Can we classify cancer using cell signaling?

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Single Cell Profiling of Potentiated Phospho-Protein Networks in Cancer Cells

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Central hypotheses (big ideas)

- “Alterations to signaling genes would cause leukemic cells to react in an inappropriate or sensitized manner to environmental inputs and this differential signaling can be read out by flow cytometry.”

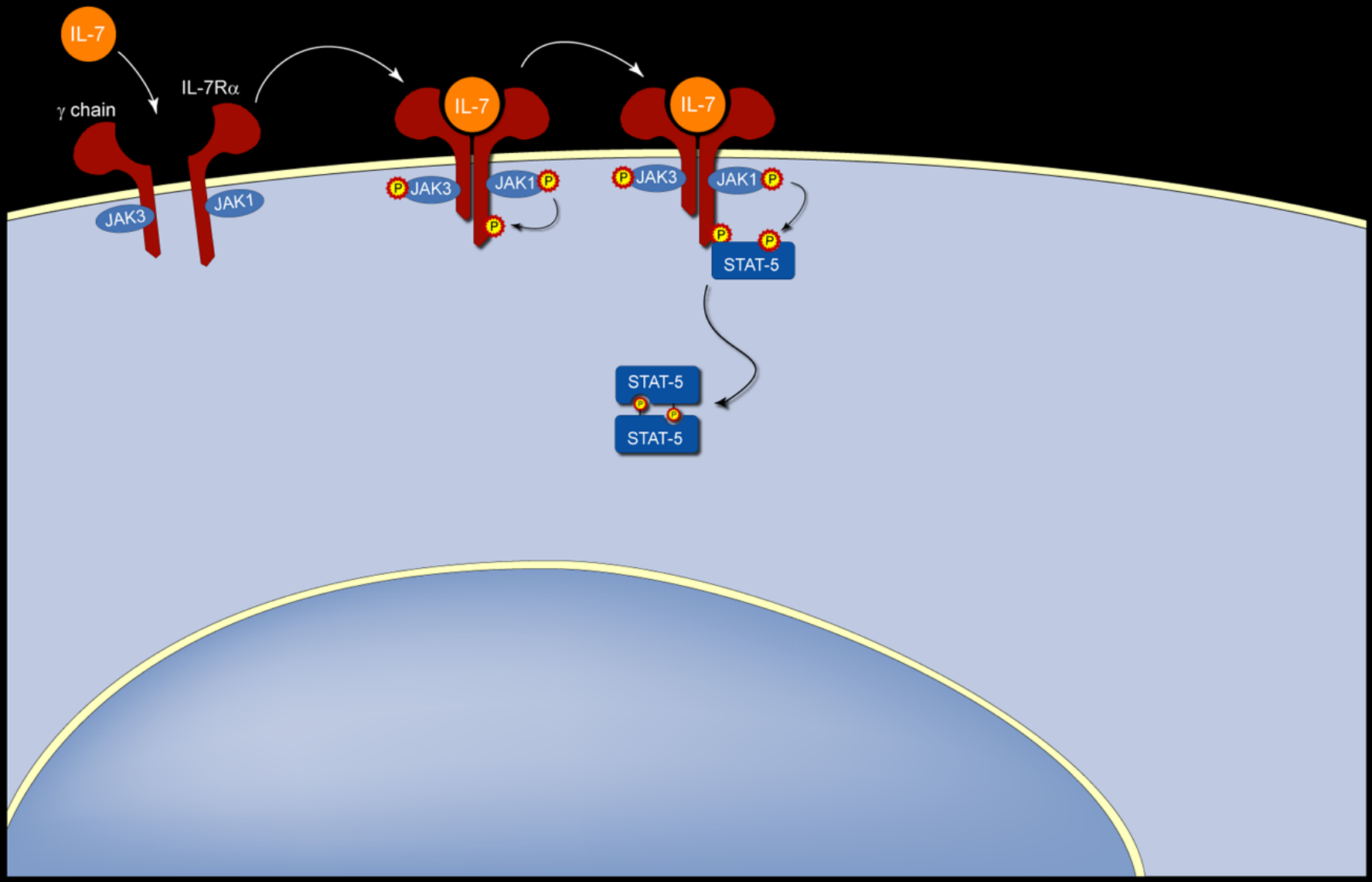
- Classification of patients by this differential cell signaling will reveal groups of patients with shared clinical outcomes and identify signaling events driving leukemia aggressiveness.

Background & rationale:
Signaling => Cancer

Why measure signaling?

(in healthy cells, cancer, and other human diseases)

Cell Signaling Programs Cell Function and Fate



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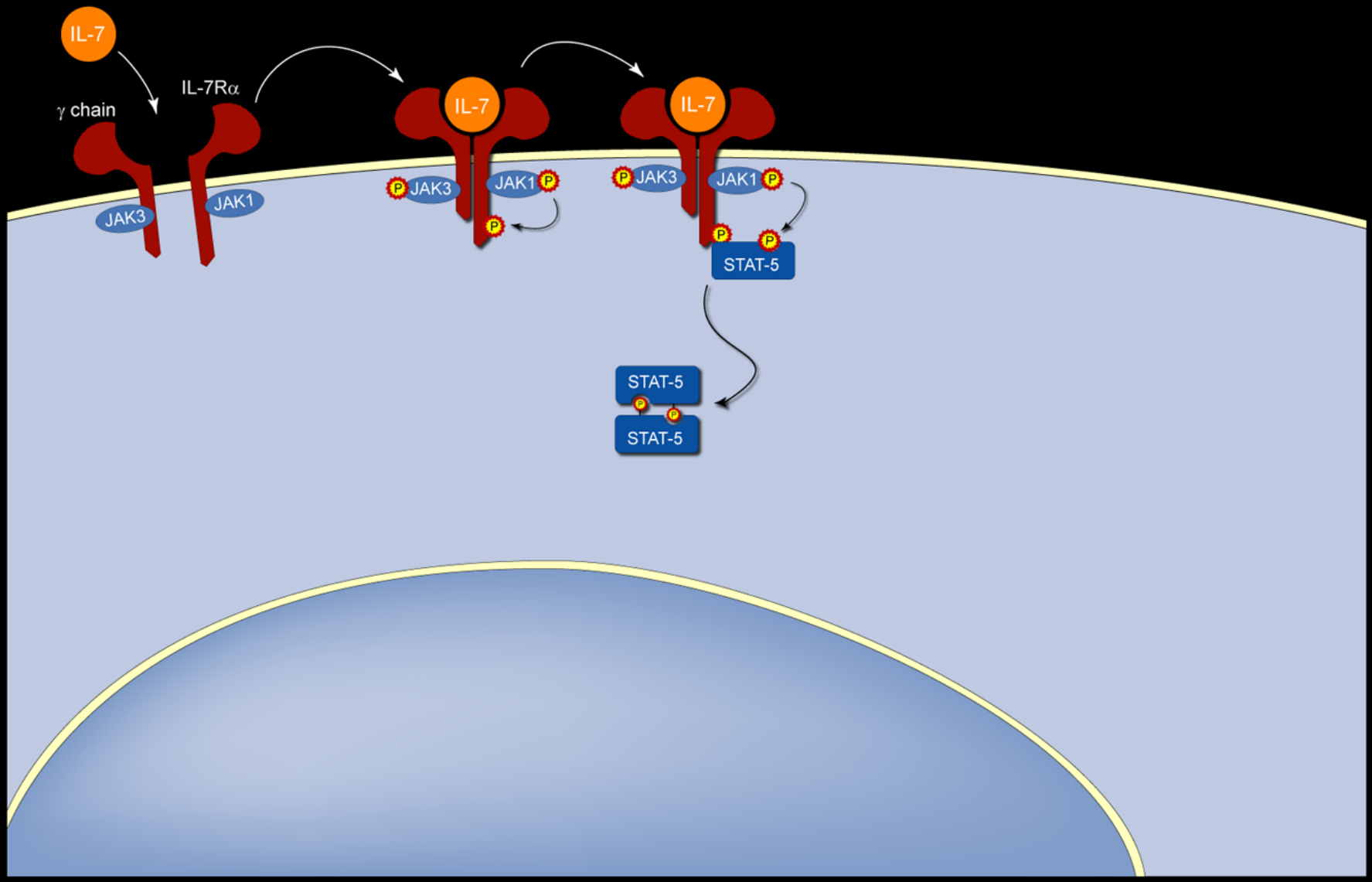
Cell Signaling Programs Cell Function and Fate



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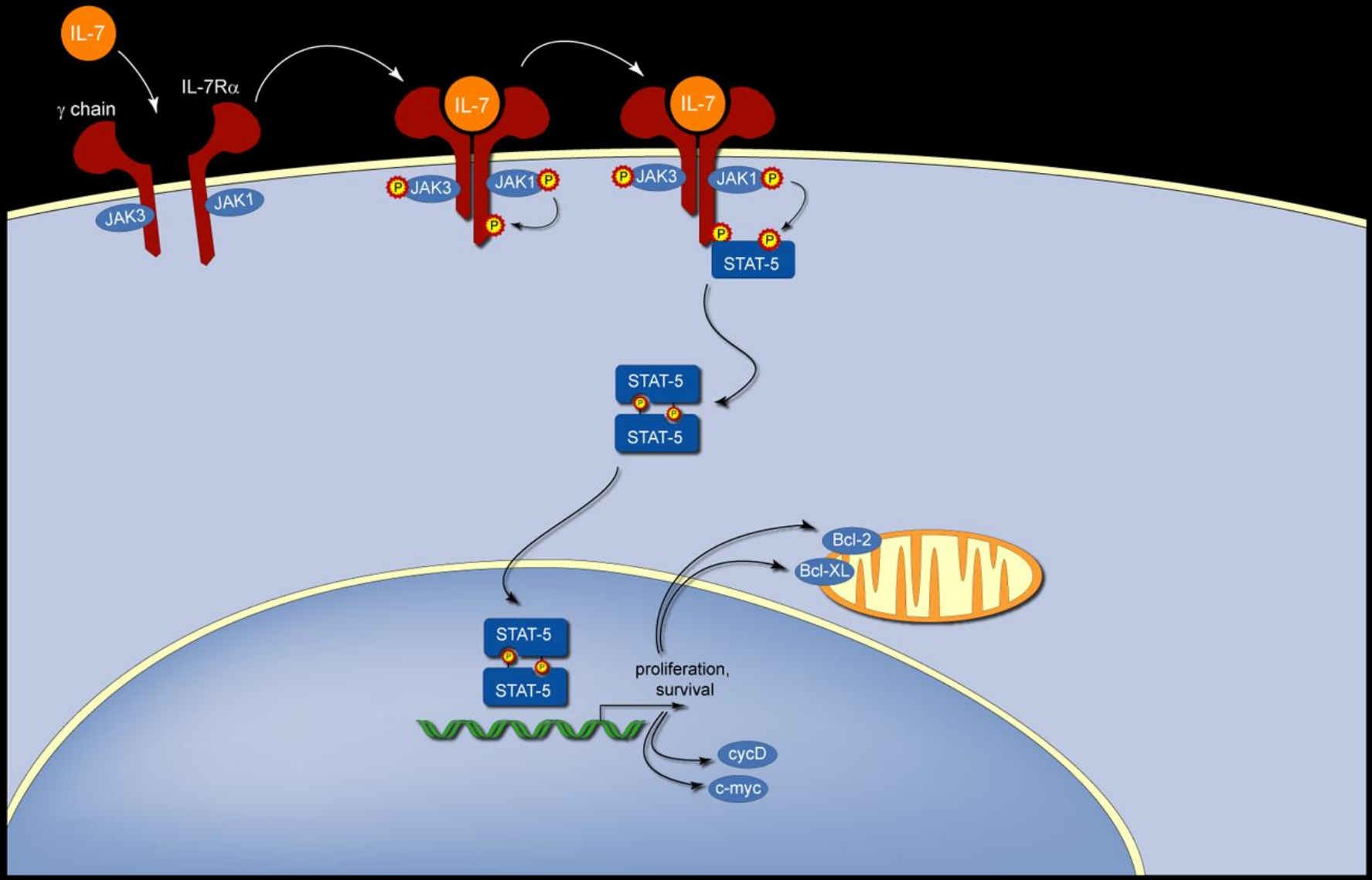

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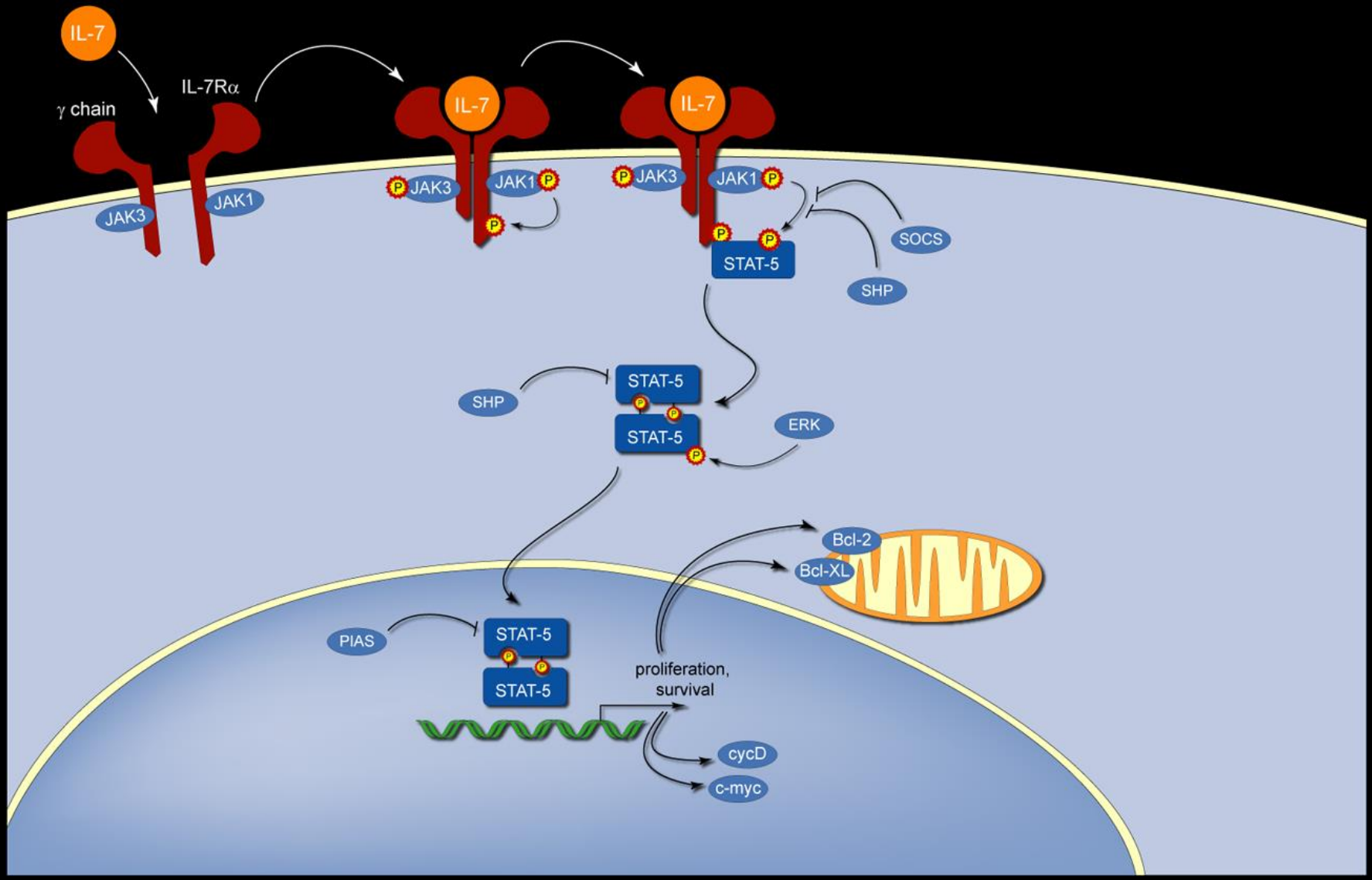
Cell Signaling Programs Cell Function and Fate



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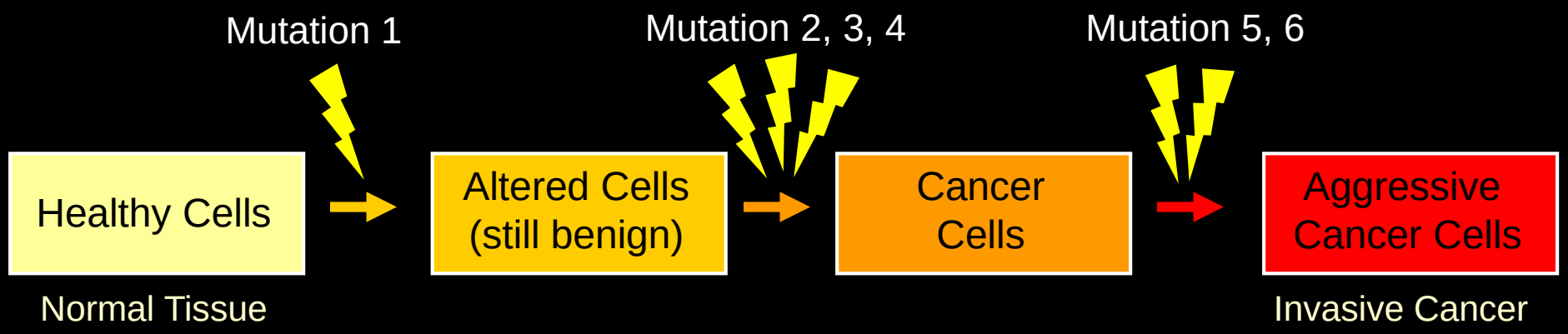
Changes to Cell Signaling are Important Steps in Cancer Progression



