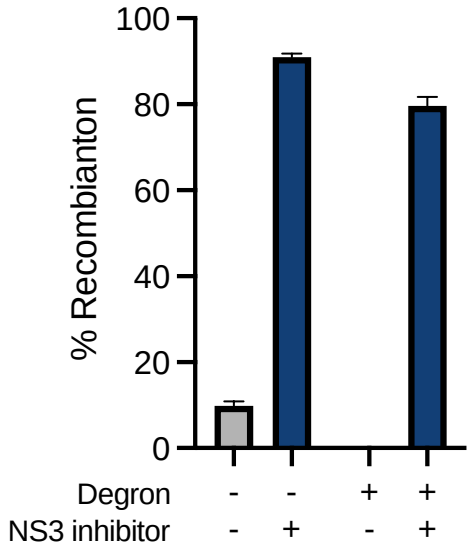


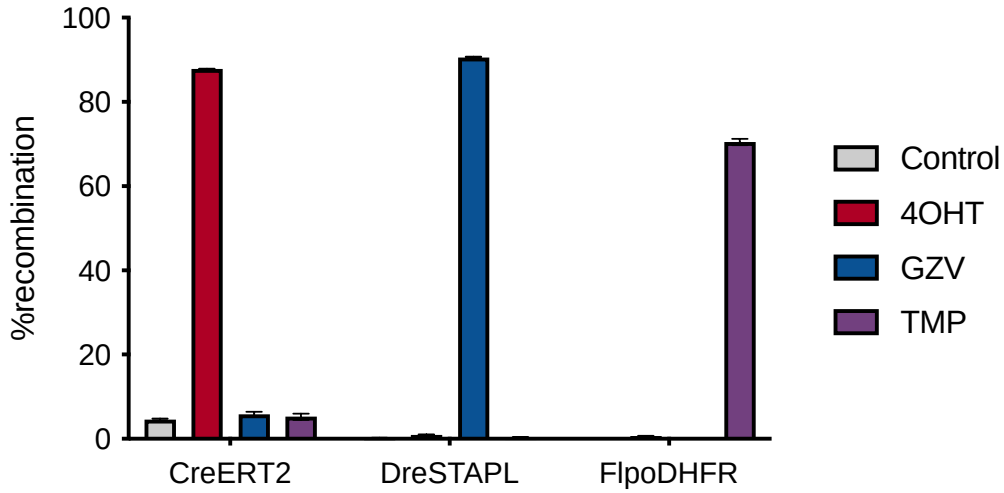
Generating **O**ncogenic **L**oci by **D**re **I**nversion – **Lox** deletion/reversion

Spectrum of AML alleles in progress. Newer alleles go WT-> MUT -> WT

Inducible recombinases - Dre-StaPL
Stabilized Peptide Linkage with a deprotected degron

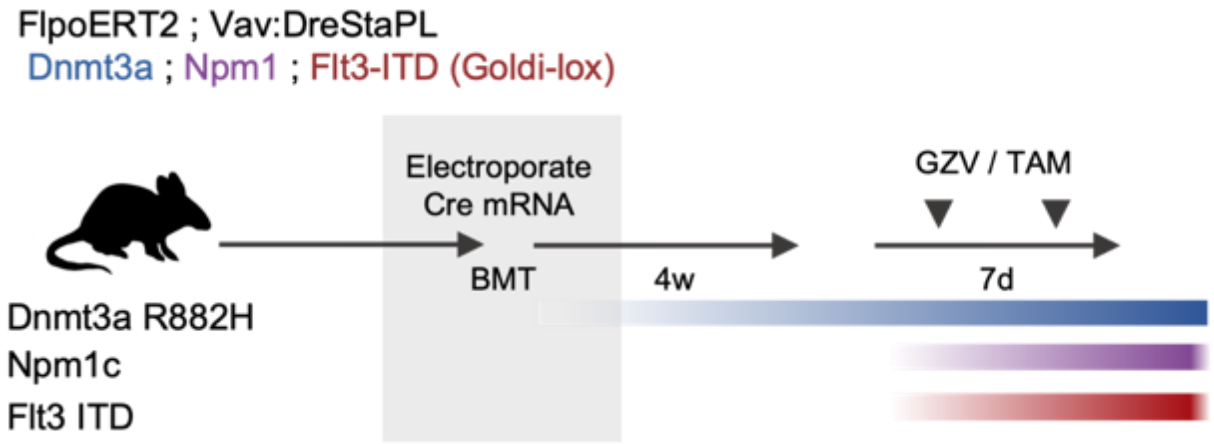


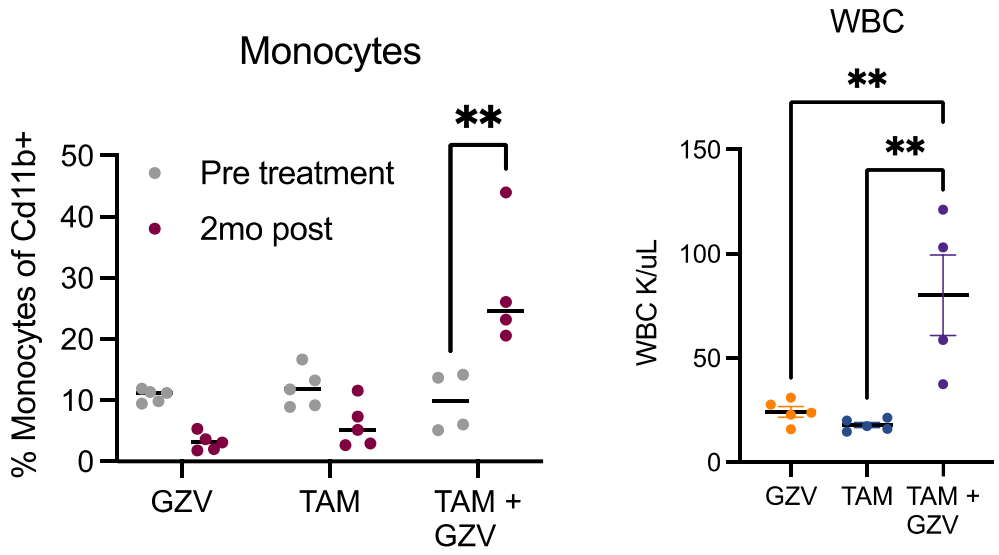




- NS3 protease splits Dre, inhibition of NS3 leads to recombination
- ODC degron next to NS3 cut site leads to degradation of N-terminus of Dre.
- DHFR degron fusion to Flpo for Trimethoprim (TMP) stabilization
- Three orthogonal tools: CreER, FlpoDHFR, DreSTAPL
- Made two different knock-ins for Dre-StaPL: Vav (low efficiency<1%), CAG (high efficiency – 40%+

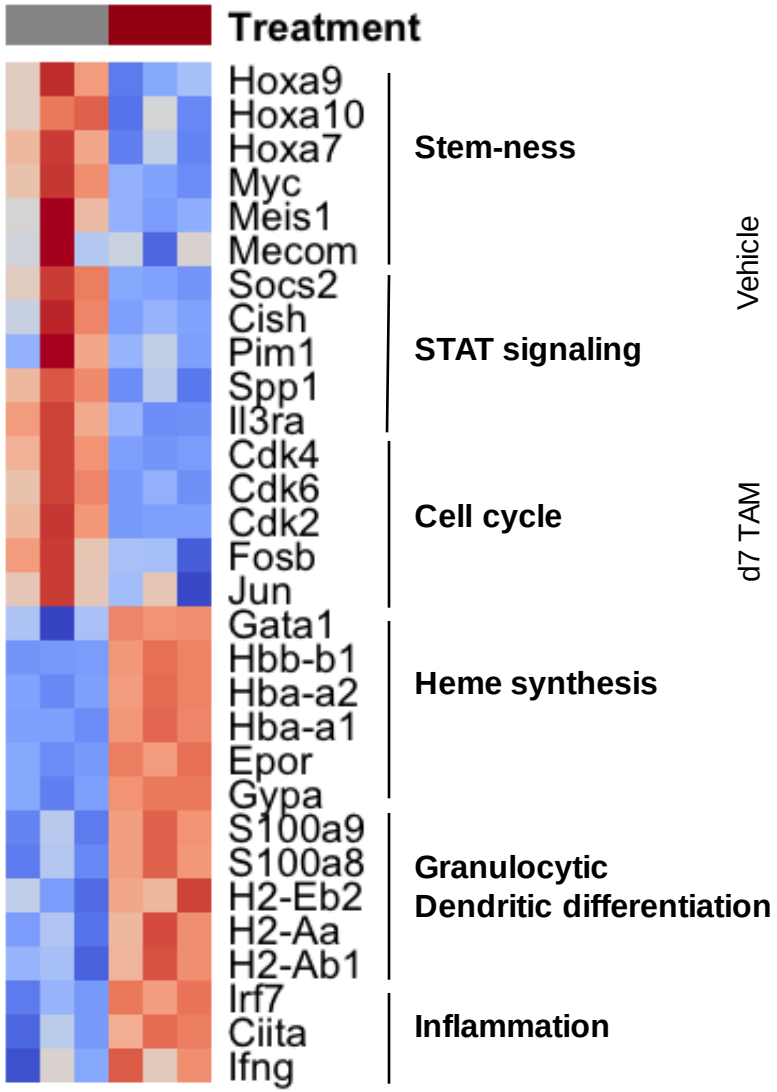
Multi-recombinase induction of triple mutant AML