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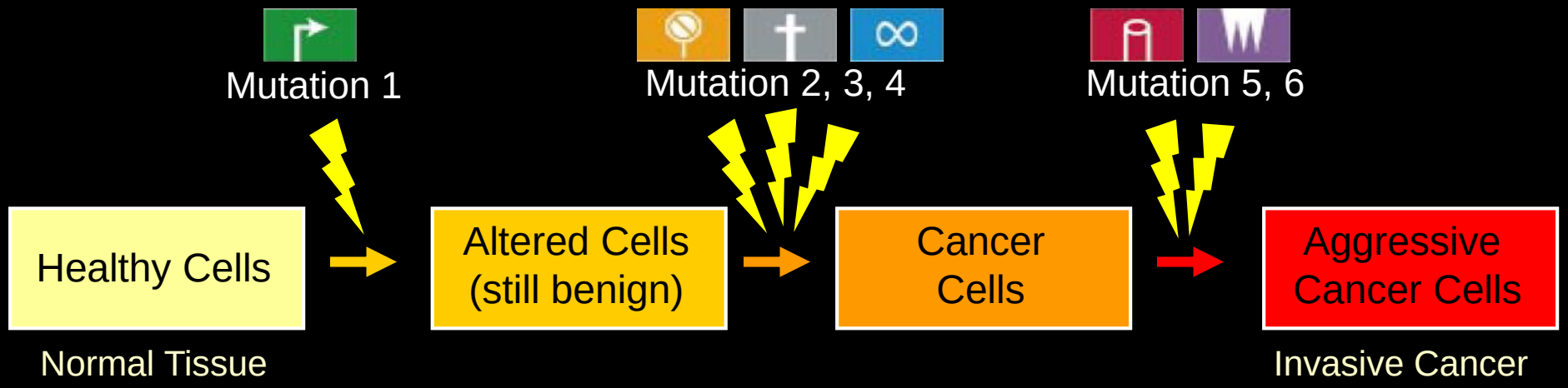
Lessons from Hereditary Colorectal Cancer

Kenneth W. Kinzler* and Bert Vogelstein*†
*The Johns Hopkins Oncology Center
†Howard Hughes Medical Institute
424 North Bond Street
Baltimore, Maryland 21231

Kinzler and Vogelstein, *Cell* 1996

Changes to Cell Signaling are Important Steps in Cancer Progression



Acquired Capability

- Self-sufficient growth
- Insensitive to anti-growth
- Evading cell death
- Limitless replication potential
- Growing blood vessels
- Tissue invasion

Cell, Vol. 100, 57–70, January 7, 2000, Copyright ©2000 by Cell Press

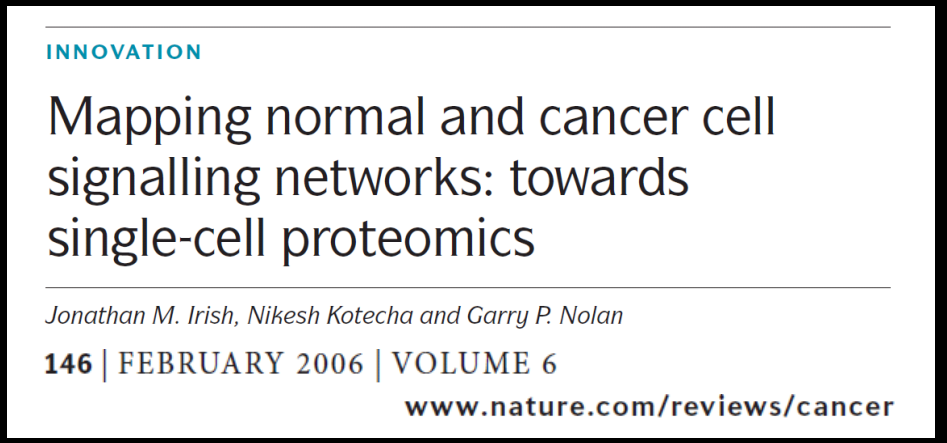
The Hallmarks of Cancer

Douglas Hanahan* and Robert A. Weinberg†
*Department of Biochemistry and Biophysics and Hormone Research Institute
University of California at San Francisco
San Francisco, California 94143
†Whitehead Institute for Biomedical Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02142

Kinzler and Vogelstein, *Cell* 1996

Hanahan and Weinberg, *Cell* 2000

Changes to Cell Signaling are Important Steps in Cancer Progression



Acquired Capability	Example Signaling Alteration
Self-sufficient growth	↑ RAS/RAF/ERK signaling
Insensitive to anti-growth	↓ STAT1, PTEN signaling
Evading cell death	↑ STAT5, ↓ p53 signaling
Limitless replication potential	↑ AKT signaling
Growing blood vessels	↑ VEGF signaling
Tissue invasion	↑ EGFR, WNT signaling

} Altered signaling supports cancer cell survival, aggressive behavior

Kinzler and Vogelstein, *Cell* 1996 Hanahan and Weinberg, *Cell* 2000 Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006

Blocking Malignant Signaling Can Kill Cancer Cells

>95% of chronic myelogenous leukemia (CML) patients have a 'BCR-ABL' gene mutation that alters cell signaling

In CML, BCR-ABL mutation alters signaling } CML cell survival, aggressive behavior

The New England Journal of Medicine

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VOLUME 344 APRIL 5, 2001 NUMBER 14

EFFICACY AND SAFETY OF A SPECIFIC INHIBITOR OF THE BCR-ABL TYROSINE KINASE IN CHRONIC MYELOID LEUKEMIA

BRIAN J. DRUKER, M.D., MOSHE TALPAZ, M.D., DEBRA J. RESTA, R.N., BIN PENG, PH.D., ELISABETH BUCHDUNGER, PH.D., JOHN M. FORD, M.D., NICHOLAS B. LYDON, PH.D., HAGOP KANTARJIAN, M.D., RENAUD CAPDEVILLE, M.D., SAYURI OHNO-JONES, B.S., AND CHARLES L. SAWYERS, M.D.

Druker et al., *New Eng J Med* 2001

Blocking Malignant Signaling Can Kill Cancer Cells

In CML,
BCR-ABL mutation
alters signaling

} CML cell survival,
aggressive behavior

Druker et al., *New Eng J Med* 2001

Blocking Malignant Signaling Can Kill Cancer Cells

Block BCR-ABL with Gleevec,
shut down altered cancer cell signaling

In CML,
BCR-ABL mutation
alters signaling

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aggressive behavior

Druker et al., *New Eng J Med* 2001

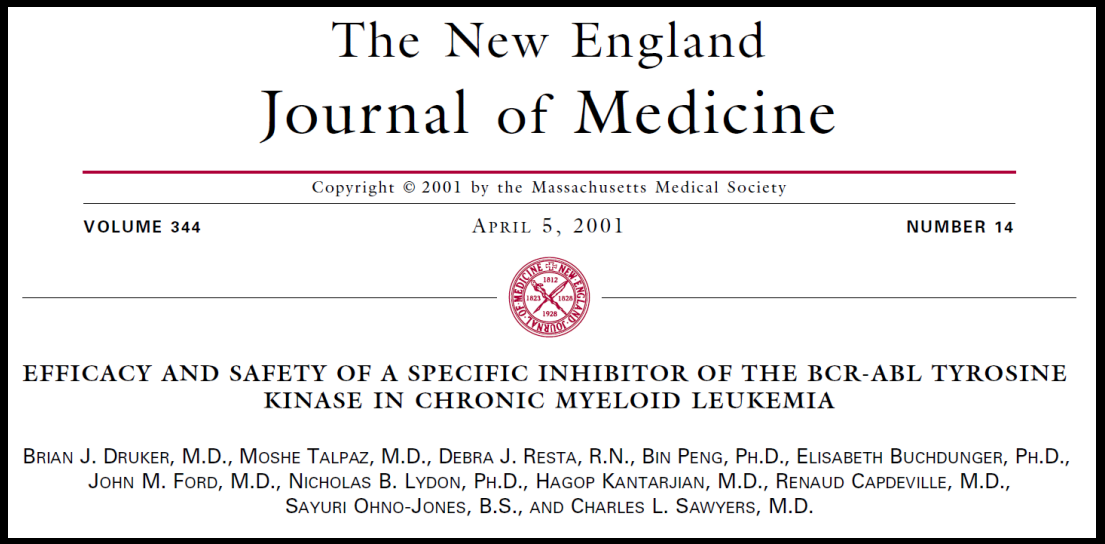
Blocking Malignant Signaling Can Kill Cancer Cells

Block BCR-ABL with Gleevec,
shut down altered cancer cell signaling

In CML,
BCR-ABL mutation
alters signaling

CML cell survival,
aggressive behavior

Leukemia cells died



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Druker et al., *New Eng J Med* 2001

Can we generalize the ‘targeted therapy approach’
by identifying driving signaling events in other cancers?

Can tumors be described in terms of cell signaling?

Study Design:

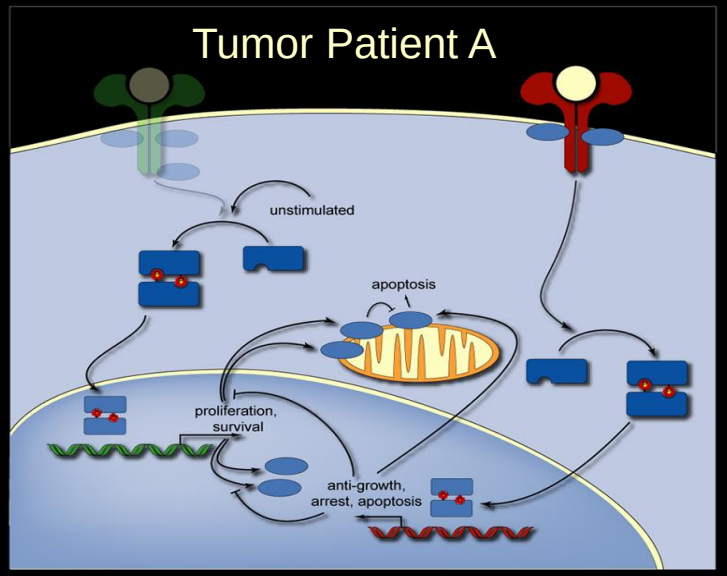
- Map signaling mechanisms across tumors and construct a signaling taxonomy.

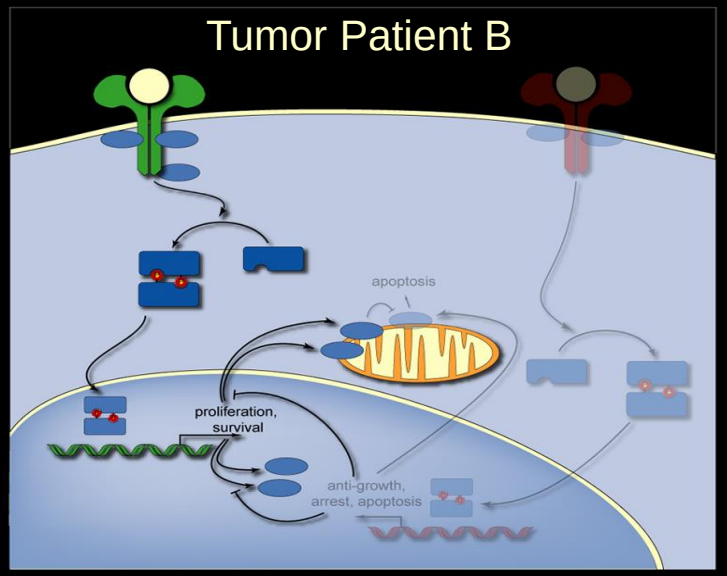
Hypotheses:

- 1) Heritable changes to cancer cells will detectably modify signaling networks.
- 2) Patients whose tumors share mechanisms of proliferative signaling will respond similarly to tumor cell killing.

Rationale:

- Signaling mutations are common, vary across tumors, and contribute to pathology.
- Will rigorously describe molecular differences among tumors.
- Will inform drug development and individual assessment of therapy and risk.





Constructing a Toolset to Probe Signaling

Combine strengths from multiple disciplines…

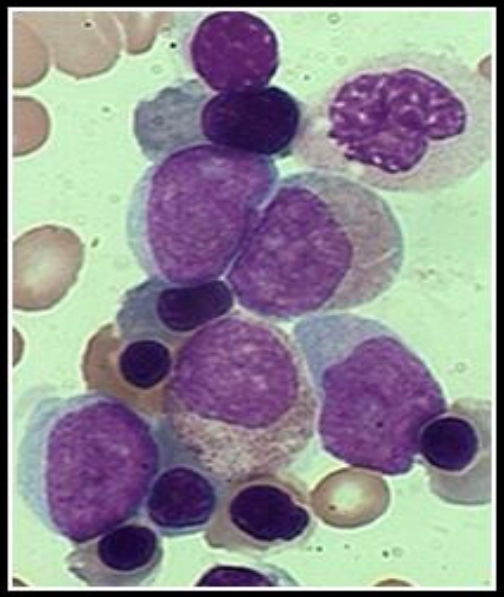
- Immunology: Measure events at the individual cell level
- Molecular Biology: Monitor signaling biochemistry (phosphorylation)
- Genomics: Detect and display numerous events, statistical tools

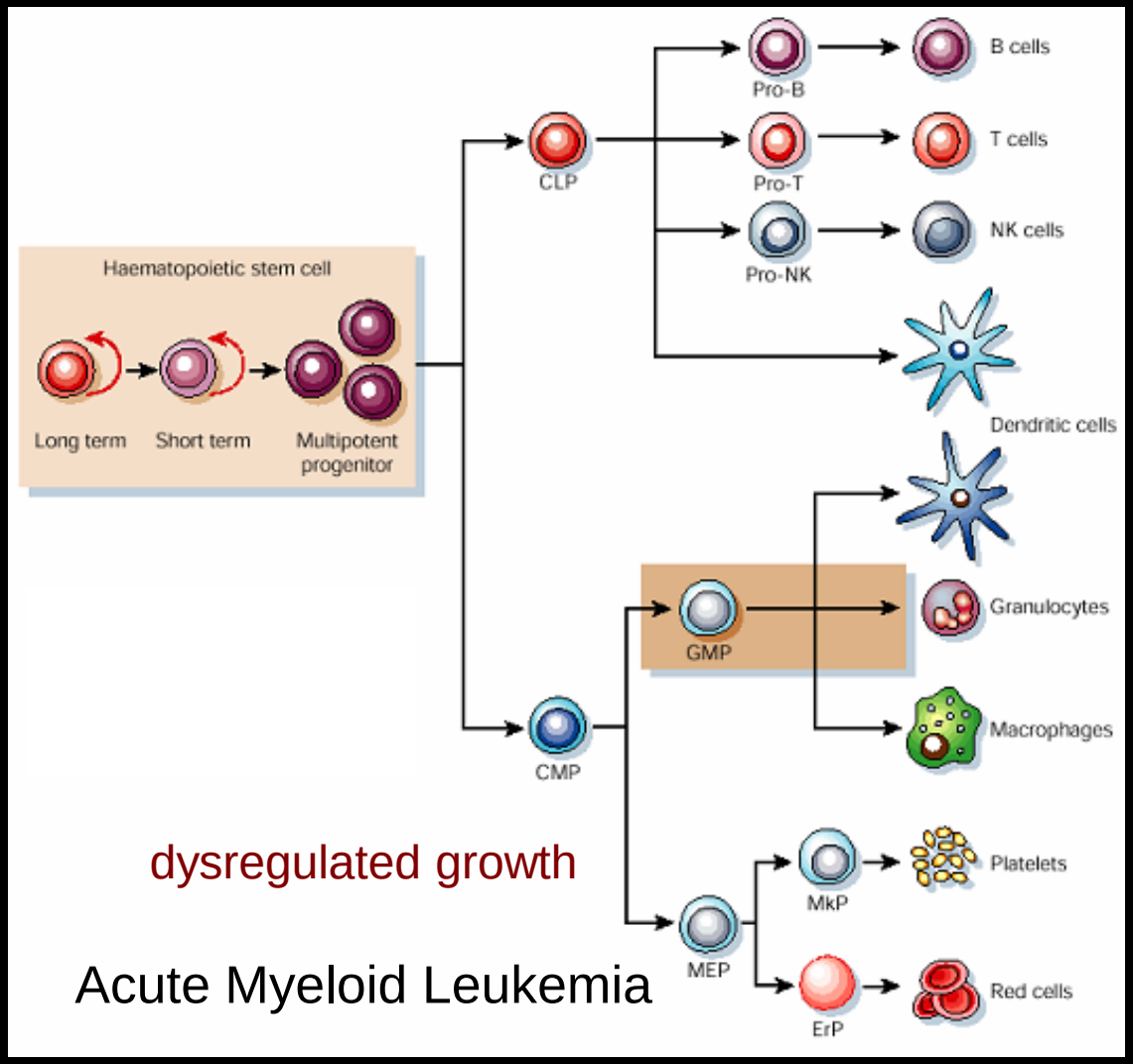
… to ask new questions about tumor signaling mechanisms

Background: Acute Myeloid Leukemia

Acute Myeloid Leukemia

AML blasts



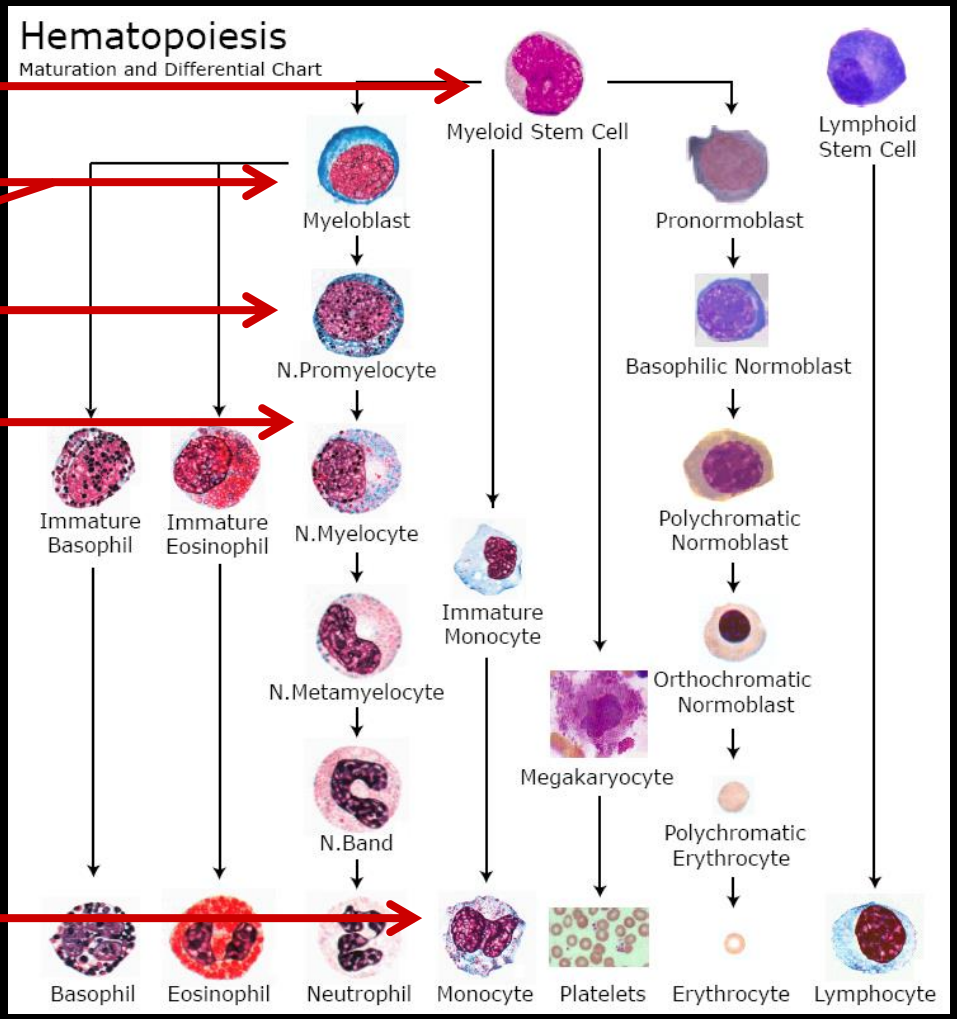


Reya and Weissman, Nature 2001

Classic AML Classification

FAB (primarily morphology)

- M0 – undifferentiated AML
- M1 – myeloblastic, immature
- M2 – myeloblastic, mature
- M3 – promyelocytic
- M4 – myelomonocytic
- M5 – monocytic



Cytogenetics + Flt3 mutation

- translocations, deletions, etc.
- frequent alterations to signaling genes
- many patients intermediate risk
- mechanism of pathology not well understood.

AML Induction Chemotherapy

Course 1

- Cytarabine (Ara-C) (pyrimidine analog, DNA synthesis inhibitor)
- An anthracycline (e.g., daunorubicin or idarubicin, DNA binding Topoisomerase II inhibitors)

Response

- Frequent relapse and < 50% treatment efficacy
- Only used with younger, healthier patients due to associated toxicity.

Leukemia free survival at 5 years: < 26% +/- 8%

Gajewski *et al.*, Blood 1997; Bruserud *et al.*, Oncologist 2000

Mechanisms of AML Oncogenesis

1) A proliferative advantage, often from aberrant signal transduction

- **Flt-3** mutations
- Increased STAT activity

2) Inhibition of apoptosis and differentiation

- **Bcl-2** family expression
- Inactivation of p53 pathway?

1. Classify / stratify patient risk based on signaling potential?
2. Identify signaling profiles linked with chemotherapy resistance?
3. Link signaling profiles with oncogene expression?

Arrayed phospho-specific flow cytometry, response panel profiles

New terms used in/around this manuscript

- Biosignature – For a disease, the biosignature includes those features that vary more in the disease than in controls

- Potentiated / Attenuated – Strengthened / Weakened
  - "Interrogating the potentiation of signaling pathways" = stimulating a network to reveal its signaling potential

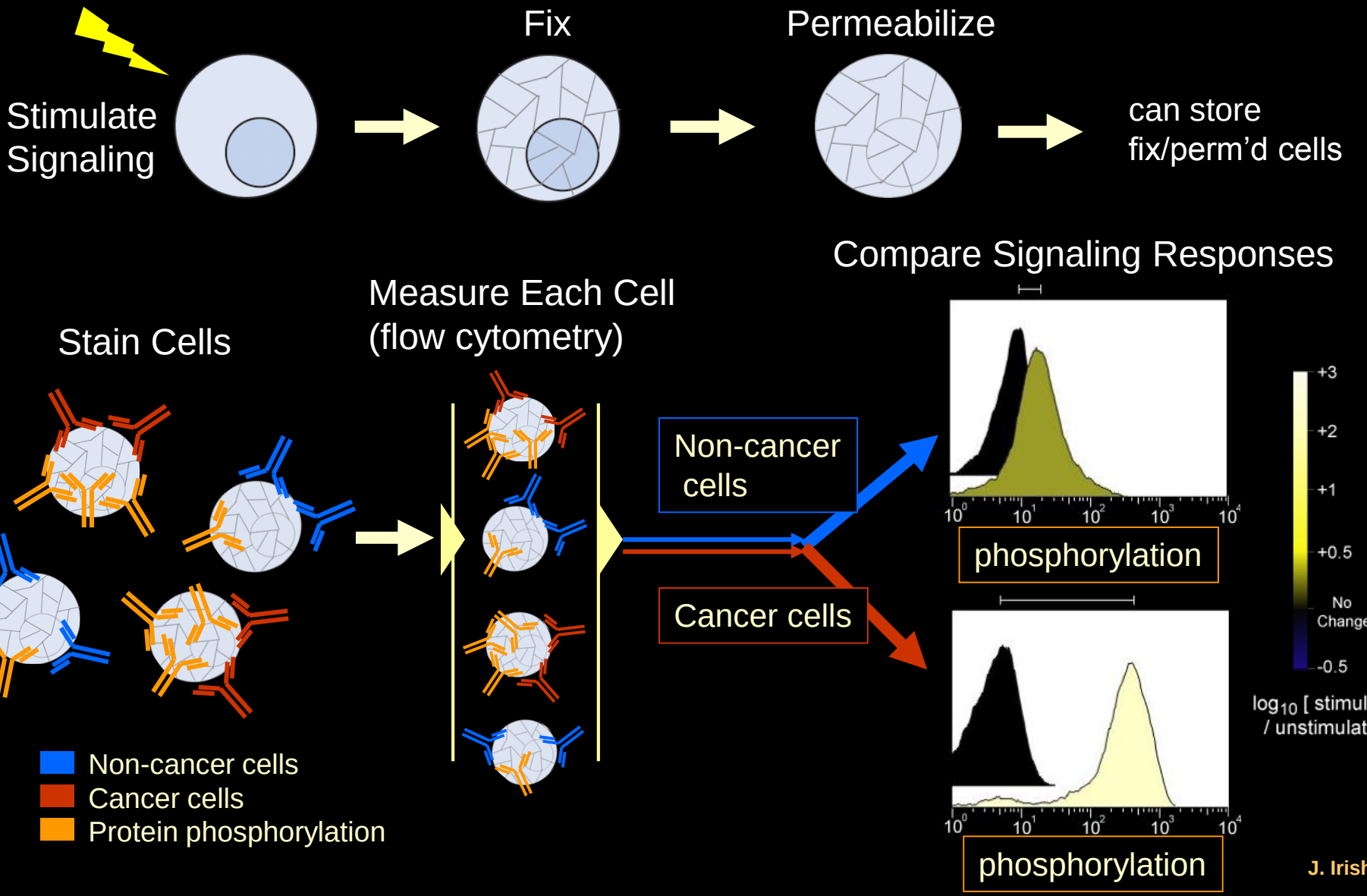
- Signaling Node & State – A signaling event.
  - A signaling node can be a protein, like STAT1. The state of the signaling node might be phosphorylation of Y701 at 15 minutes following 20 ng/mL IFNγ. For more information, see Irish et al. Nature Reviews Cancer 2006.

- Unsupervised vs. Supervised
  - Whether the features used to classify were selected based on prior knowledge of their ability to classify

- Arrayed flow cytometry
  - An array is a systematic arrangement of objects, usually in rows and columns.
  - Early way of referring to showing aggregate data in a heat map

Tools: Phospho-specific flow cytometry (phospho-flow)

Flow Cytometry Measures Signaling in Every Cell within a Sample



Background: Phospho-specific flow cytometry

Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications

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Received 7 November 2003; accepted 10 November 2003

CLINICAL IMMUNOLOGY

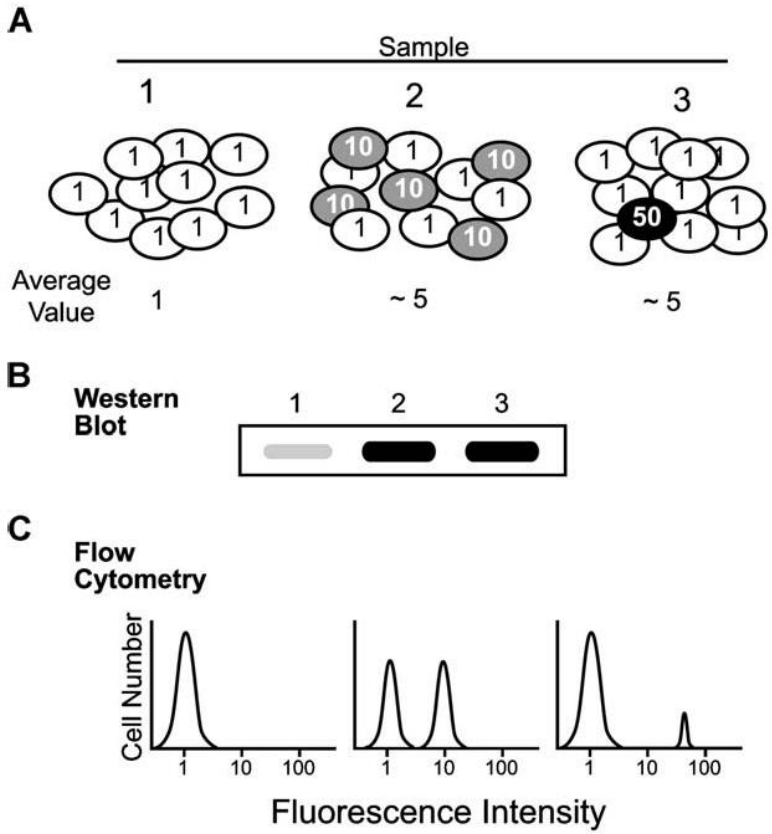
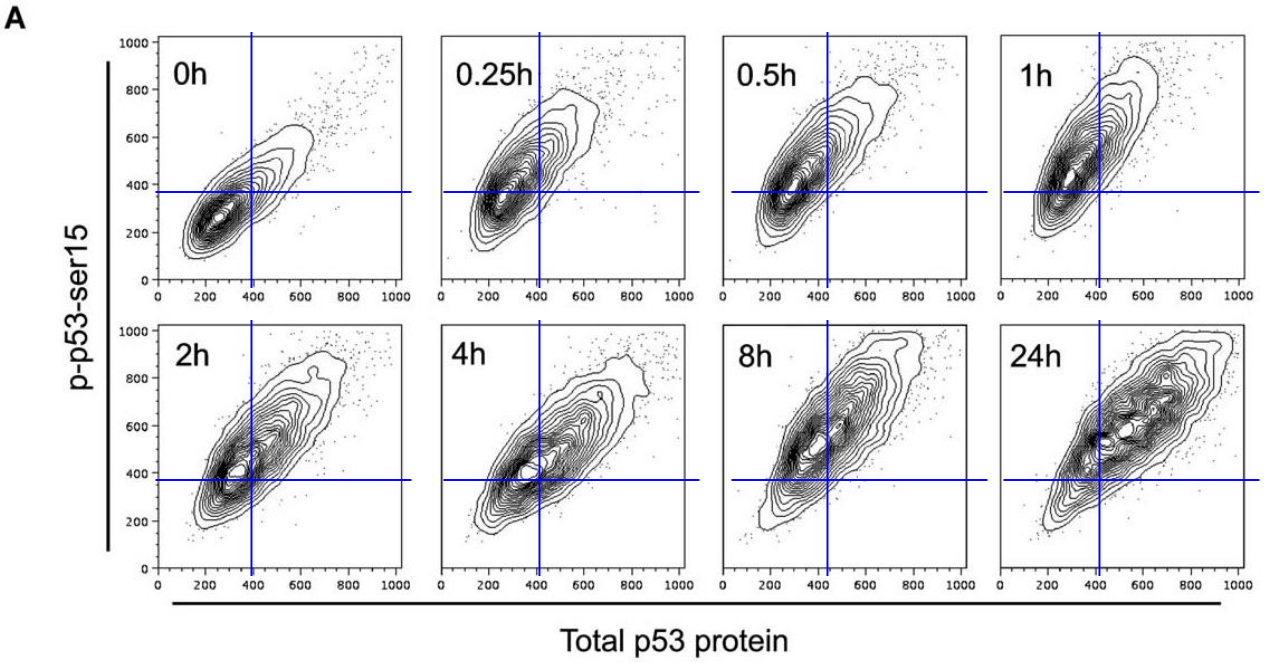


Fig. 1. The advantages of single cell analysis. (A) In this hypothetical experiment, three samples are obtained that contain a protein of interest at 1, 10, or 50 copies per cell as indicated. The average number of protein molecules per cell is 1 for sample 1, and 5 for both samples 2 and 3. (B) When these cell populations are analyzed by Western blotting, samples 2 and 3 will show darker bands but will appear identical to one another. (C) When the samples are stained for the protein with fluorescently labeled antibodies and analyzed by flow cytometry, however, one can clearly see that sample 2 contains cells in two distinct populations that are equally represented, while in sample 3, only about 1 in 10 cells has an elevated level of protein. This kind of heterogeneity in the samples could be due to different cell types (i.e., immune cells), or because of all-or-none type signaling responses.

Flow cytometry can measure both phospho- and total protein levels in single cells

Cells: GM0536 / GM536 (lymphoblastoid CD19+ precursor B cells transformed by EBV, ATM+/+ p53+/+, derived from healthy cells)

Stim: 8 Gray of γ IR



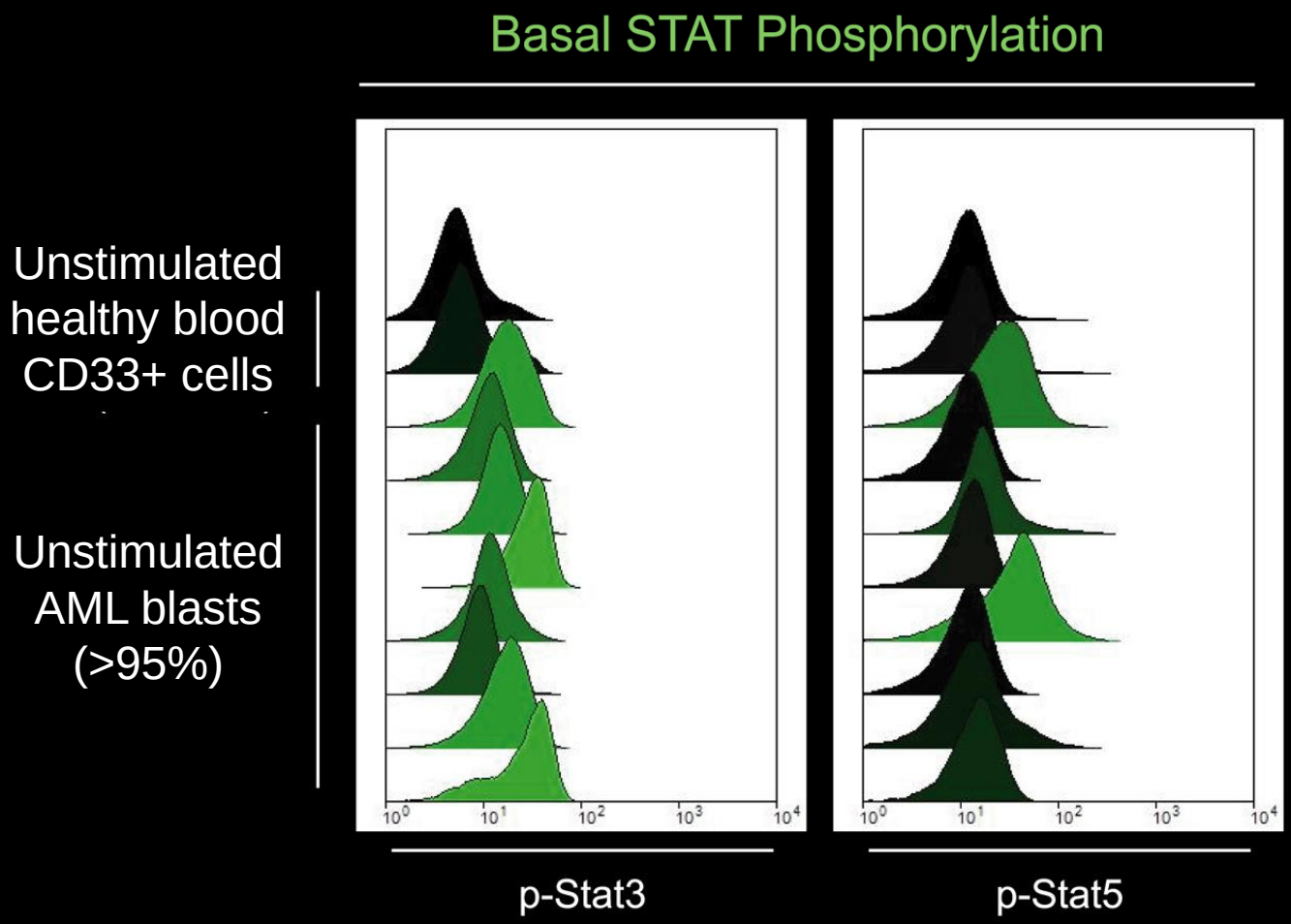
B

Time after 8Gy IR:	0h	0.25h	0.5h	1h	2h	4h	8h	24h	
Fold change in median:	0.00	0.90	1.14	1.46	1.38	1.26	1.92	2.12	p-p53-serine15
	0.00	0.16	0.40	0.40	0.92	1.24	1.34	2.00	Total p53 protein

Fig. 5. Analysis of p53 phosphorylation and total protein levels shows p53 phosphorylation at serine15 precedes accumulation of total p53 protein. (A) GM0536 lymphoblastoid cells were treated with 8 Gy of gamma irradiation and p53 phosphorylation at serine15 and total p53 protein levels were monitored over time following irradiation and compared on a per cell basis. (B) Quantitation of the change in median fluorescence (log_2 converted) of the population over time showed rapid induction of phosphorylation in the first hour followed by a more gradual accumulation of total p53 protein. Similar analyses can be performed on cancerous cells to determine p53 status and activation.