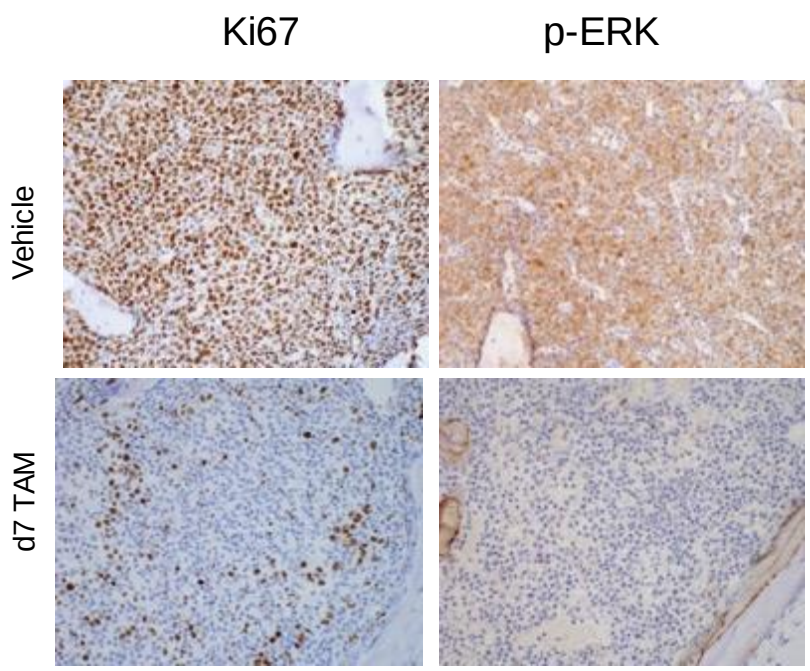


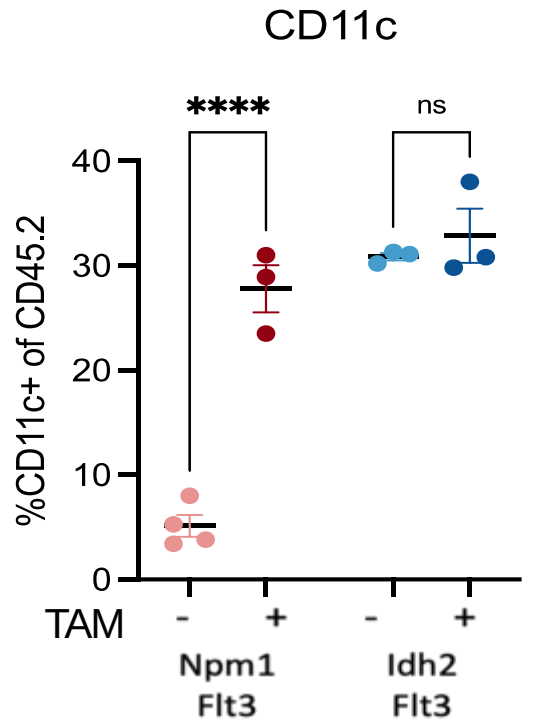


- Integration of FlpoERT2 and DreSTAPL allow for facile evaluation of mutation order as well as allele burden (titration of TAM or GZV)
- **Forward thinking:**
  - Expression of Dre /Flp in different cell types for cell of transformation/origin studies.

Ftl3 deletion decreases proliferation and MAPK signaling

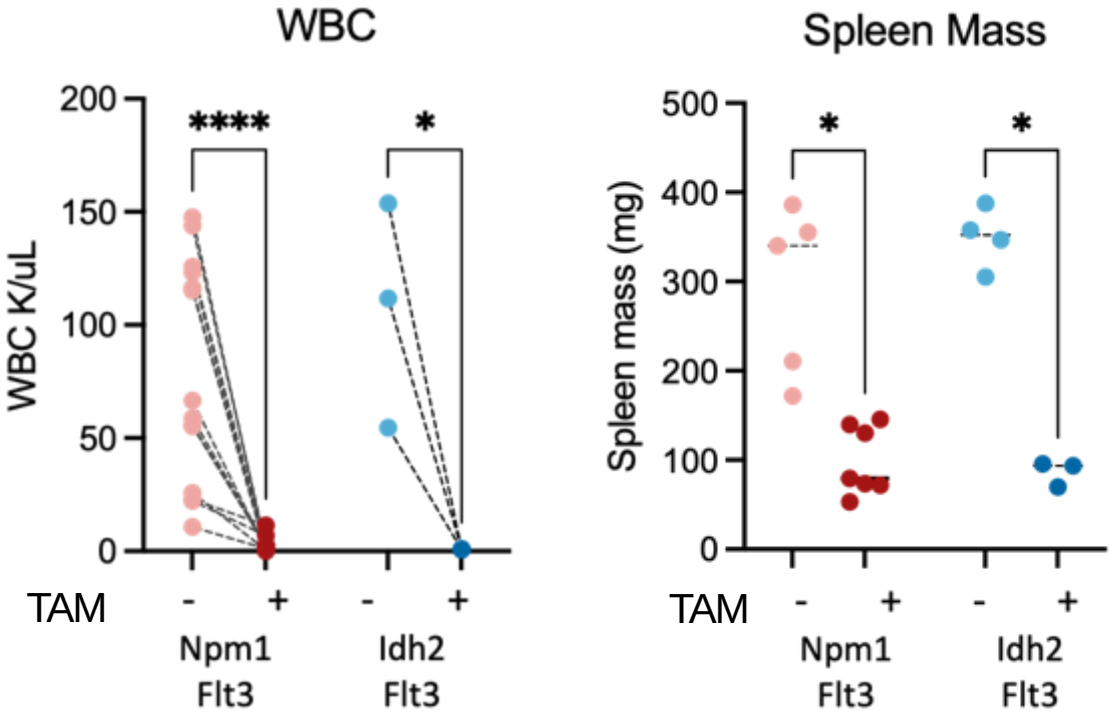


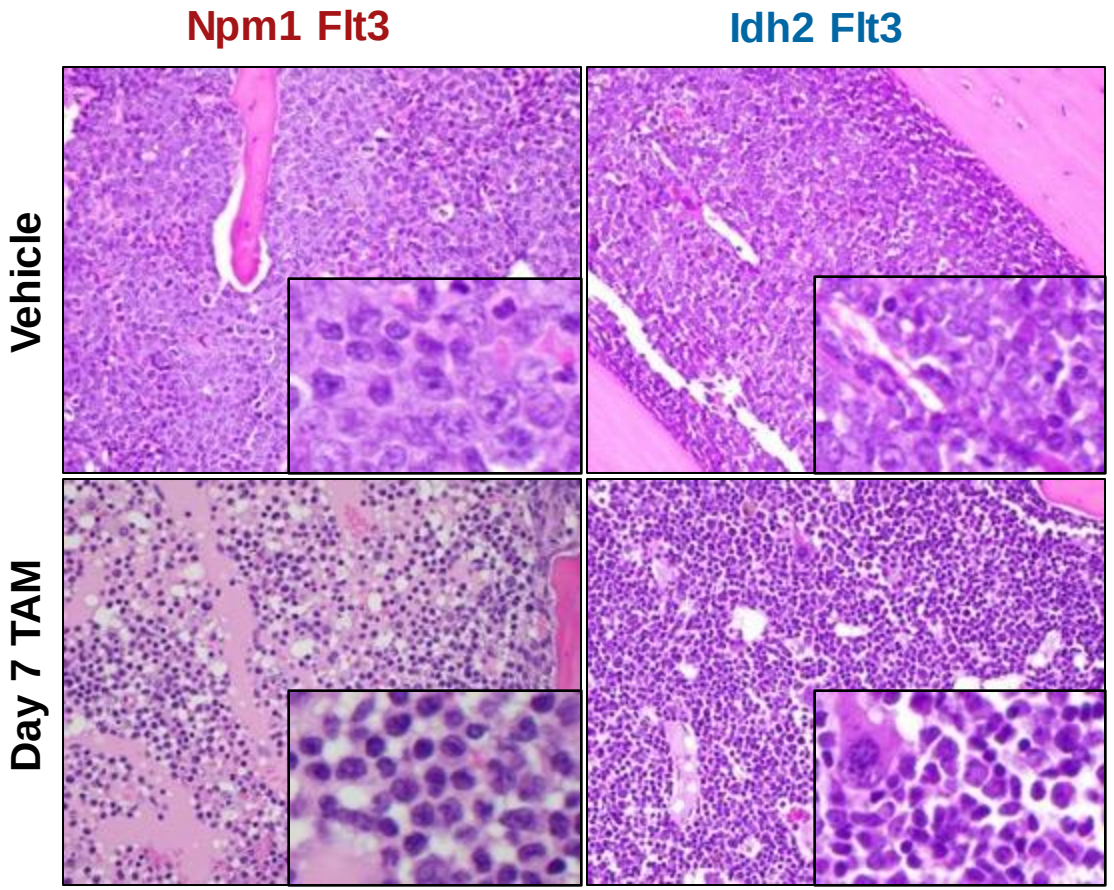




- Shutdown in proliferation and phospho-ERK signaling
- Increased apoptosis *in vivo* (CC3) and *ex vivo* (annexin V)
- Increased differentiation towards dendritic and granulocytic cells

FLT3 deletion results in acute disease regression and differentiation





- Cre activation (tamoxifen) results in acute disease regression (7 days)
- Decrease in spleen mass, bone marrow cellularity, cKIT+ in peripheral blood, and chimerism
- See relapsed leukemia: some AML cells with lack of FLT3-IITD reversal (escape), some FLT3-independent