Background: What was known prior?

Basal (constitutive) phosphorylation is common in AML



Basal p-STAT5 in AML is not associated with FLT3 mutation

Flow cytometric measurement of phosphorylated STAT5 in AML: lack of specific association with FLT3 internal tandem duplications

Monica Pallis*, Claire Seedhouse, Martin Grundy, Nigel Russell

Division of Haematology, Clinical Sciences Building, University of Nottingham and Nottingham City Hospital, Nottingham NG5 1PB, UK

Received 15 August 2002; accepted 21 December 2002

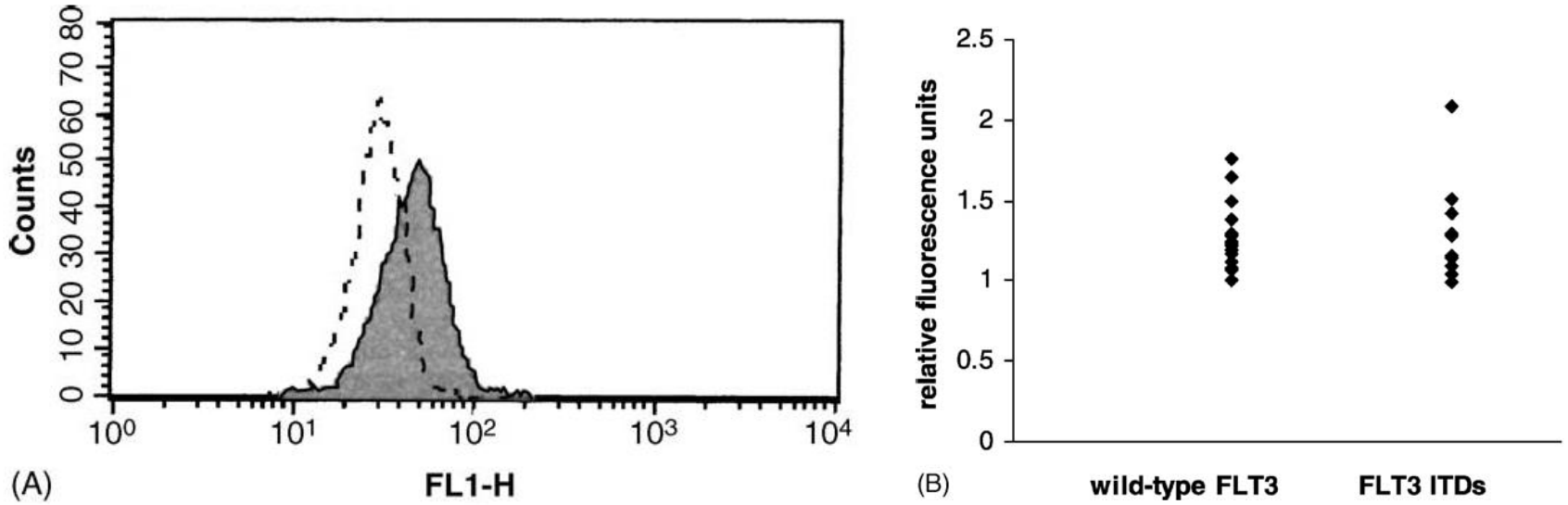
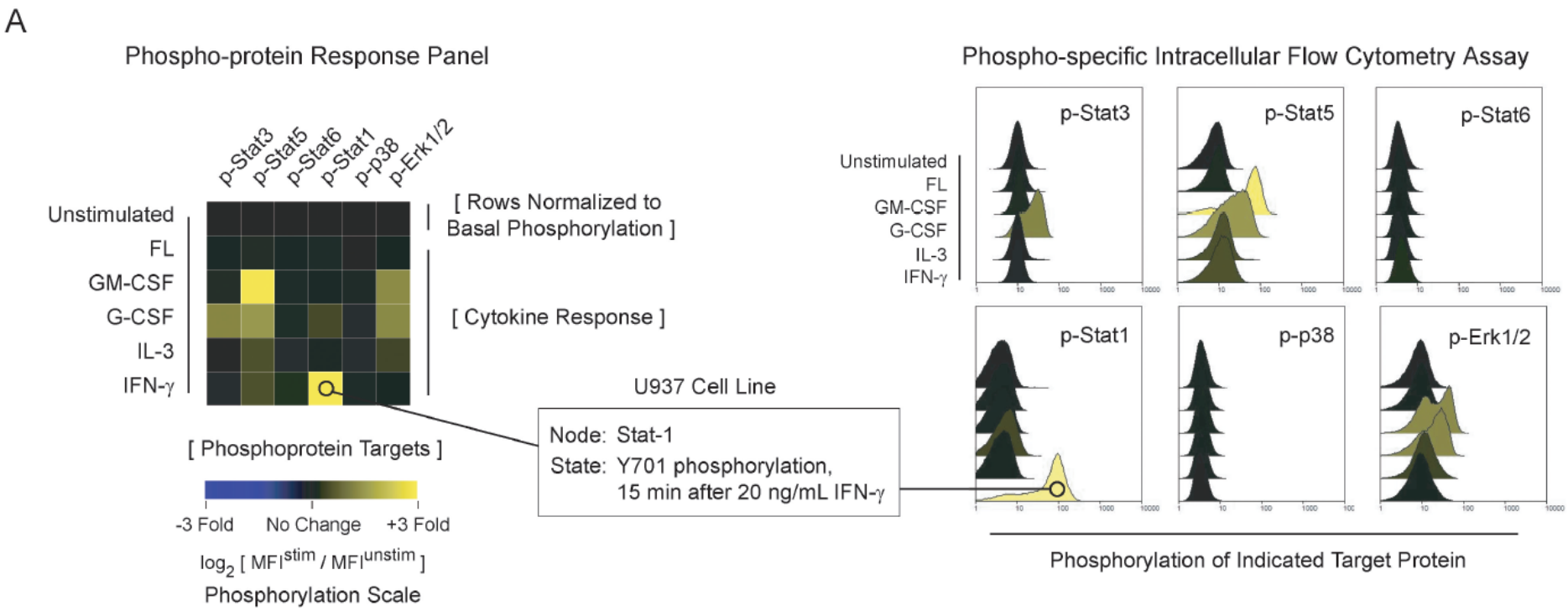


Fig. 2. Phosphorylated STAT5 expression in primary AML cells. (A) An example of phosphorylated STAT5 expression in an AML sample. The shaded histogram indicates phosphorylated STAT5 fluorescence and the dotted-line histogram represents isotype control fluorescence. Test/control fluorescence = 1.51 RFU; (B) phosphorylated STAT5 expression in 28 AML samples. Note the lack of any obvious cut-off point between positive and negative fluorescence and also the lack of differential distribution between FLT3 wildtype ($n = 17$) and FLT3 mutant ($n = 11$) samples.

Figures

Figure 1A: Creation of a 6 x 6 phospho-flow cytokine response panel



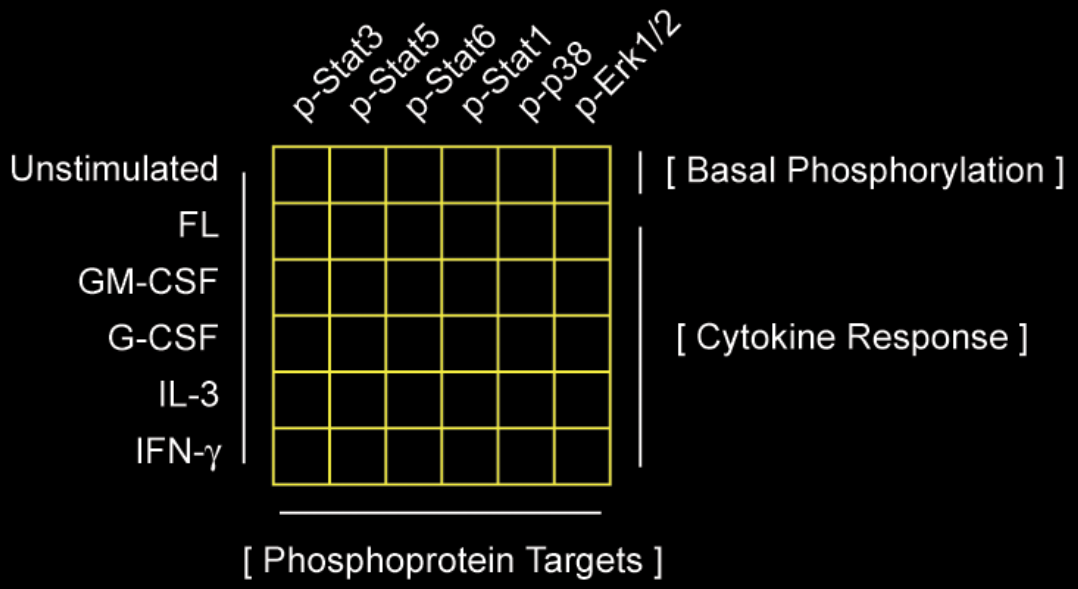
U937 cell line: myeloid histiocytic lymphoma

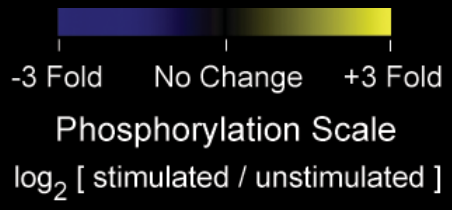
Figure 1. A Cytokine Response Panel Reveals Potentiated Signal Transduction Nodes in Primary Acute Myeloid Leukemias

(A) Stimulation states, shown in rows, included unstimulated or 20 ng/ml of FL, GM-CSF, G-CSF, IL-3, or IFNγ. Target phosphorylations were detected using phospho-specific antibodies for Stat1, Stat3, Stat5, Stat6, p38, and Erk1/2, shown in columns. Each square in the grid represents the response of one phosphorylation site to one condition. The relationship between the grid and the flow cytometry data on which it is based is diagrammed for U937 cells.

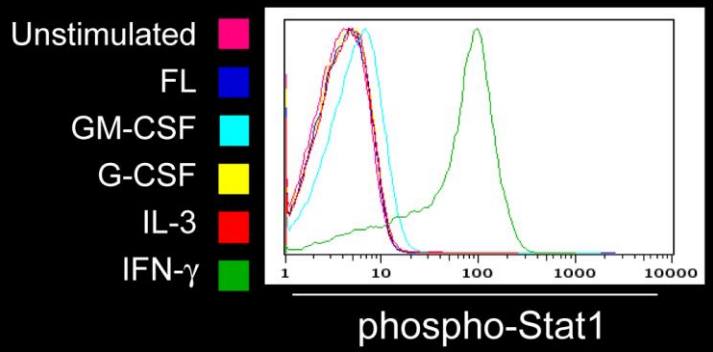
(B) Representative cytokine response panels of the HL-60 AML cell line, normal $CD33^+$ leukocytes, and six AML patient samples. Repeat experiments using these AML blasts yielded similar results ($n = 3$), and variation among normal, healthy donors was minimal ($n = 6$). The response to stimulation at each signaling node is calculated as $\log_2$ (MFI $^{\text{stimulated}}$ / MFI $^{\text{unstimulated}}$).

A 36-Spot Cytokine Response Panel

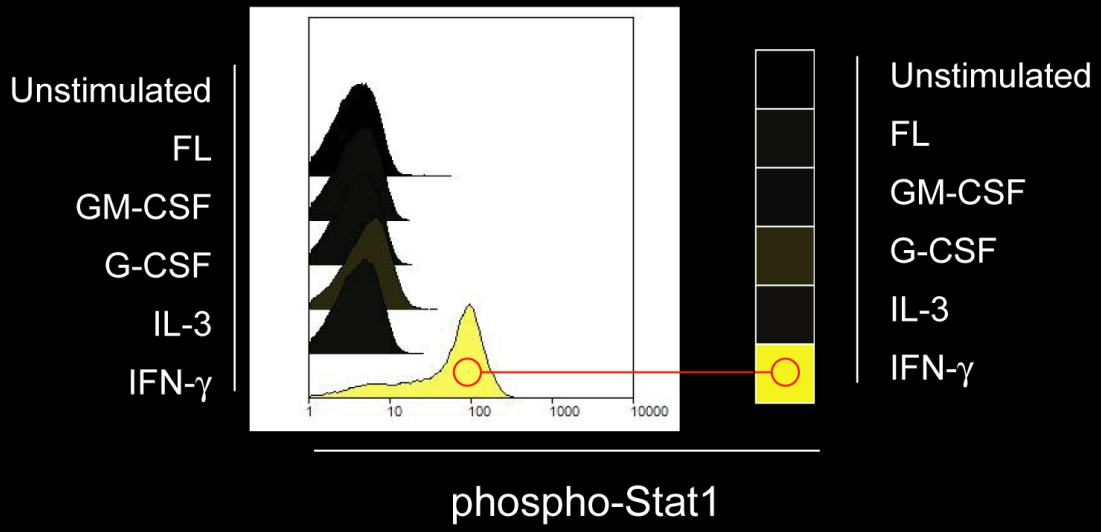


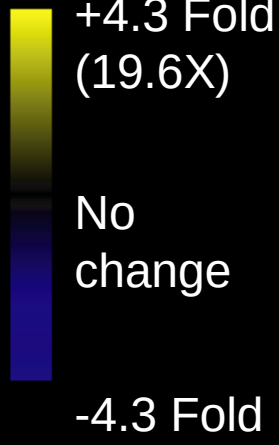


Arraying Flow Cytometry Experiments



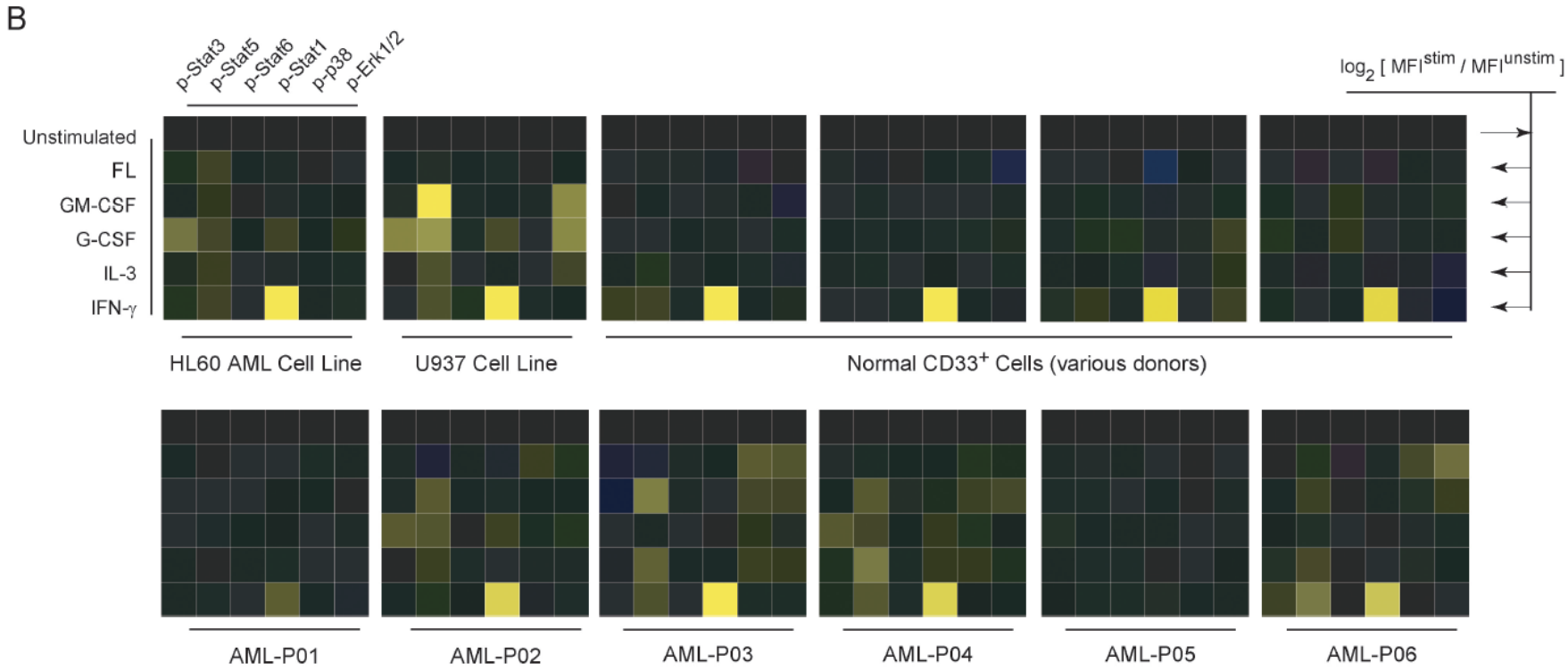
$$x' = \log_2\left(\frac{x_{experimental}}{x_{basal}}\right)$$