How else can we use reversible murine models? Testing the JAK2 hypothesis

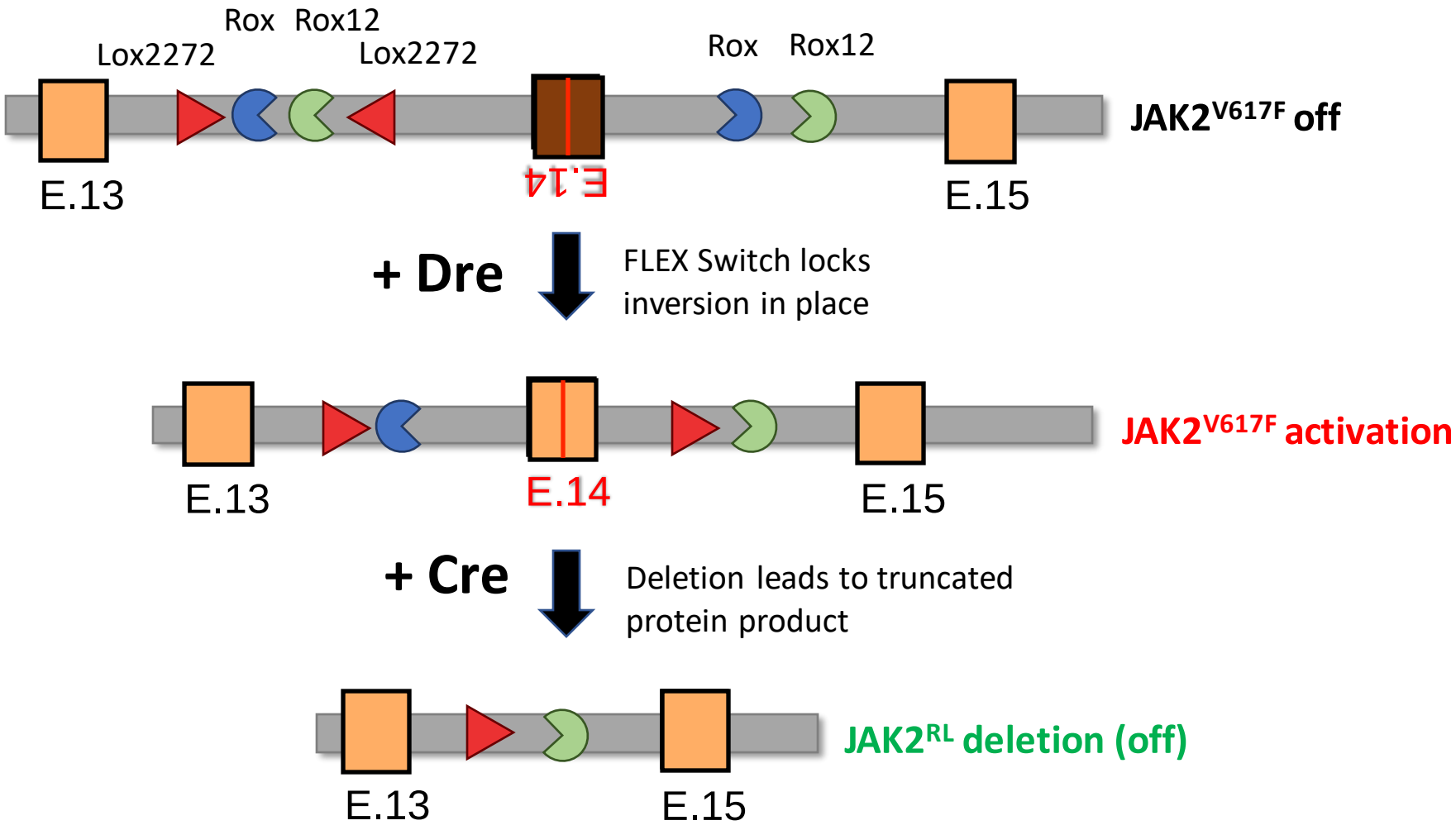
- MPN mutant clone persists in the presence of chronic JAK2 kinase inhibition
- This may be due, at least in part, to the presence of other disease-initiating alleles (e.g. *TET2*, *ASXL1*, *EZH2*, etc)
- However, JAK2-driven pre-clinical models argue still other factors contribute:
  - JAK2V617F knock-in model: Disease initiating cells are resistant to JAK2 inhibition (Mullally et al. 2010)
  - MPLW515L bone marrow transplant model: No decrease in allele burden (Koppikar et al. Blood 2010)
- No identified second-site mutations in patients treated with chronic JAK2 inhibition
- Lack of an initial clone-specific response argues for inherent insensitivity/survival in setting of current JAK inhibitors (“persistence”)

Hypotheses

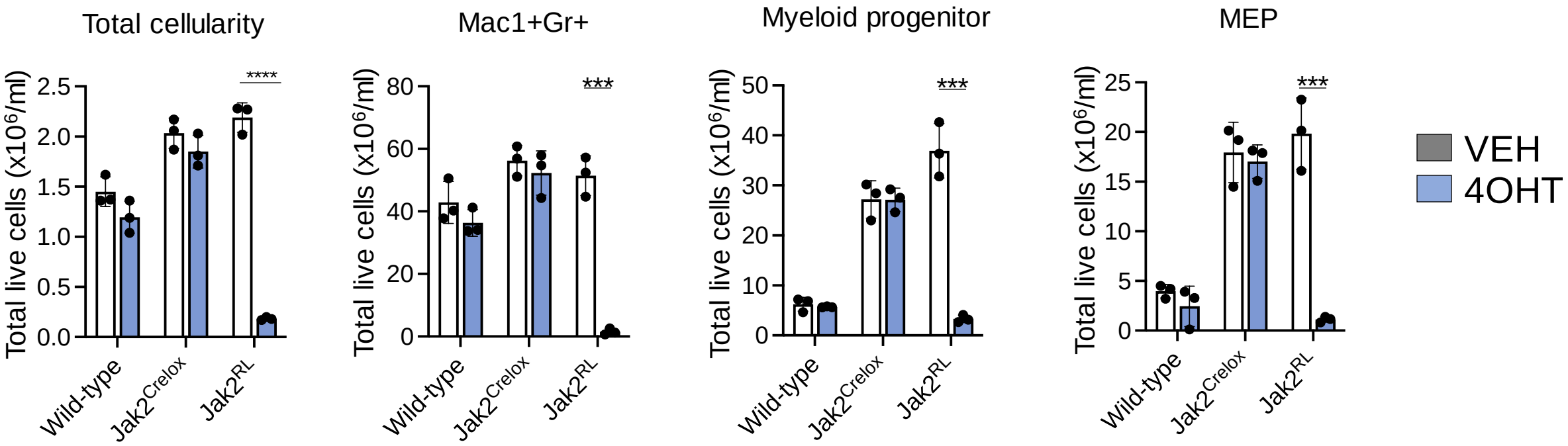
- Deletion of mutant JAK2^{V617F} abrogates the MPN phenotype and reduces mutant clonal fraction supporting the role of improved mutant-specific JAK2 inhibition in MPNs

- Reliance on JAK/STAT signaling is affected by cooperating disease alleles (i.e. *TP53*, *ASXL1*, *TET2*, etc) and influences response to JAKi therapy

Design of a reversible *Jak2*^{V617F} *Rox-Lox* (JAK2^{RL}) knock-in/knock-out allele



Jak2V617F Deletion Ex Vivo Abrogates JAK2-Mutant Fitness

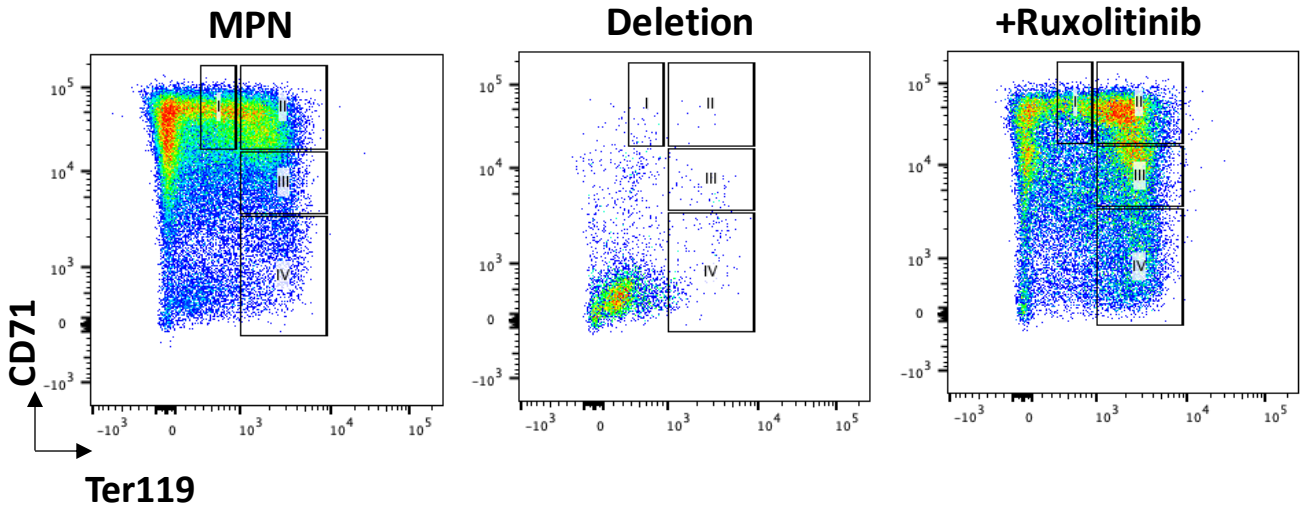


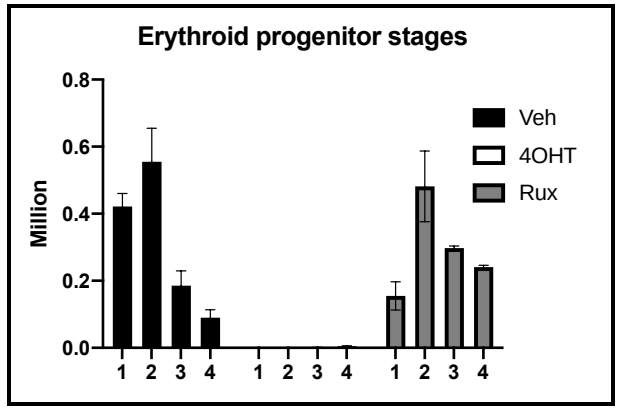
Jak2^{V617F} deletion ablates Erythropoietin-independent growth *in vitro*

Culture lin-neg *Jak2*^{RL}
MPN cells over BMECs

ETOH (Vehicle)
1uM 4OHT
250nM Rux

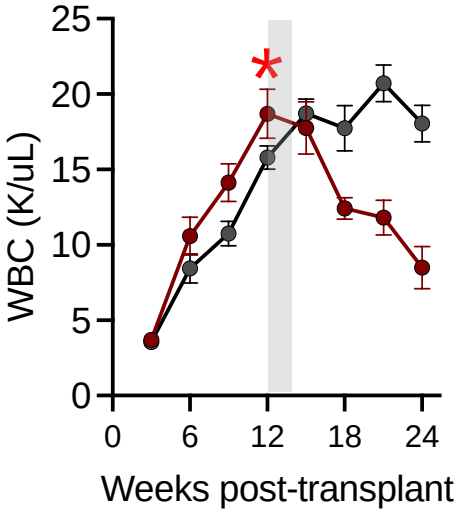
Readout @ Day 7:
- CD71/Ter119
- HSPC
- Mature myeloid



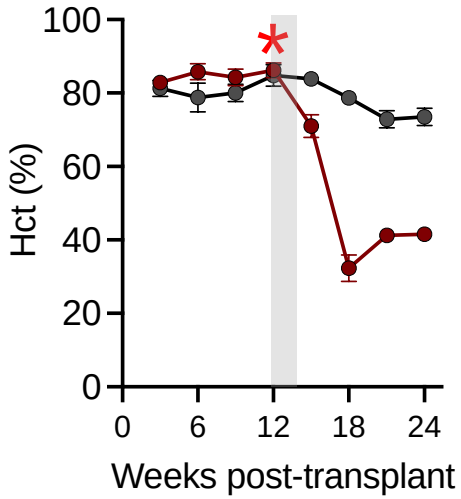


Jak2^{VF} Deletion Abrogates Pathognomonic MPN Features *In Vivo*

WBC



Hematocrit

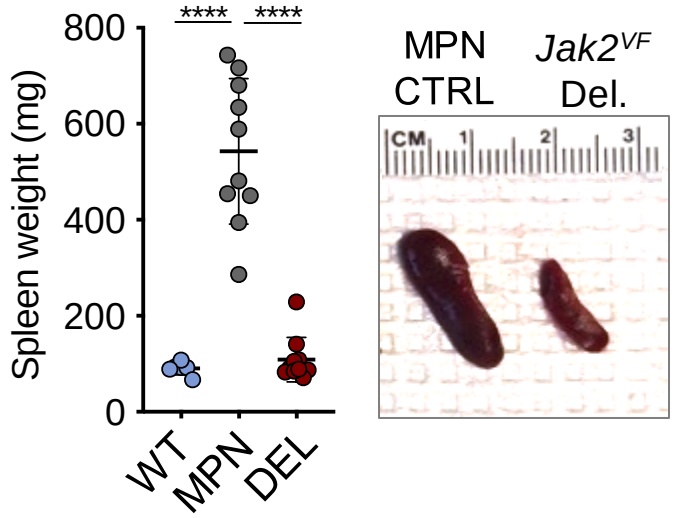


* TAM to delete *Jak2*^{VF}

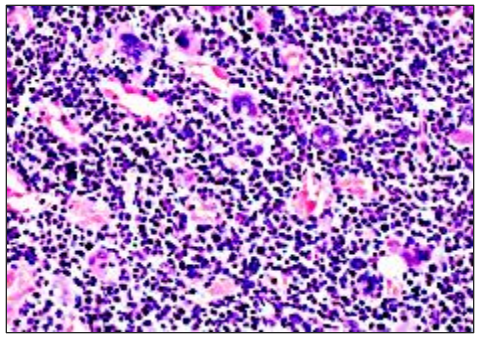
MPN

Jak2^{VF} Deletion

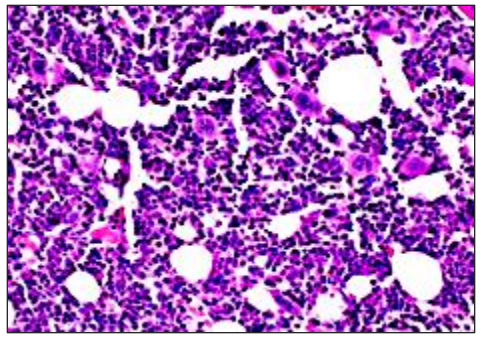
Spleen weights



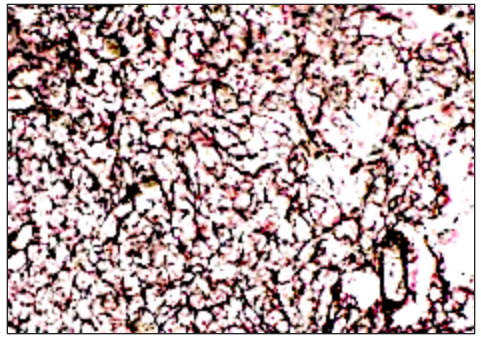
MPN CTRL



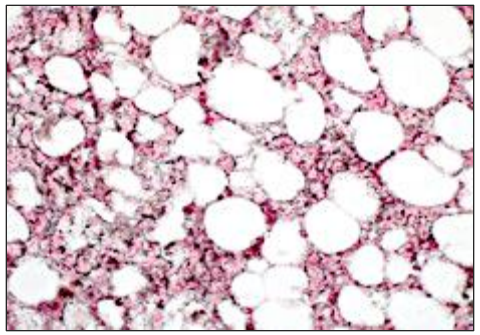
Jak2^{VF} deletion



MPN CTRL

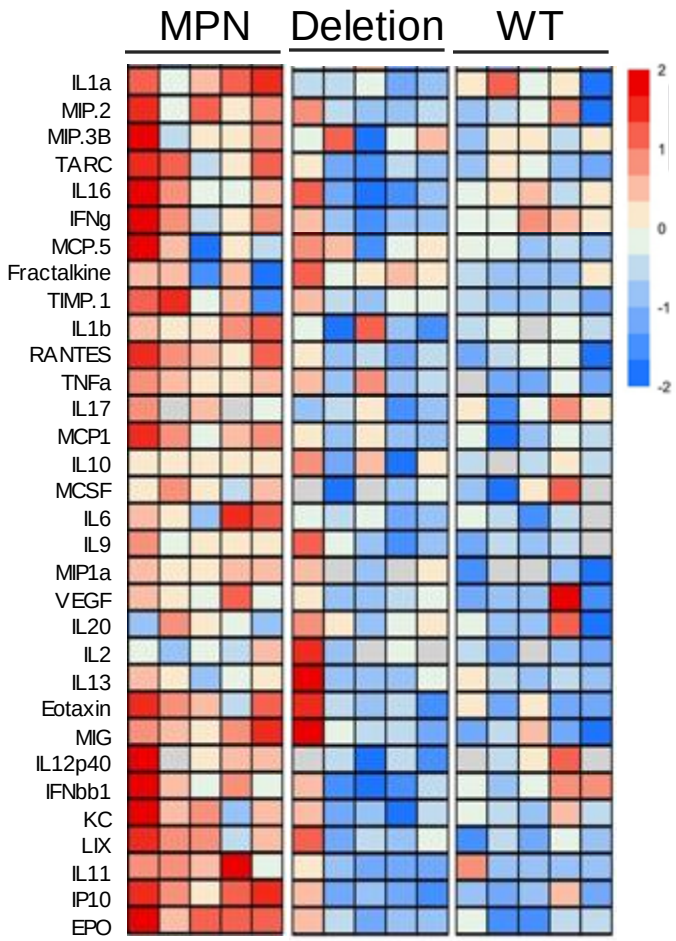


Jak2^{VF} deletion

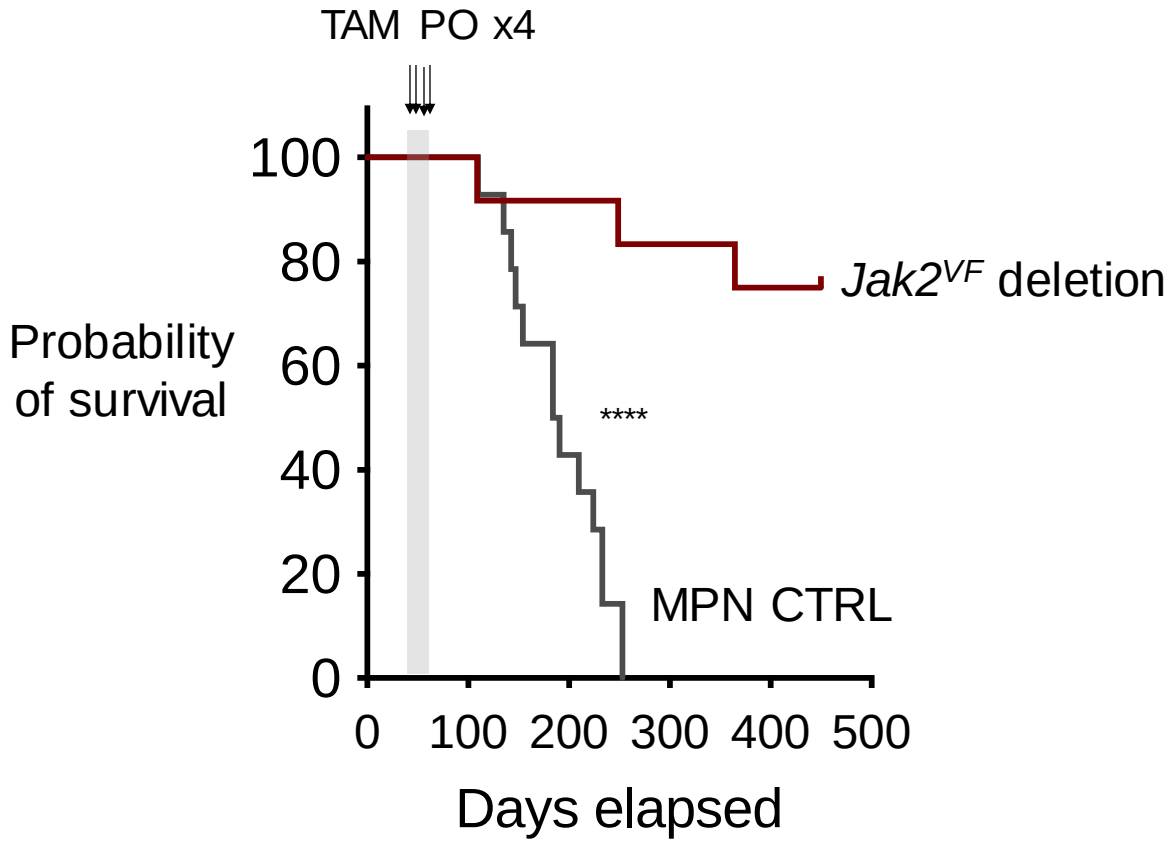


Jak2^{VF} Deletion Reduces Pro-inflammatory Cytokine Production and Prolongs Overall Survival