Scale: $\log_2$ (E / B)

Figure 1B: Individual AML patients display unique signaling profiles



HL60: acute promyelocytic leukemia (APML) cell line

CD33: In the same sialoadhesin family as CD22, contains ITIMs, expressed on early myeloid lineage cells

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Expansion to 30 AML Patient Samples

- We applied the cytokine response panel to 30 AML patient samples
- Goal: survey both the basal phosphorylation and the cytokine response in AML patient samples.
- Find statistically significant differences between patients and use these to define and classify signaling network subgroups (that correlate with prognosis…)

Figure 2A: Identification of an AML ‘biosignature’

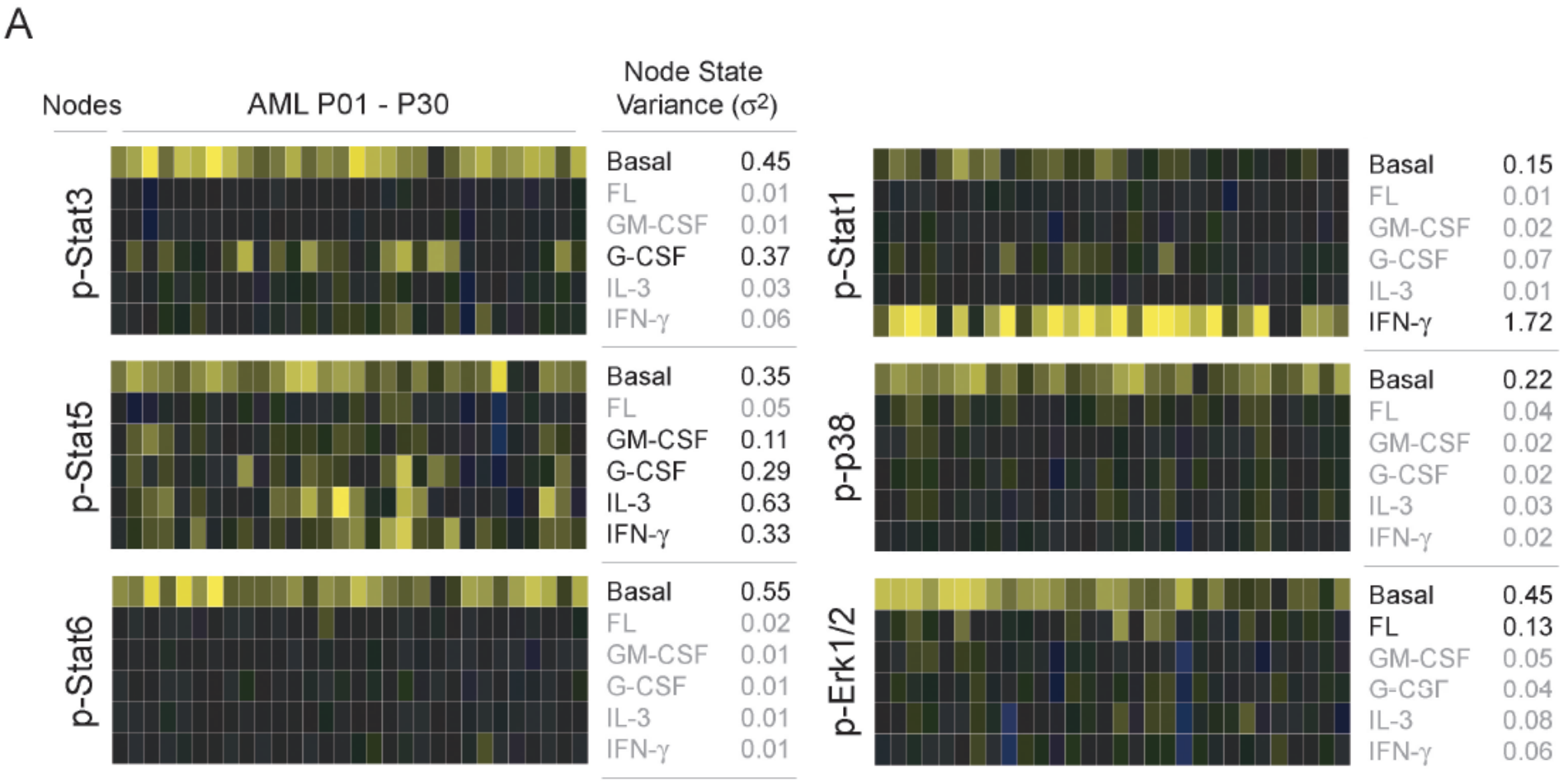


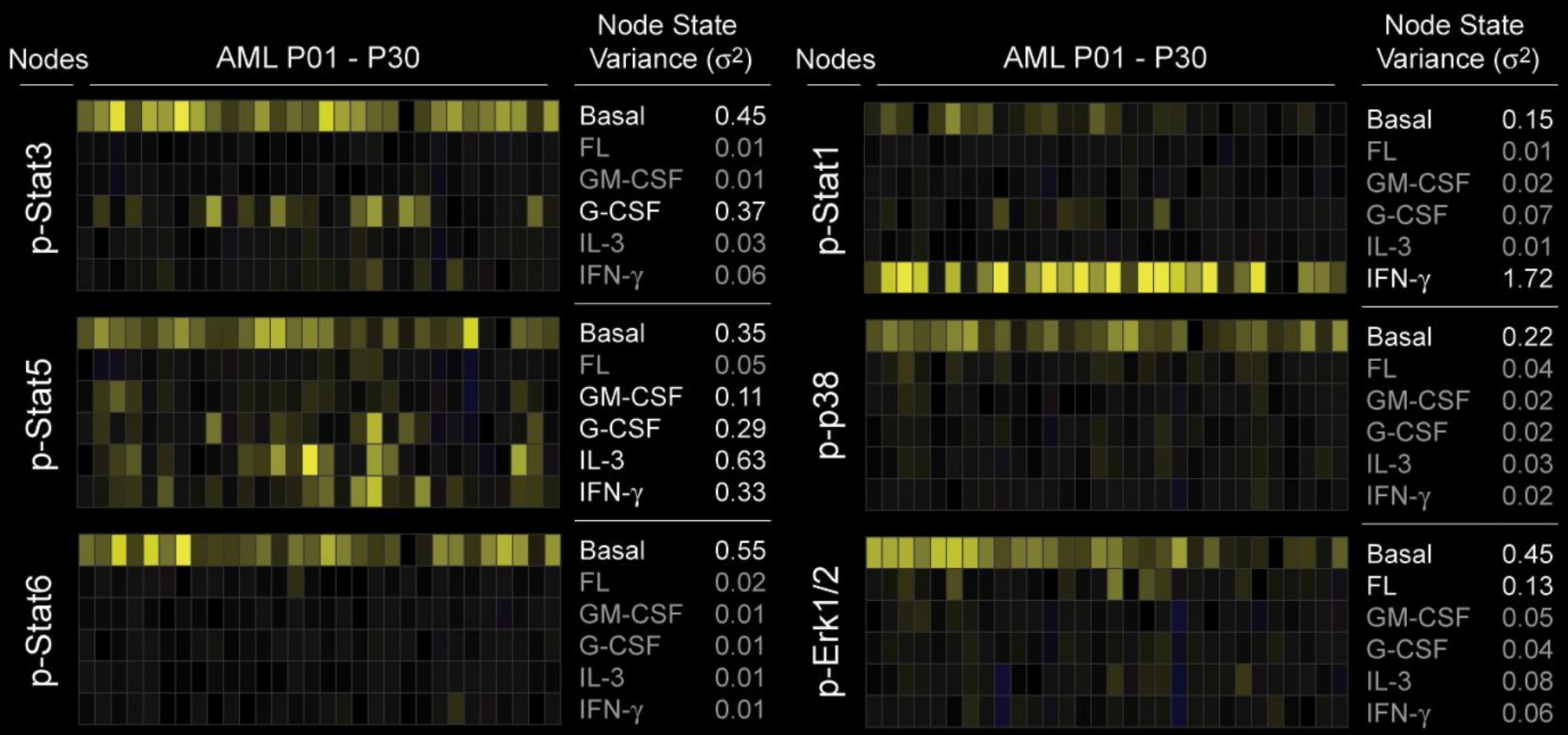
Figure 2. Basal and Potentiated Signaling Nodes that Varied among Cancer Samples Were Used to Define an AML Biosignature

(A) The cytokine response panel of 30 total AML patient samples. The first row of each has been colored to show the variation in the basal phosphorylation (relative to the minimum among the AML blasts). Of 900 cytokine responses assayed, 93 (10.3%) displayed a detectable phosphorylation increase following stimulation (greater than 0.55-fold on a $\log_2$ scale).

(B) Significant cytokine responses were restricted primarily to the 7/30 cytokine response nodes with a variance across cancers greater than 0.1 (yellow circles).

(C) A graph of the absolute median plotted against the variance for each node state indicates the signal-to-noise threshold.

Cytokine Responses of 30 AML Samples



Cytokine Responses of 30 AML Samples

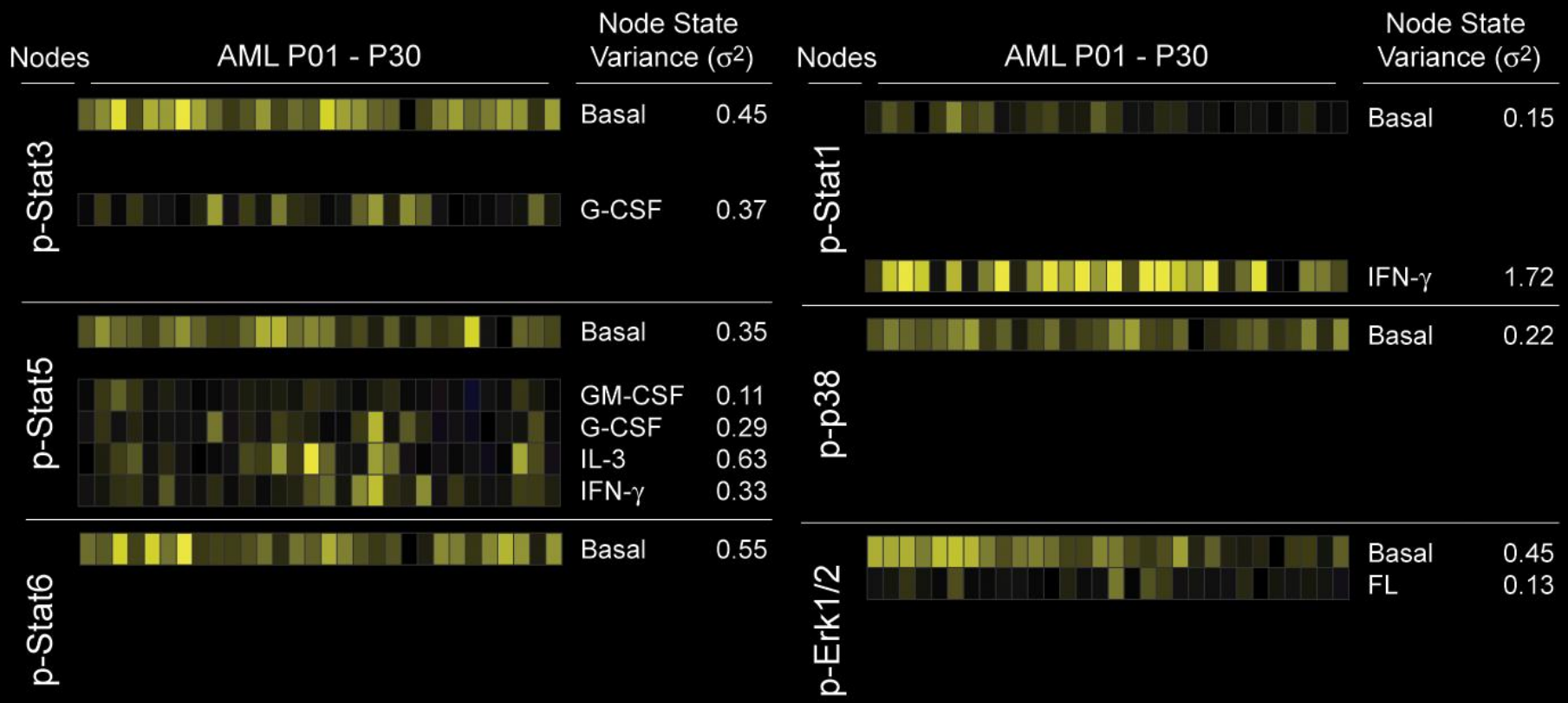


Figure 2B: 13 Signaling node states displayed significant variance

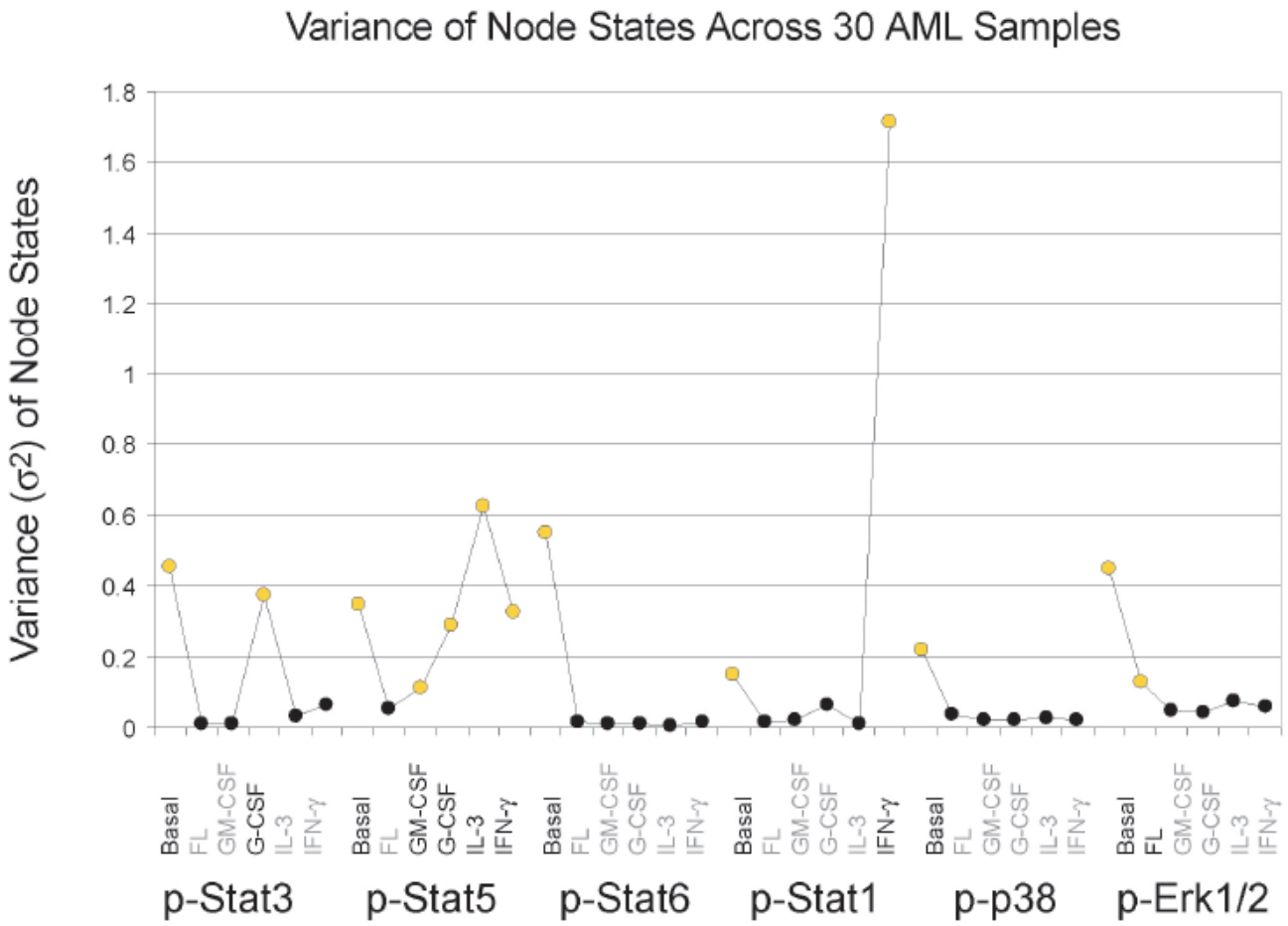


Figure 2. Basal and Potentiated Signaling Nodes that Varied among Cancer Samples Were Used to Define an AML Biosignature

(A) The cytokine response panel of 30 total AML patient samples. The first row of each has been colored to show the variation in the basal phosphorylation (relative to the minimum among the AML blasts). Of 900 cytokine responses assayed, 93 (10.3%) displayed a detectable phosphorylation increase following stimulation (greater than 0.55-fold on a $\log_2$ scale).