Serum cytokine levels

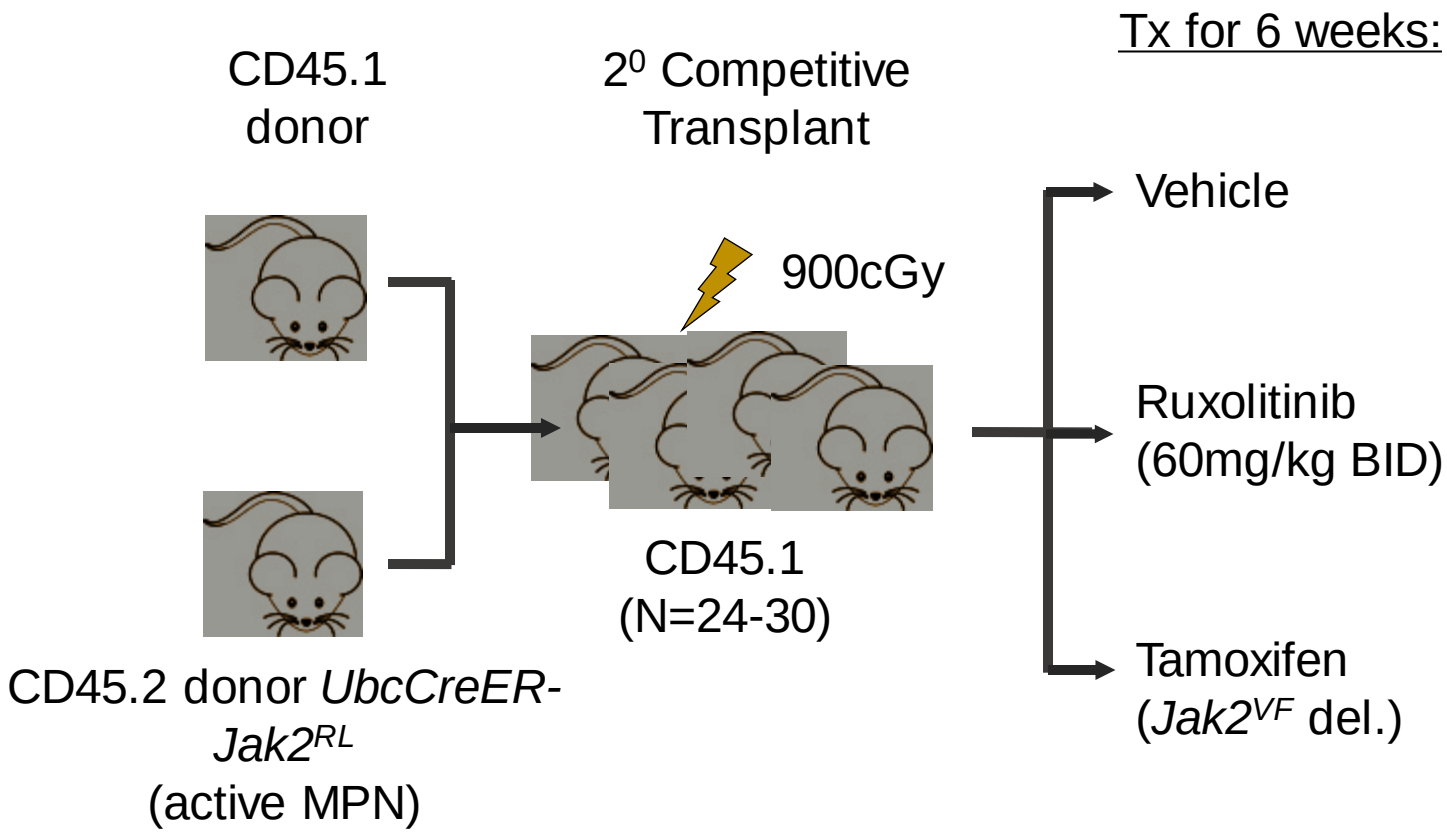


Survival Analysis

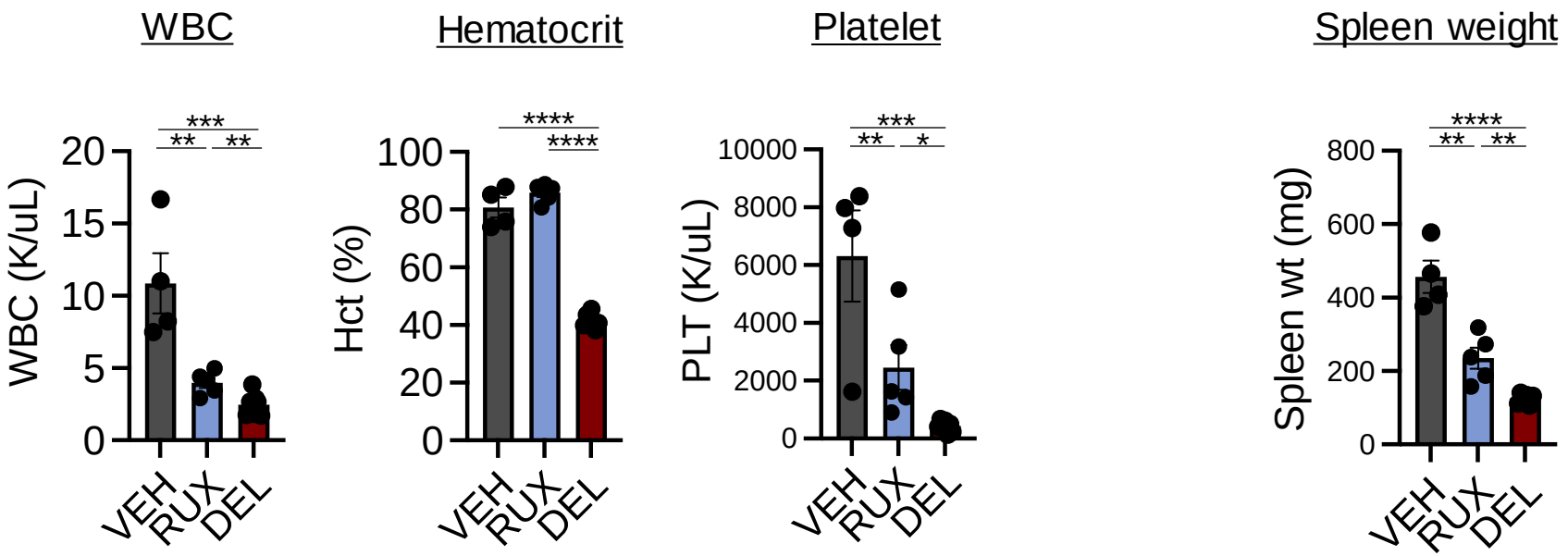


How Does *Jak2*^{VF} Deletion Compare to Ruxolitinib

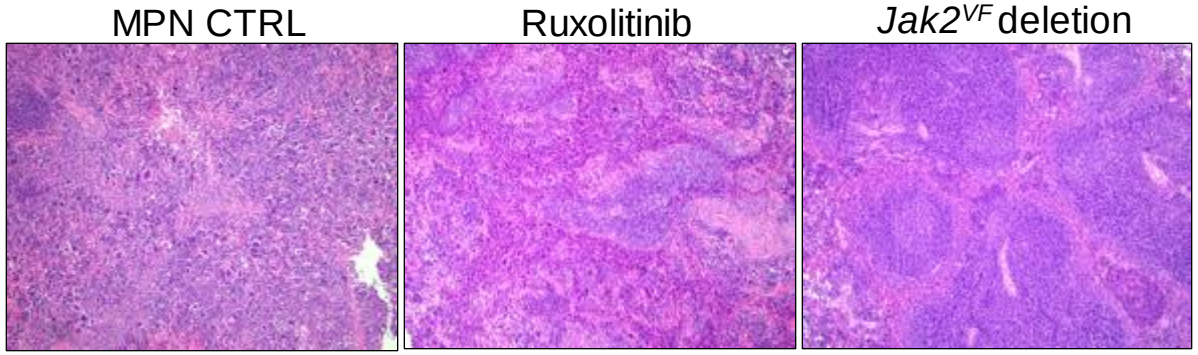
Schematic of the competitive transplant drug trial:



Jak2^{VF} Deletion is Superior to Ruxolitinib Therapy in Attenuating MPN Disease Parameters

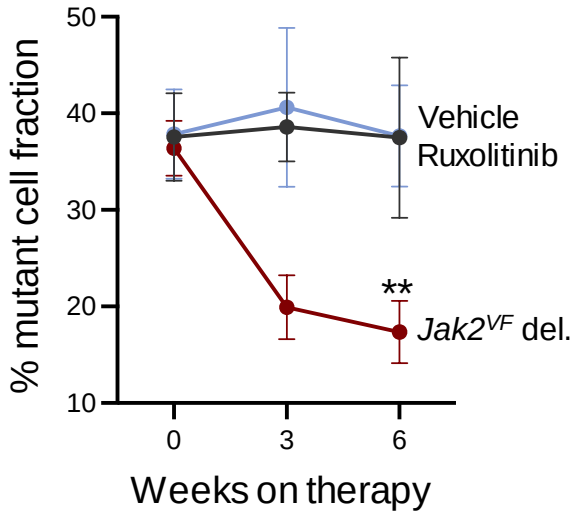


Spleen

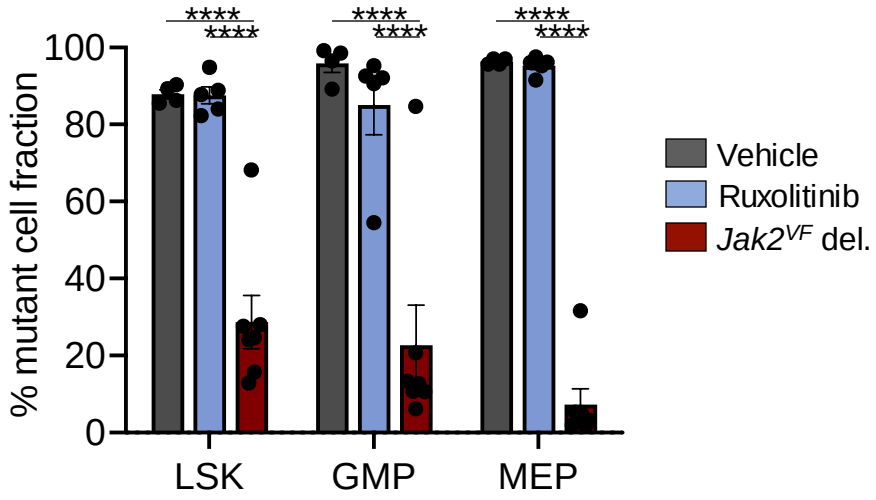


Jak2^{VF} Deletion Reduces Mutant Clonal Fraction and Depletes MPN Stem Cells

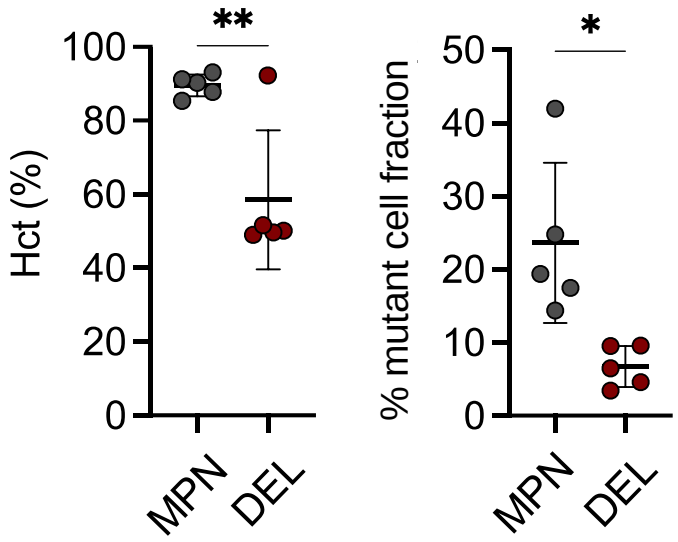
Mutant fraction (PB)



Mutant fraction (BM HSPCs)

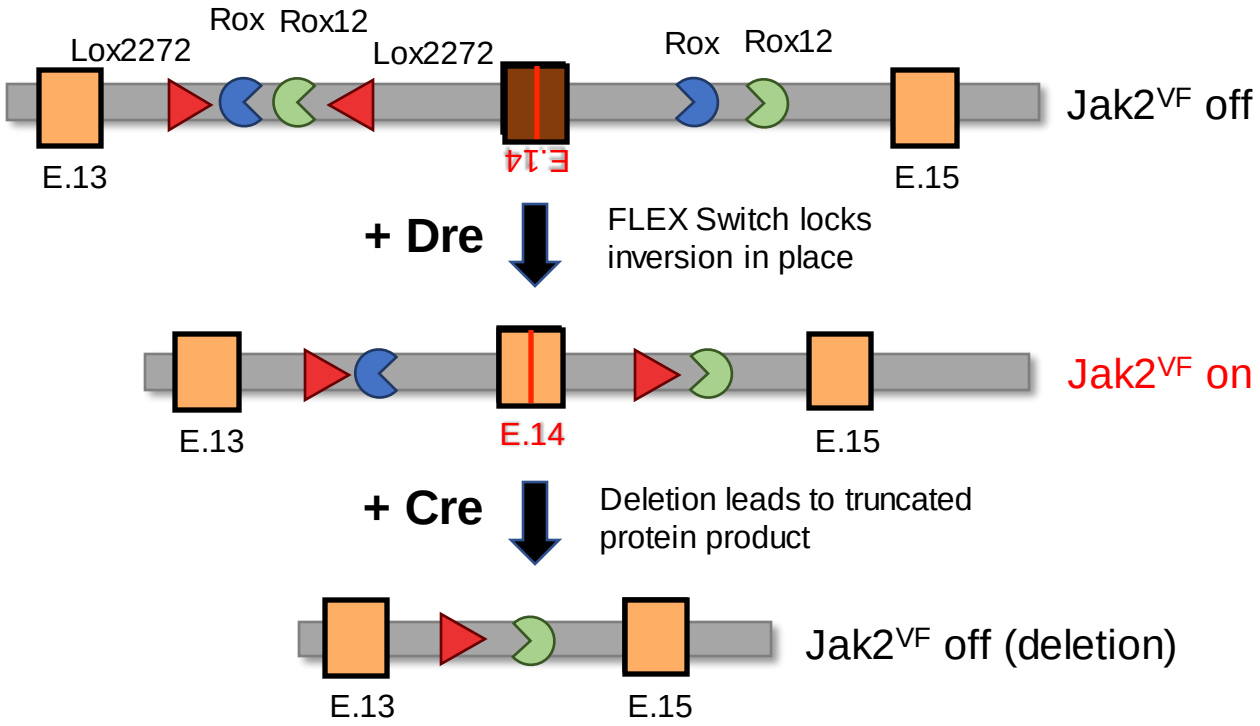


Secondary transplant

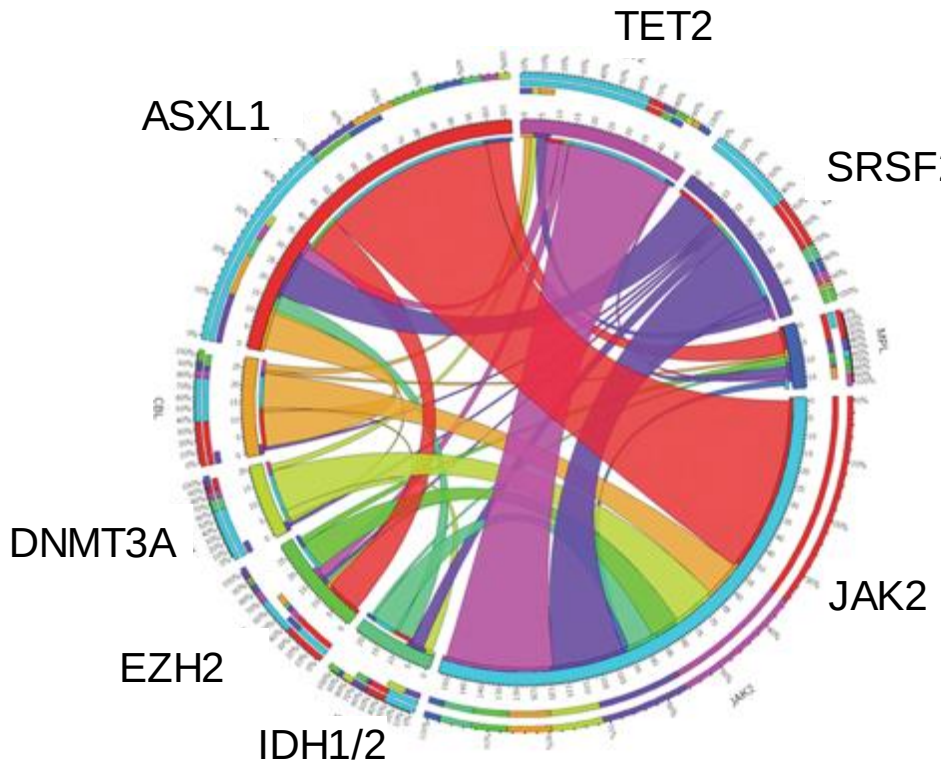


Dre/Cre System Allows Us to Evaluate *Jak2^{VF}* Oncogenic Dependency In Context of Co-occurring Mutations

Jak2^{RL} on/off allele

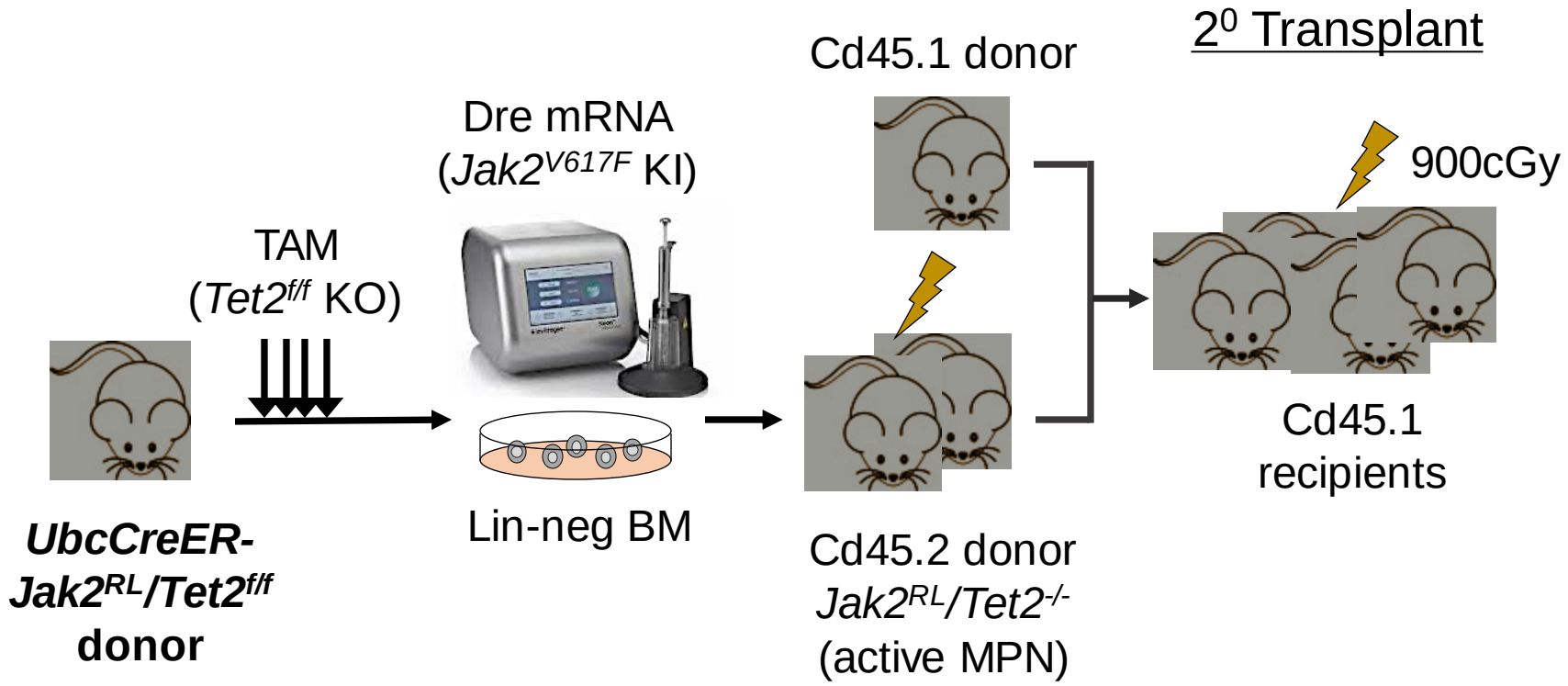


Common co-occurring mutations



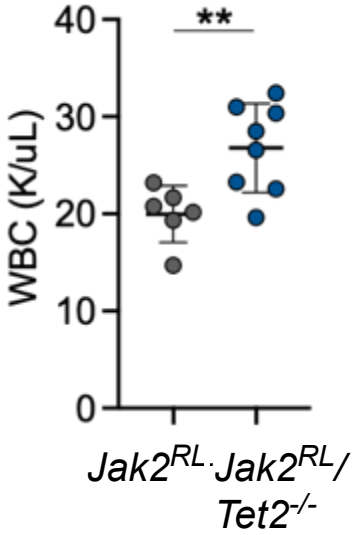
Vannucchi et al, *Leukemia* 2013
Chen et al, *Blood* 2015
Ortmann et al, *NEJM* 2015