(B) Significant cytokine responses were restricted primarily to the 7/30 cytokine response nodes with a variance across cancers greater than 0.1 (yellow circles).

(C) A graph of the absolute median plotted against the variance for each node state indicates the signal-to-noise threshold.

Figure 2C: Some high magnitude signaling events were not significantly variable in AML

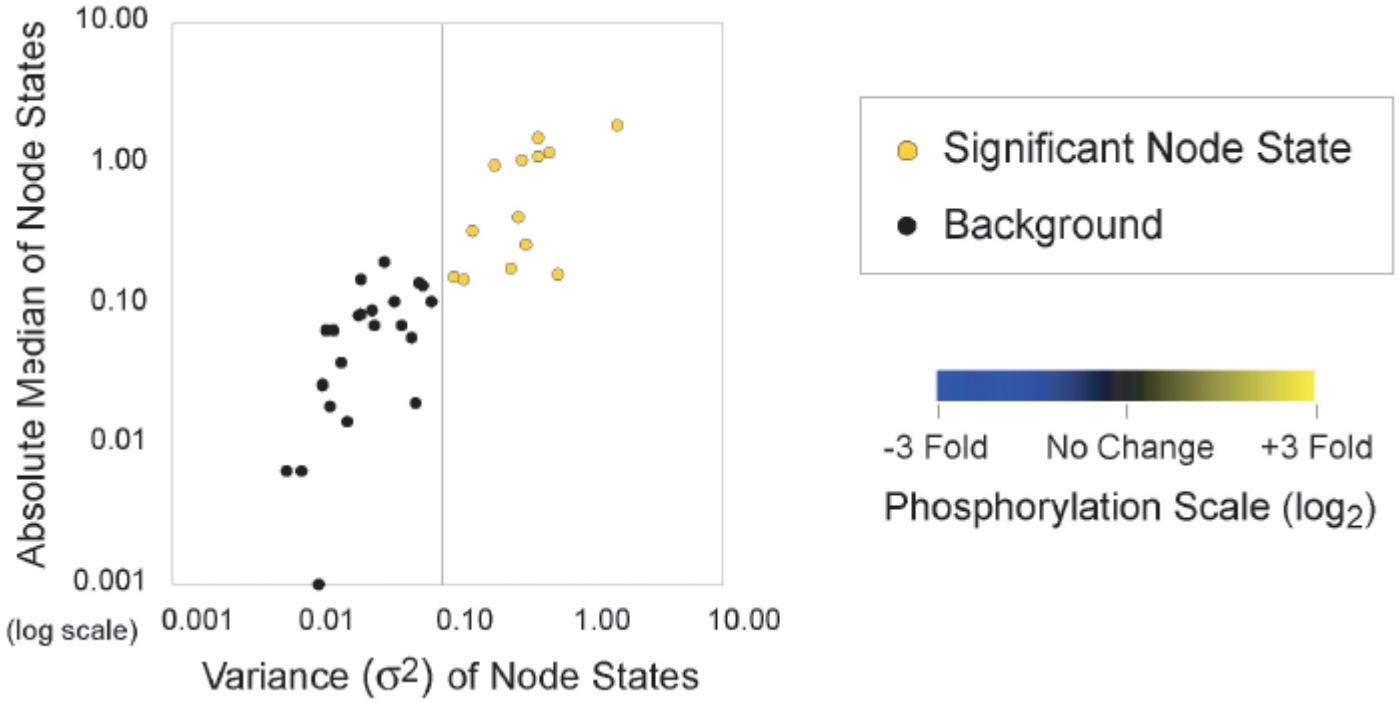


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Figure 6A: Filtering by variance identifies an AML biosignature

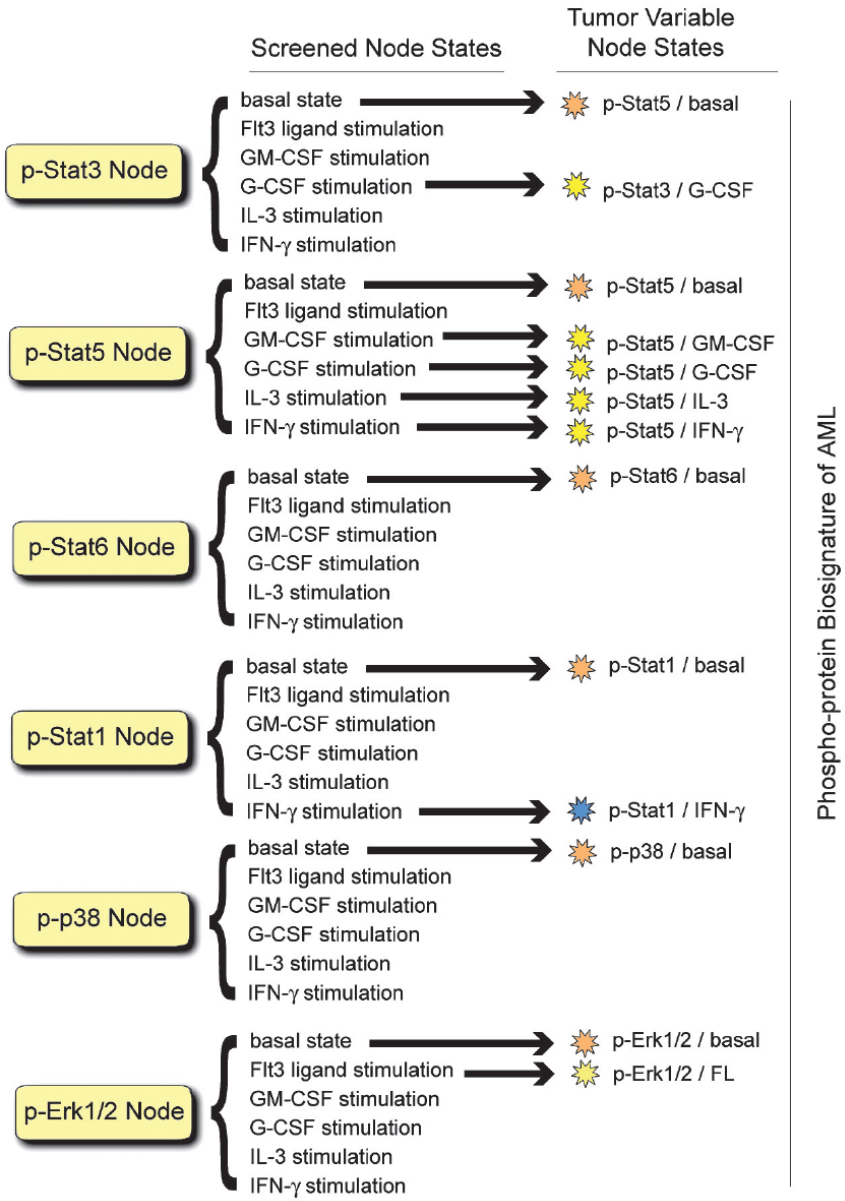


Figure 3: Grouping AML patients by signaling stratifies multiple clinical features

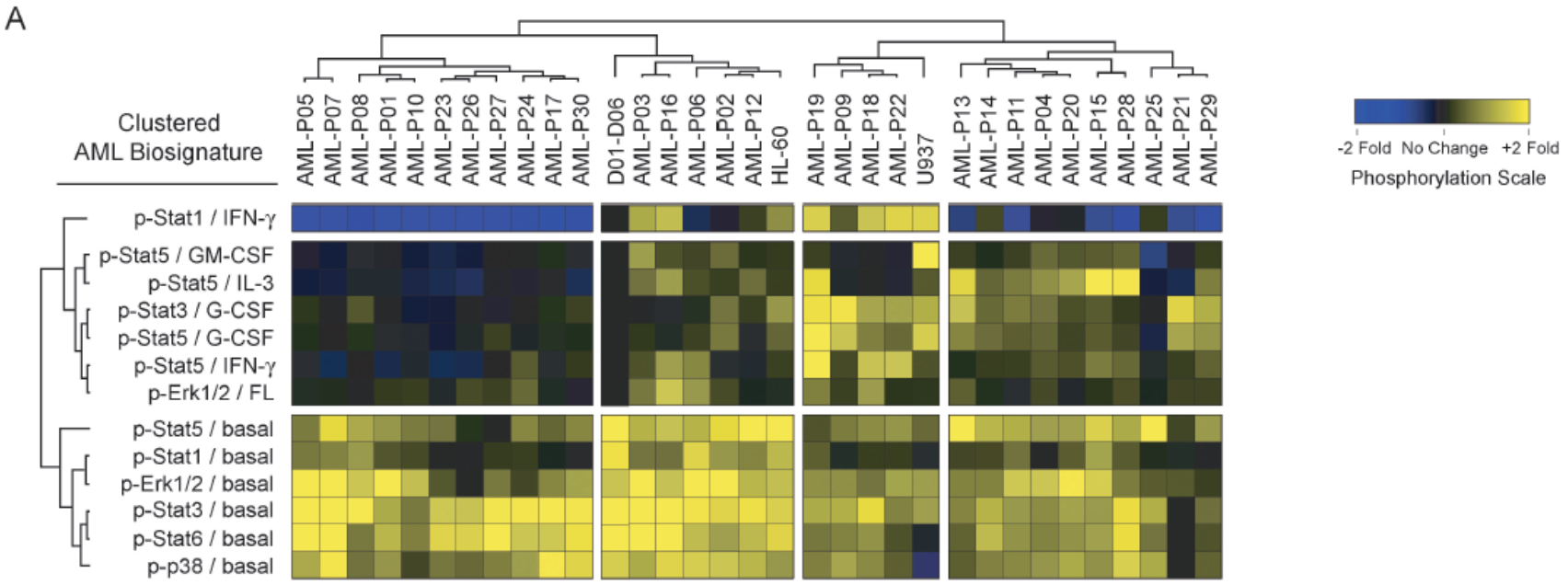
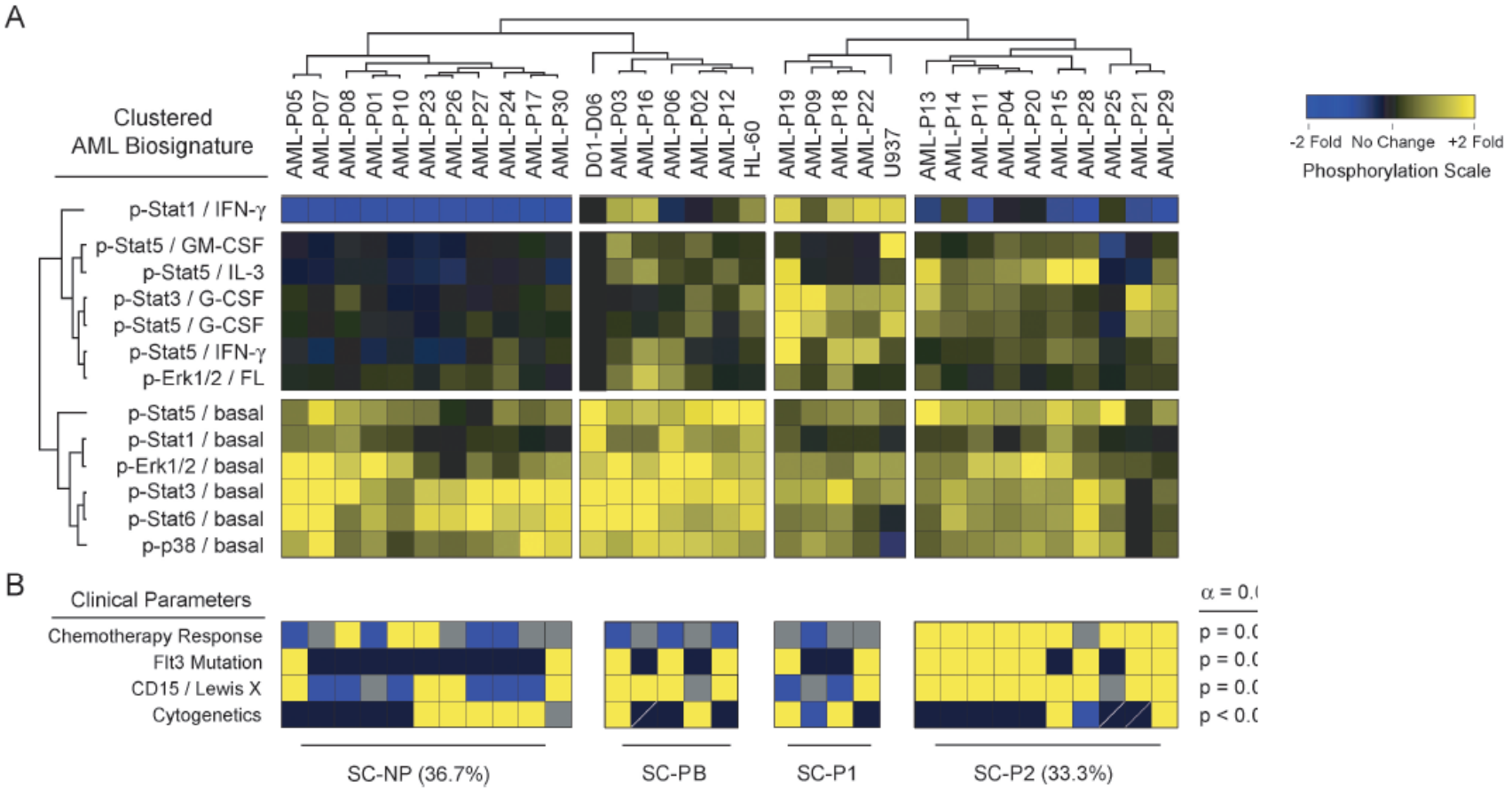


Figure 3. AML Patients Grouped by Signal Transduction Biosignature Form Four Groups that Exhibit Significant Correlations to Clinical Prognostic Markers

(A) The 13-parameter biosignatures of differentiated $CD33^{+}$ myeloid cells from six normal blood donors (D01 – D06), U937 and HL-60 cancer cell lines, and 30 AML patient samples were grouped according to similarity using hierarchical clustering. The heat map for AML and cancer cell line cytokine responses was scaled by subtracting donor sample medians to provide a dynamic color range. As shown previously, basal responses are relative to the minimum among AML samples.

(B) Four main groups of AML patients were identified based on the similarity of their signal transduction biosignatures. We designated these groups with signaling cluster (SC) nomenclature based on the signaling that defined them and mapped several clinical markers within the identified patient groups.

Figure 3: Grouping AML patients by signaling stratifies multiple clinical features



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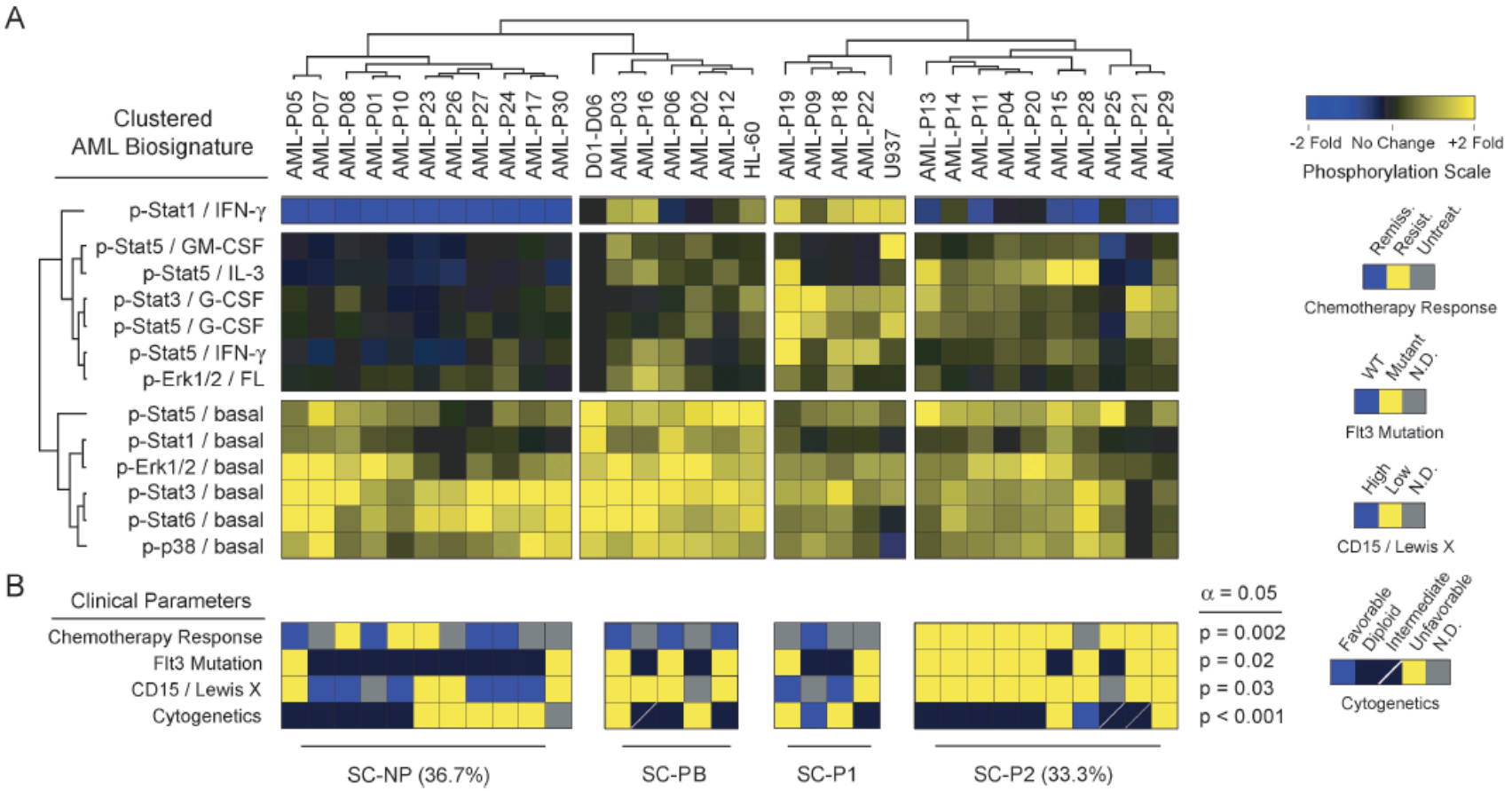

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Did other signaling events matter?
Did we miss important features?