Assessing *Jak2*^{VF} Dependency in the Setting of *Tet2* Loss

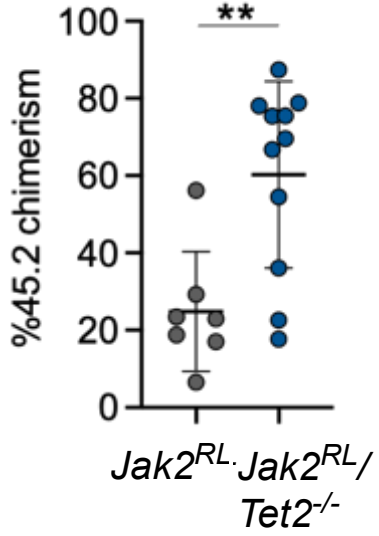


Tet2 Loss, Followed by *Jak2*^{VF} Knock-in, Accelerates MPN *In Vivo* And Ehnances Self Renewal

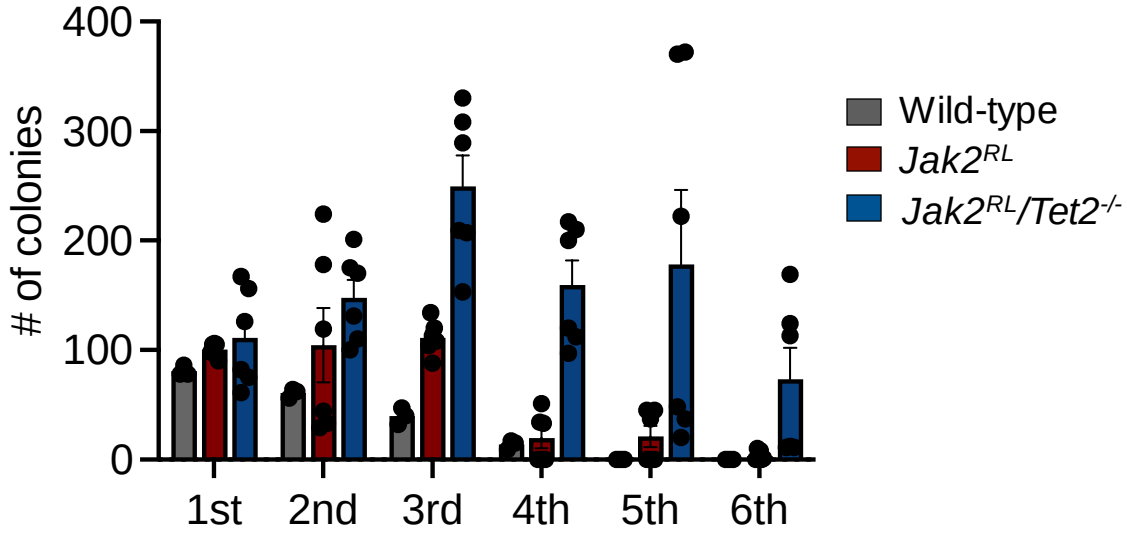
WBC



PB mutant cell fraction

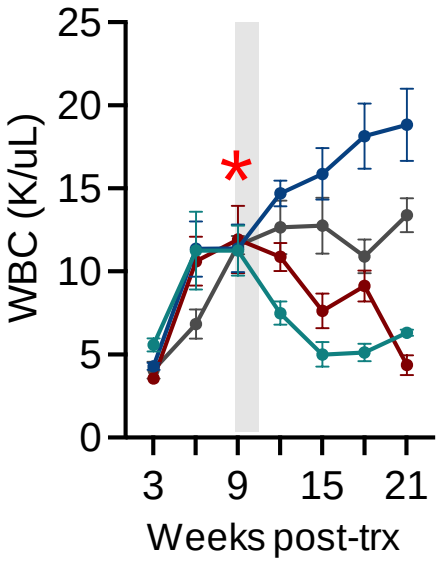


Serially replating assay

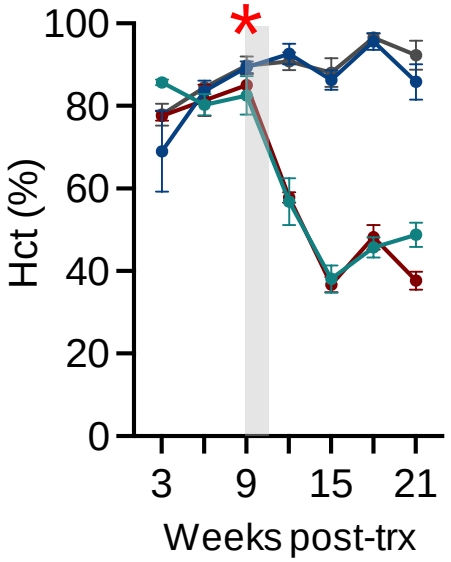


Jak2^{VF}/*Tet2*$^{-/-}$ MPN Cells Remain Dependent on JAK/STAT Signaling

WBC



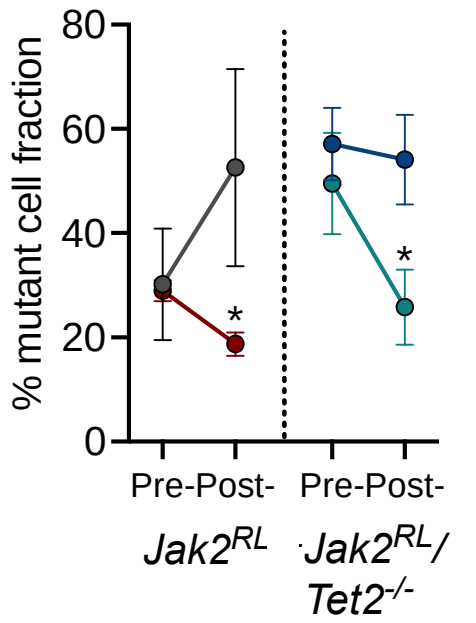
Hematocrit



* Induce *Jak2*^{VF} del.

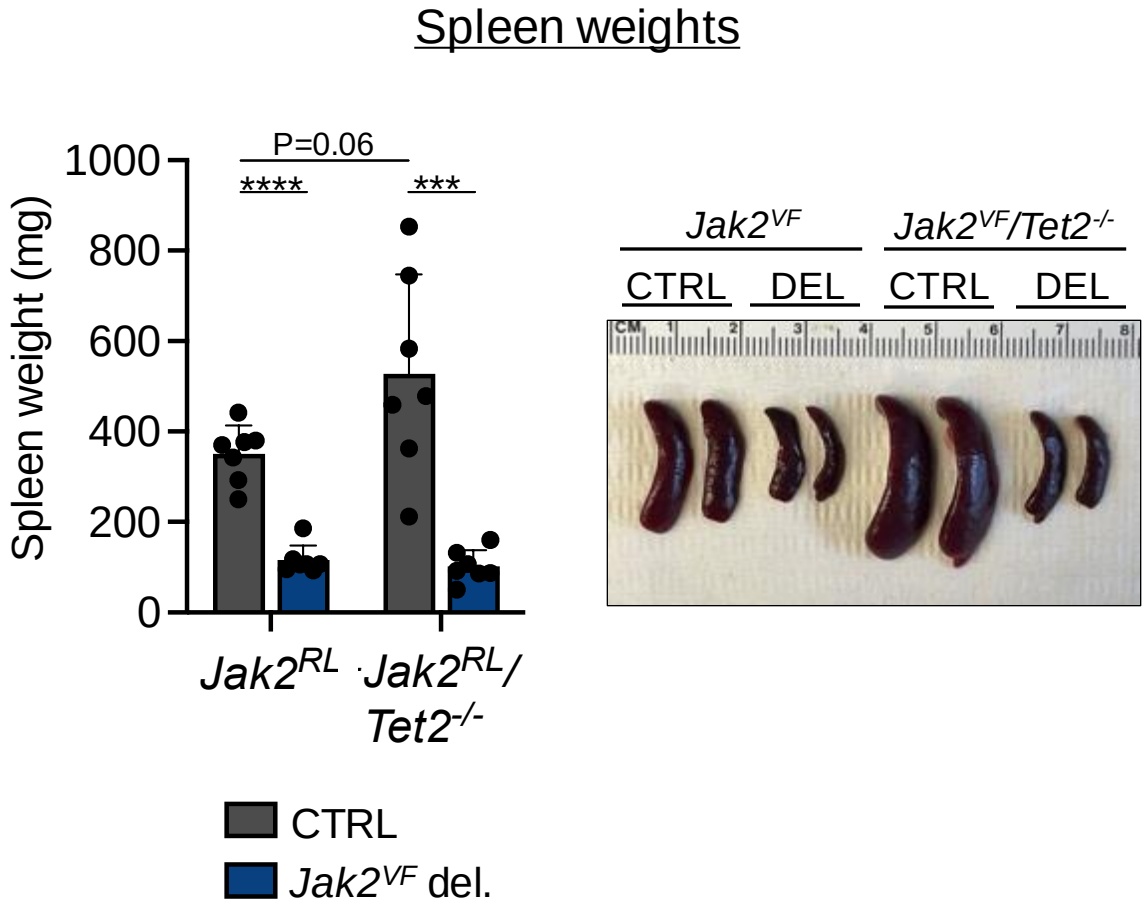
- *Jak2*^{RL} CTRL
- *Jak2*^{RL} TAM
- *Jak2*^{RL}/*Tet2*$^{-/-}$ CTRL
- *Jak2*^{RL}/*Tet2*$^{-/-}$ TAM