Supp Table 3: IL-3 ► p-ERK & G-CSF ► p-STAT1 were next on the list (including them in the clustering didn't change the 4 main cluster groups)

Supplementary Table 3 - Values of all 36 node states for all 30 AML patient samples.*

Rank	Variance	Name	Description	GWEIGHT	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30
		EWEIGHT			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1.716	p-Stat1-IFNg	biosignature	1	1.89	0.16	1.20	0.14	2.44	0.37	2.43	1.07	0.45	2.20	0.81	0.27	0.60	0.32	0.75	1.41	1.78	1.50	1.63	0.10	0.76	1.68	2.19	1.30	0.21	2.65	2.60	1.00	1.15	1.72
2	0.627	p-Stat5-IL-3	biosignature	1	0.12	0.23	0.64	0.92	0.21	0.36	0.25	0.12	0.08	0.26	0.73	0.61	1.70	0.54	2.76	1.05	0.05	0.01	1.76	1.14	0.31	0.14	0.31	0.16	0.23	0.40	0.11	1.91	0.75	0.38
3	0.551	p-Stat6-basal	biosignature	1	1.31	1.08	2.57	0.79	2.58	1.39	3.44	0.70	0.79	0.76	0.95	1.35	0.53	1.38	1.09	2.11	1.53	0.95	0.69	0.95	0.00	0.38	1.66	1.50	0.66	1.63	2.19	1.79	0.39	1.82
4	0.454	p-Stat3-basal	biosignature	1	1.19	1.67	2.80	0.88	2.13	1.88	3.19	2.04	1.23	0.70	1.01	1.83	0.82	1.28	1.03	2.63	1.96	1.76	1.21	1.13	0.00	0.79	1.53	1.91	1.23	1.48	1.89	1.79	0.62	1.89
5	0.450	p-Erk1/2-basal	biosignature	1	2.12	2.05	2.13	1.52	2.31	2.38	2.25	1.52	0.92	1.42	1.53	1.31	0.79	0.79	1.60	1.45	0.85	0.66	0.92	1.94	0.44	1.12	0.42	0.31	0.47	0.00	0.70	0.74	0.25	1.13
6	0.372	p-Stat3-G-CSF	biosignature	1	0.03	0.66	0.03	0.64	0.18	0.13	0.03	0.43	1.88	0.19	0.74	0.23	1.45	0.56	0.45	0.05	0.17	1.14	1.84	0.36	1.69	1.13	0.18	0.01	0.03	0.04	0.16	0.21	1.23	0.27
7	0.346	p-Stat5-basal	biosignature	1	0.99	1.76	1.27	1.08	0.73	1.28	1.76	1.18	0.74	0.73	1.23	2.11	2.24	1.31	1.63	1.45	0.56	0.88	0.39	1.09	0.30	0.97	0.66	0.84	2.61	0.17	0.00	1.19	1.02	0.84
8	0.328	p-Stat5-IFNg	biosignature	1	0.30	0.07	0.38	0.45	0.06	0.84	0.33	0.02	0.33	0.11	0.23	0.11	0.15	0.23	0.73	1.07	0.07	1.47	2.17	0.37	0.23	1.49	0.33	0.46	0.08	0.32	0.02	0.56	0.53	0.20
9	0.289	p-Stat5-G-CSF	biosignature	1	0.05	0.70	0.20	0.55	0.16	0.26	0.01	0.17	1.49	0.12	0.49	0.05	0.83	0.60	0.47	0.12	0.13	0.77	2.32	0.29	1.14	0.56	0.18	0.09	0.26	0.09	0.26	0.30	0.97	0.14
10	0.221	p-p38-basal	biosignature	1	0.97	1.52	1.21	0.97	1.18	1.60	1.91	0.65	1.14	0.31	0.86	1.31	0.51	0.78	0.93	1.66	1.93	0.87	0.77	1.21	0.00	0.51	0.69	1.06	1.21	0.57	0.77	1.61	0.53	1.69
11	0.152	p-Stat1-basal	biosignature	1	0.40	0.98	0.66	0.00	0.70	1.66	0.79	1.02	0.11	0.34	0.65	0.80	0.31	0.32	1.11	0.63	0.10	0.22	0.50	0.48	0.11	0.22	0.00	0.23	0.14	0.03	0.20	0.45	0.04	0.05
12	0.130	p-Erk1/2-FL	biosignature	1	0.20	0.34	0.69	0.32	0.12	1.00	0.14	0.03	0.24	0.23	0.06	0.10	0.51	0.16	0.17	1.52	0.11	1.04	0.77	0.10	0.28	0.17	0.08	0.56	0.10	0.34	0.25	0.36	0.28	0.14
13	0.112	p-Stat5-GM-CSF	biosignature	1	0.11	0.59	1.06	0.58	0.16	0.30	0.19	0.06	0.12	0.20	0.25	0.18	0.27	0.15	0.47	0.38	0.16	0.03	0.25	0.42	0.14	0.15	0.28	0.01	0.62	0.22	0.01	0.55	0.21	0.06
14	0.076	p-Erk1/2-IL-3		1	0.01	0.05	0.29	0.23	0.10	0.09	0.29	0.14	0.65	0.03	0.01	0.17	0.13	0.18	0.04	0.56	0.21	0.40	0.42	0.55	0.12	0.19	0.18	0.66	0.22	0.09	0.08	0.16	0.04	0.29
15	0.067	p-Stat1-G-CSF		1	0.13	0.36	0.00	0.28	0.12	0.01	0.03	0.10	0.86	0.03	0.26	0.20	0.57	0.43	0.36	0.01	0.23	0.11	0.95	0.04	0.19	0.17	0.11	0.03	0.10	0.10	0.09	0.14	0.14	0.16
16	0.062	p-Stat3-IFNg		1	0.30	0.12	0.21	0.04	0.05	0.21	0.26	0.16	0.14	0.36	0.03	0.09	0.08	0.07	0.14	0.12	0.05	0.32	0.64	0.05	0.13	0.31	0.49	0.56	0.06	0.39	0.19	0.05	0.01	0.04
17	0.058	p-Erk1/2-IFNg		1	0.14	0.04	0.19	0.14	0.07	0.02	0.47	0.06	0.55	0.32	0.25	0.21	0.28	0.01	0.02	0.28	0.03	0.25	0.28	0.58	0.27	0.09	0.08	0.18	0.08	0.45	0.07	0.03	0.25	0.06
18	0.055	p-Stat5-FL		1	0.10	0.18	0.17	0.22	0.14	0.35	0.01	0.04	0.32	0.11	0.34	0.08	0.01	0.18	0.26	0.45	0.17	0.69	0.67	0.20	0.01	0.19	0.18	0.13	0.39	0.03	0.25	0.01	0.18	0.16
19	0.046	p-Erk1/2-GM-CSF		1	0.01	0.12	0.44	0.49	0.04	0.35	0.29	0.04	0.12	0.18	0.10	0.26	0.17	0.17	0.06	0.13	0.03	0.01	0.12	0.61	0.14	0.01	0.10	0.10	0.26	0.11	0.01	0.11	0.19	0.18
20	0.041	p-Erk1/2-G-CSF		1	0.01	0.20	0.11	0.08	0.01	0.07	0.31	0.19	0.12	0.18	0.01	0.34	0.24	0.14	0.10	0.18	0.21	0.24	0.40	0.47	0.22	0.18	0.21	0.02	0.13	0.28	0.19	0.02	0.14	0.10
21	0.035	p-p38-FL		1	0.24	0.46	0.81	0.37	0.12	0.59	0.28	0.29	0.55	0.18	0.19	0.05	0.25	0.33	0.55	0.54	0.15	0.49	0.32	0.06	0.47	0.32	0.17	0.58	0.58	0.16	0.23	0.31	0.24	0.32
22	0.030	p-Stat3-IL-3		1	0.11	0.01	0.15	0.24	0.11	0.19	0.05	0.01	0.05	0.24	0.16	0.14	0.20	0.11	0.36	0.12	0.12	0.37	0.21	0.06	0.06	0.23	0.33	0.07	0.02	0.19	0.15	0.12	0.24	0.15
23	0.028	p-p38-IL-3		1	0.14	0.18	0.48	0.42	0.16	0.29	0.22	0.25	0.10	0.04	0.10	0.05	0.22	0.16	0.53	0.39	0.21	0.21	0.54	0.02	0.15	0.21	0.01	0.32	0.45	0.21	0.10	0.25	0.49	0.30
24	0.024	p-p38-G-CSF		1	0.03	0.22	0.27	0.25	0.01	0.05	0.21	0.10	0.29	0.03	0.03	0.27	0.16	0.09	0.34	0.14	0.05	0.18	0.44	0.22	0.26	0.05	0.03	0.16	0.27	0.17	0.01	0.04	0.21	0.16
25	0.023	p-p38-IFNg		1	0.02	0.10	0.17	0.18	0.02	0.05	0.18	0.25	0.14	0.18	0.17	0.05	0.12	0.03	0.16	0.21	0.20	0.14	0.19	0.35	0.01	0.05	0.09	0.22	0.48	0.17	0.01	0.04	0.08	0.13
26	0.023	p-p38-GM-CSF		1	0.18	0.18	0.55	0.47	0.08	0.27	0.19	0.09	0.01	0.16	0.08	0.12	0.08	0.09	0.14	0.22	0.14	0.17	0.21	0.16	0.15	0.20	0.03	0.17	0.49	0.10	0.01	0.17	0.23	0.23
27	0.019	p-Stat1-GM-CSF		1	0.13	0.13	0.02	0.22	0.06	0.12	0.08	0.08	0.00	0.08	0.12	0.37	0.03	0.12	0.08	0.03	0.29	0.04	0.24	0.10	0.01	0.24	0.09	0.05	0.04	0.01	0.09	0.01	0.23	0.03
28	0.017	p-Stat6-FL		1	0.04	0.10	0.07	0.11	0.12	0.19	0.01	0.12	0.12	0.03	0.07	0.05	0.05	0.56	0.07	0.02	0.02	0.01	0.12	0.06	0.14	0.03	0.08	0.05	0.05	0.15	0.11	0.04	0.02	0.15
29	0.015	p-Stat6-IFNg		1	0.13	0.00	0.06	0.18	0.22	0.10	0.10	0.21	0.10	0.18	0.09	0.16	0.18	0.14	0.17	0.23	0.35	0.10	0.26	0.01	0.10	0.12	0.22	0.64	0.19	0.11	0.16	0.19	0.09	0.18
30	0.014	p-Stat1-FL		1	0.14	0.06	0.21	0.21	0.13	0.05	0.01	0.25	0.27	0.03	0.02	0.01	0.14	0.22	0.21	0.01	0.35	0.22	0.17	0.03	0.03	0.10	0.22	0.16	0.03	0.12	0.10	0.13	0.01	0.13
31	0.013	p-Stat6-G-CSF		1	0.07	0.04	0.00	0.06	0.08	0.06	0.01	0.08	0.18	0.05	0.04	0.08	0.09	0.06	0.14	0.08	0.12	0.08	0.21	0.20	0.08	0.04	0.26	0.05	0.01	0.21	0.18	0.05	0.00	0.17
32	0.012	p-Stat3-GM-CSF		1	0.05	0.07	0.33	0.08	0.10	0.06	0.08	0.07	0.05	0.16	0.01	0.02	0.02	0.03	0.03	0.19	0.01	0.01	0.04	0.06	0.06	0.19	0.28	0.03	0.14	0.06	0.16	0.16	0.06	0.12
33	0.012	p-Stat1-IL-3		1	0.05	0.10	0.01	0.21	0.08	0.10	0.08	0.15	0.01	0.11	0.15	0.21	0.15	0.08	0.07	0.29	0.02	0.10	0.03	0.06	0.06	0.03	0.10	0.01	0.28	0.01	0.04	0.06	0.15	0.16
34	0.012	p-Stat3-FL		1	0.10	0.03	0.25	0.07	0.08	0.02	0.05	0.05	0.07	0.03	0.12	0.03	0.01	0.02	0.08	0.01	0.02	0.15	0.20	0.03	0.05	0.08	0.21	0.02	0.12	0.06	0.21	0.08	0.09	0.16
35	0.009	p-Stat6-GM-CSF		1	0.00	0.10	0.09	0.13	0.06	0.03	0.10	0.00	0.04	0.01	0.08	0.13	0.00	0.09	0.03	0.06	0.10	0.03	0.13	0.18	0.08	0.05	0.13	0.01	0.05	0.13	0.30	0.04	0.13	0.06
36	0.007	p-Stat6-IL-3		1	0.12	0.08	0.08	0.16	0.08	0.01	0.01	0.00	0.08	0.03	0.04	0.01	0.01	0.14	0.08	0.01	0.01	0.05	0.10	0.10	0.01	0.05	0.14	0.12	0.13	0.12	0.09	0.12	0.05	0.04

* Significance of node states to this system was determined by looking at the variance of node states across tumors. Rank was determined by variance of a node state across tumors. Complete linkage hierarchical clusterings are shown in Figure 3 and Supplementary Figure 1. The inner cells of this table can be copied and pasted as text to make a Stanford format file readable by the MeV software used for hierarchical clustering and produced the order indicated in Supplementary Table 1 . Clustering for this manuscript employed the significant node states flagged as "biosignature."

Supp Table 3: IL-3 ► p-ERK & G-CSF ► p-STAT1 were next on the list (including them in the clustering didn't change the 4 main cluster groups)

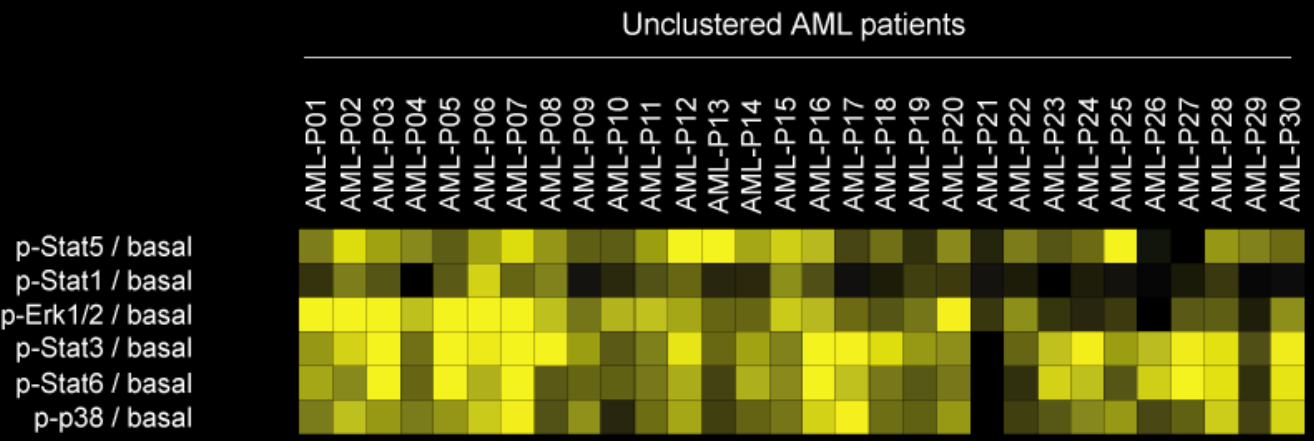
Supplementary Table 3 - Values of all 36 node states for all 30 AML patient samples.*