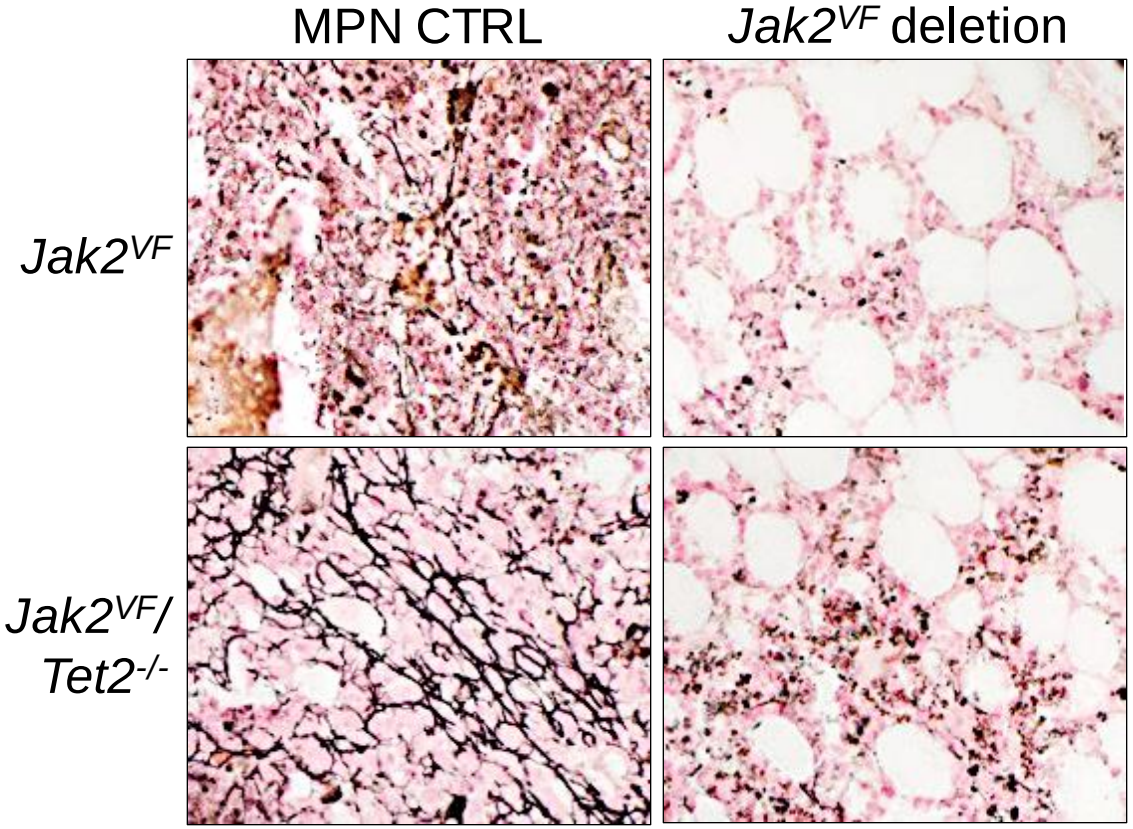
PB mutant cell fraction



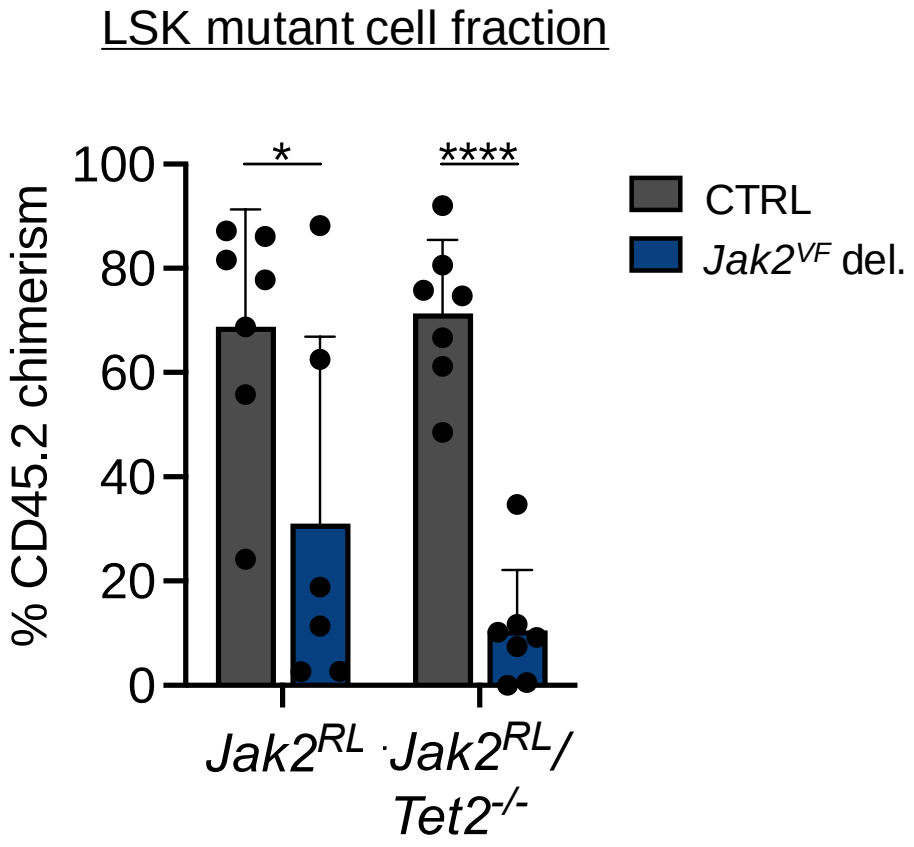
- *Jak2*^{RL} CTRL
- *Jak2*^{RL} TAM
- *Jak2*^{RL}/*Tet2*$^{-/-}$ CTRL
- *Jak2*^{RL}/*Tet2*$^{-/-}$ TAM

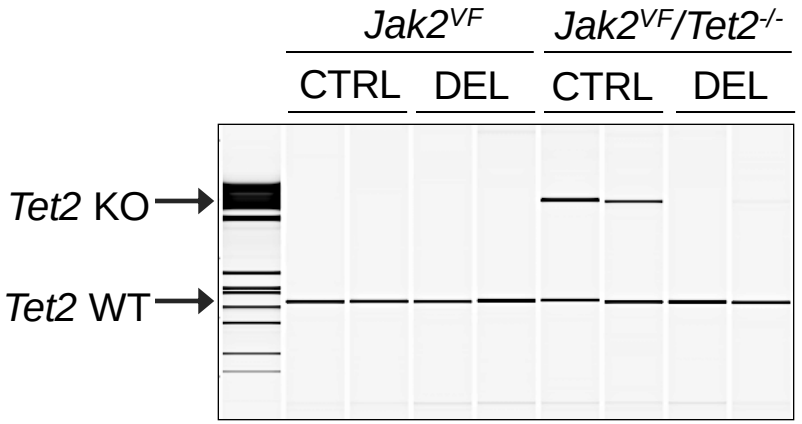
Jak2^{VF} Reversal Normalizes Splenomegaly and Fibrosis of *Jak2*^{VF}/*Tet2*$^{-/-}$ MPN

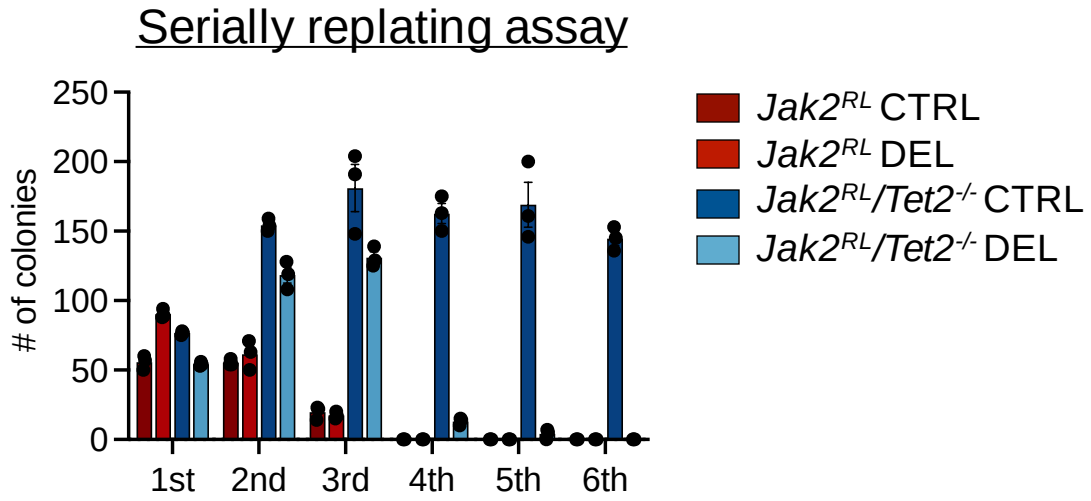




Jak2^{VF} Reversal Reduces MPN Clonal Fitness in *Jak2*^{VF}/*Tet2*$^{-/-}$ Double-Mutant MPN Including in MPN Stem Cells







Conclusions

- We can map the cascade of genetic and epigenetic events which drive malignant transformation

- We can model clonal evolution with increasing accuracy and develop better preclinical leukemia (cancer) models for biologic and therapeutic studies

- Novel preclinical systems can be used to identify genetic/pharmacologic dependencies in clones with defined genotypes->identify therapeutic targets which abrogate myeloid transformation and/or target specific mutations/clones in leukemia patients