Rank	Variance	Name	Description	GWEIGHT	P06	P07	P08	P09	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30
		EWEIGHT			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1.716	p-Stat1-IFNg	biosignature	1	0.37	2.43	1.07	0.45	2.20	0.81	0.27	0.60	0.32	0.75	1.41	1.78	1.50	1.63	0.10	0.76	1.68	2.19	1.30	0.21	2.65	2.60	1.00	1.15	1.72
2	0.627	p-Stat5-IL-3	biosignature	1	0.36	0.25	0.12	0.08	0.26	0.73	0.61	1.70	0.54	2.76	1.05	0.05	0.01	1.76	1.14	0.31	0.14	0.31	0.16	0.23	0.40	0.11	1.91	0.75	0.38
3	0.551	p-Stat6-basal	biosignature	1	1.39	3.44	0.70	0.79	0.76	0.95	1.35	0.53	1.38	1.09	2.11	1.53	0.95	0.69	0.95	0.00	0.38	1.66	1.50	0.66	1.63	2.19	1.79	0.39	1.82
4	0.454	p-Stat3-basal	biosignature	1	1.88	3.19	2.04	1.23	0.70	1.01	1.83	0.82	1.28	1.03	2.63	1.96	1.76	1.21	1.13	0.00	0.79	1.53	1.91	1.23	1.48	1.89	1.79	0.62	1.89
5	0.450	p-Erk1/2-basal	biosignature	1	2.38	2.25	1.52	0.92	1.42	1.53	1.31	0.79	0.79	1.60	1.45	0.85	0.66	0.92	1.94	0.44	1.12	0.42	0.31	0.47	0.00	0.70	0.74	0.25	1.13
6	0.372	p-Stat3-G-CSF	biosignature	1	0.13	0.03	0.43	1.88	0.19	0.74	0.23	1.45	0.56	0.45	0.05	0.17	1.14	1.84	0.36	1.69	1.13	0.18	0.01	0.03	0.04	0.16	0.21	1.23	0.27
7	0.346	p-Stat5-basal	biosignature	1	1.28	1.76	1.18	0.74	0.73	1.23	2.11	2.24	1.31	1.63	1.45	0.56	0.88	0.39	1.09	0.30	0.97	0.66	0.84	2.61	0.17	0.00	1.19	1.02	0.84
8	0.328	p-Stat5-IFNg	biosignature	1	0.84	0.33	0.02	0.33	0.11	0.23	0.11	0.15	0.23	0.73	1.07	0.07	1.47	2.17	0.37	0.23	1.49	0.33	0.46	0.08	0.32	0.02	0.56	0.53	0.20
9	0.289	p-Stat5-G-CSF	biosignature	1	0.26	0.01	0.17	1.49	0.12	0.49	0.05	0.83	0.60	0.47	0.12	0.13	0.77	2.32	0.29	1.14	0.56	0.18	0.09	0.26	0.09	0.26	0.30	0.97	0.14
10	0.221	p-p38-basal	biosignature	1	1.60	1.91	0.65	1.14	0.31	0.86	1.31	0.51	0.78	0.93	1.66	1.93	0.87	0.77	1.21	0.00	0.51	0.69	1.06	1.21	0.57	0.77	1.61	0.53	1.69
11	0.152	p-Stat1-basal	biosignature	1	1.66	0.79	1.02	0.11	0.34	0.65	0.80	0.31	0.32	1.11	0.63	0.10	0.22	0.50	0.48	0.11	0.22	0.00	0.23	0.14	0.03	0.20	0.45	0.04	0.05
12	0.130	p-Erk1/2-FL	biosignature	1	1.00	0.14	0.03	0.24	0.23	0.06	0.10	0.51	0.16	0.17	1.52	0.11	1.04	0.77	0.10	0.28	0.17	0.08	0.56	0.10	0.34	0.25	0.36	0.28	0.14
13	0.112	p-Stat5-GM-CSF	biosignature	1	0.30	0.19	0.06	0.12	0.20	0.25	0.18	0.27	0.15	0.47	0.38	0.16	0.03	0.25	0.42	0.14	0.15	0.28	0.01	0.62	0.22	0.01	0.55	0.21	0.06
14	0.076	p-Erk1/2-IL-3		1	0.09	0.29	0.14	0.65	0.03	0.01	0.17	0.13	0.18	0.04	0.56	0.21	0.40	0.42	0.55	0.12	0.19	0.18	0.66	0.22	0.09	0.08	0.16	0.04	0.29
15	0.067	p-Stat1-G-CSF		1	0.01	0.03	0.10	0.86	0.03	0.26	0.20	0.57	0.43	0.36	0.01	0.23	0.11	0.95	0.04	0.19	0.17	0.11	0.03	0.10	0.10	0.09	0.14	0.14	0.16
16	0.062	p-Stat1-FL		1	0.21	0.26	0.16	0.14	0.36	0.03	0.09	0.08	0.07	0.14	0.12	0.05	0.32	0.64	0.05	0.13	0.31	0.49	0.56	0.06	0.39	0.19	0.05	0.01	0.04
17	0.060	p-Stat1-GM-CSF		1	0.02	0.47	0.06	0.55	0.32	0.25	0.21	0.28	0.01	0.02	0.28	0.03	0.25	0.28	0.58	0.27	0.09	0.08	0.18	0.08	0.45	0.07	0.03	0.25	0.06
18	0.059	p-Stat3-IL-3		1	0.35	0.01	0.04	0.32	0.11	0.34	0.08	0.01	0.18	0.26	0.45	0.17	0.69	0.67	0.20	0.01	0.19	0.18	0.13	0.39	0.03	0.25	0.01	0.18	0.16
19	0.057	p-Stat6-GM-CSF		1	0.35	0.29	0.04	0.12	0.18	0.10	0.26	0.17	0.17	0.06	0.13	0.03	0.01	0.12	0.61	0.14	0.01	0.10	0.10	0.26	0.11	0.01	0.11	0.19	0.18
20	0.036	p-Stat6-IL-3		1	0.07	0.31	0.19	0.12	0.18	0.01	0.34	0.24	0.14	0.10	0.18	0.21	0.24	0.40	0.47	0.22	0.18	0.21	0.02	0.13	0.28	0.19	0.02	0.14	0.10
21	0.035	p-Stat3-FL		1	0.59	0.28	0.29	0.55	0.18	0.19	0.05	0.25	0.33	0.55	0.54	0.15	0.49	0.32	0.06	0.47	0.32	0.17	0.58	0.58	0.16	0.23	0.31	0.24	0.32
22	0.009	p-Stat6-GM-CSF		1	0.19	0.05	0.01	0.05	0.24	0.16	0.14	0.20	0.11	0.36	0.12	0.12	0.37	0.21	0.06	0.06	0.23	0.33	0.07	0.02	0.19	0.15	0.12	0.24	0.15
23	0.007	p-Stat6-IL-3		1	0.29	0.22	0.25	0.10	0.04	0.10	0.05	0.22	0.16	0.53	0.39	0.21	0.21	0.54	0.02	0.15	0.21	0.01	0.32	0.45	0.21	0.10	0.25	0.49	0.30
					0.05	0.21	0.10	0.29	0.03	0.03	0.27	0.16	0.09	0.34	0.14	0.05	0.18	0.44	0.22	0.26	0.05	0.03	0.16	0.27	0.17	0.01	0.04	0.21	0.16
					0.05	0.18	0.25	0.14	0.18	0.17	0.05	0.12	0.03	0.16	0.21	0.20	0.14	0.19	0.35	0.01	0.05	0.09	0.22	0.48	0.17	0.01	0.04	0.08	0.13
					0.27	0.19	0.09	0.01	0.16	0.08	0.12	0.08	0.09	0.14	0.22	0.14	0.17	0.21	0.16	0.15	0.20	0.03	0.17	0.49	0.10	0.01	0.17	0.23	0.23
					0.12	0.08	0.08	0.00	0.08	0.12	0.37	0.03	0.12	0.08	0.03	0.29	0.04	0.24	0.10	0.01	0.24	0.09	0.05	0.04	0.01	0.09	0.01	0.23	0.03
					0.19	0.01	0.12	0.12	0.03	0.07	0.05	0.05	0.56	0.07	0.02	0.02	0.01	0.12	0.06	0.14	0.03	0.08	0.05	0.05	0.15	0.11	0.04	0.02	0.15
					0.10	0.10	0.21	0.10	0.18	0.09	0.16	0.18	0.14	0.17	0.23	0.35	0.10	0.26	0.01	0.10	0.12	0.22	0.64	0.19	0.11	0.16	0.19	0.09	0.18
					0.05	0.01	0.25	0.27	0.03	0.02	0.01	0.14	0.22	0.21	0.01	0.35	0.22	0.17	0.03	0.03	0.10	0.22	0.16	0.03	0.12	0.10	0.13	0.01	0.13
					0.06	0.01	0.08	0.18	0.05	0.04	0.08	0.09	0.06	0.14	0.08	0.12	0.08	0.21	0.20	0.08	0.04	0.26	0.05	0.01	0.21	0.18	0.05	0.00	0.17
					0.06	0.08	0.07	0.05	0.16	0.01	0.02	0.02	0.03	0.03	0.19	0.01	0.01	0.04	0.06	0.06	0.19	0.28	0.03	0.14	0.06	0.16	0.16	0.06	0.12
					0.10	0.08	0.15	0.01	0.11	0.15	0.21	0.15	0.08	0.07	0.29	0.02	0.10	0.03	0.06	0.06	0.03	0.10	0.01	0.28	0.01	0.04	0.06	0.15	0.16
34	0.012	p-Stat3-FL		1	0.10	0.03	0.25	0.07	0.08	0.02	0.05	0.05	0.07	0.03	0.12	0.03	0.01	0.02	0.08	0.01	0.02	0.15	0.20	0.03	0.05	0.08	0.21	0.02	0.12	0.06	0.21	0.08	0.09	0.16
35	0.009	p-Stat6-GM-CSF		1	0.00	0.10	0.09	0.13	0.06	0.03	0.10	0.00	0.04	0.01	0.08	0.13	0.00	0.09	0.03	0.06	0.10	0.03	0.13	0.18	0.08	0.05	0.13	0.01	0.05	0.13	0.30	0.04	0.13	0.06
36	0.007	p-Stat6-IL-3		1	0.12	0.08	0.08	0.16	0.08	0.01	0.01	0.00	0.08	0.03	0.04	0.01	0.01	0.14	0.08	0.01	0.01	0.05	0.10	0.10	0.01	0.05	0.14	0.12	0.13	0.12	0.09	0.12	0.05	0.04

* Significance of node states to this system was determined by looking at the variance of node states across tumors. Rank was determined by variance of a node state across tumors. Complete linkage hierarchical clusterings are shown in Figure 3 and Supplementary Figure 1. The inner cells of this table can be copied and pasted as text to make a Stanford format file readable by the MeV software used for hierarchical clustering and produced the order indicated in Supplementary Table 1 . Clustering for this manuscript employed the significant node states flagged as "biosignature."

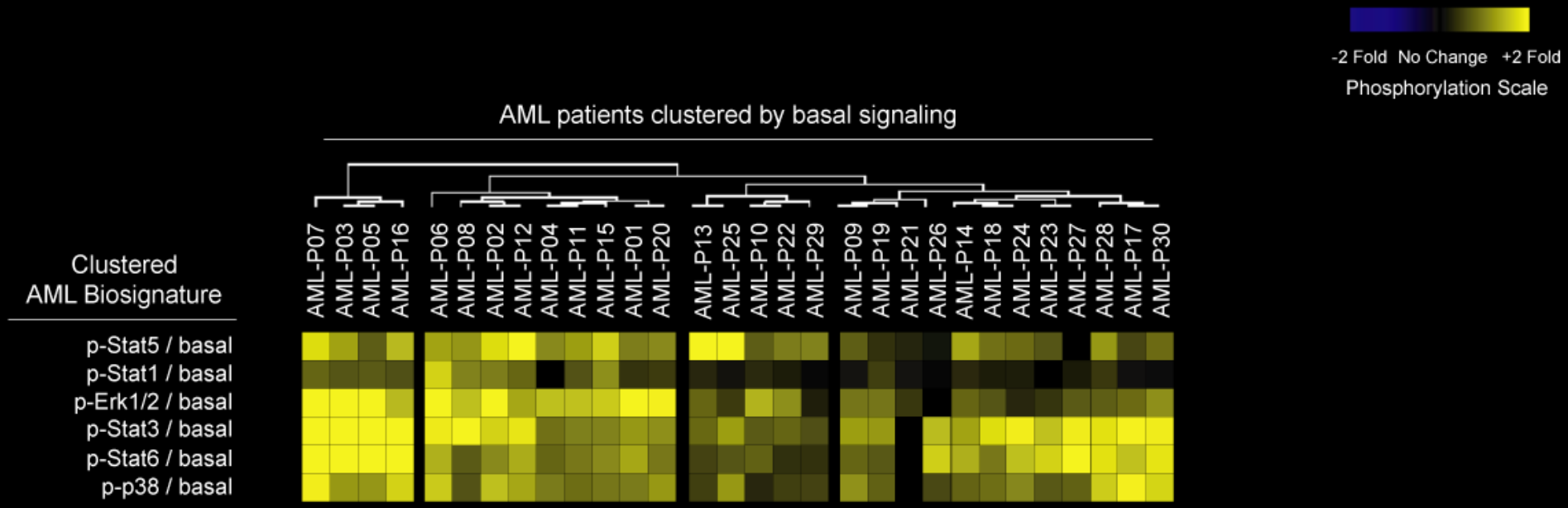
What if we had just clustered on basal signaling?

Clustering AML by Basal Signaling Alone



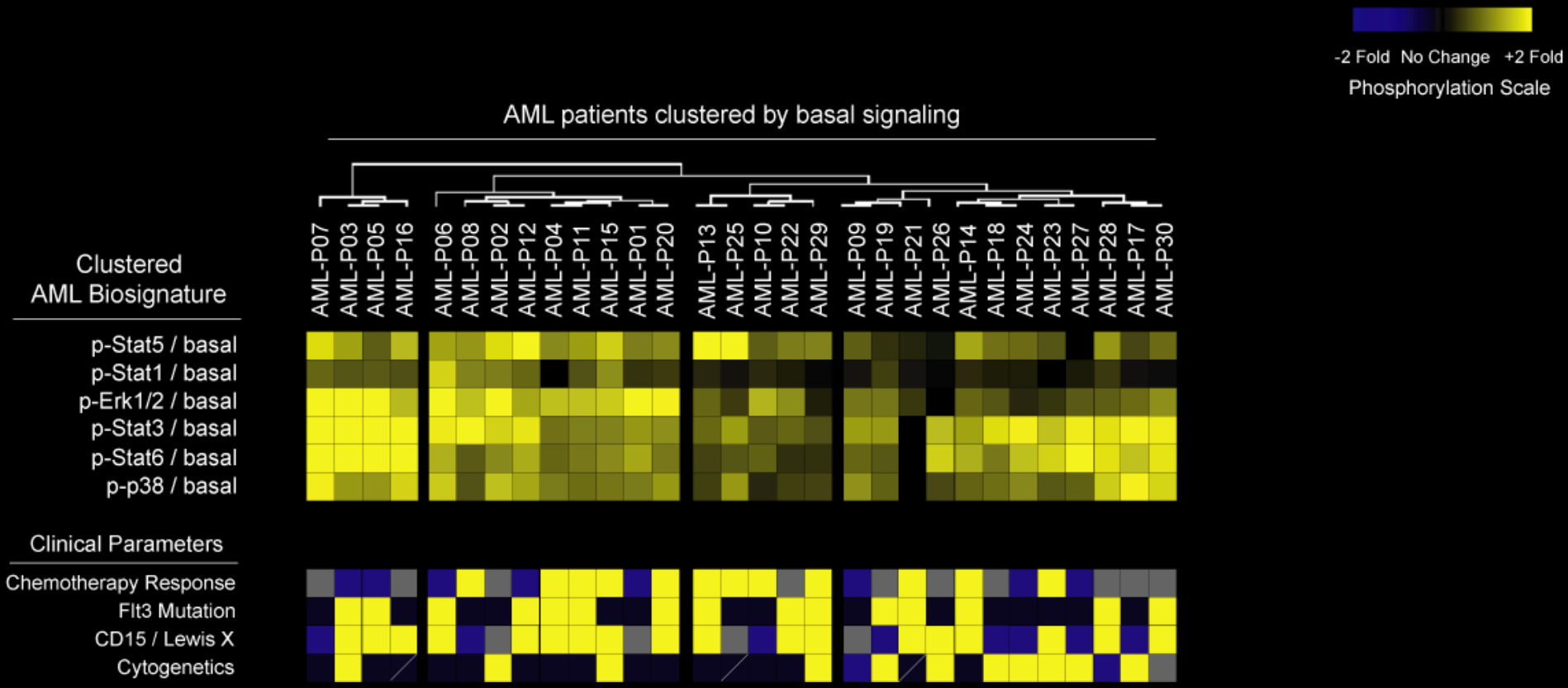
Irish et al., *Cell* 2004

Clustering AML by Basal Signaling Alone



Irish et al., *Cell* 2004

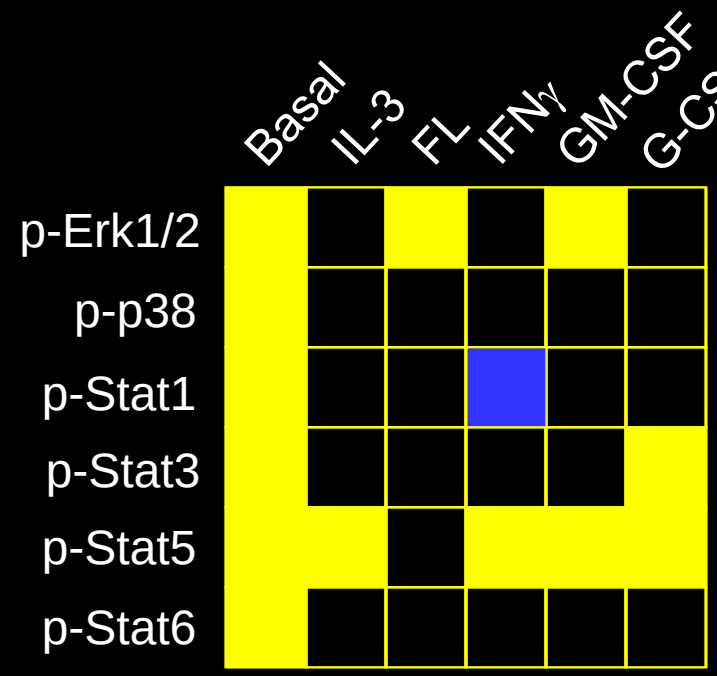
Clustering AML by Basal Signaling Alone



Irish et al., *Cell* 2004

AML Signaling Profile: Evoked Signaling

Add in signaling network inputs
upstream of available p-proteins



'Interrogating' signaling reveals:

- Potentiated (strengthened) signaling responses

- Attenuated (weakened) signaling responses

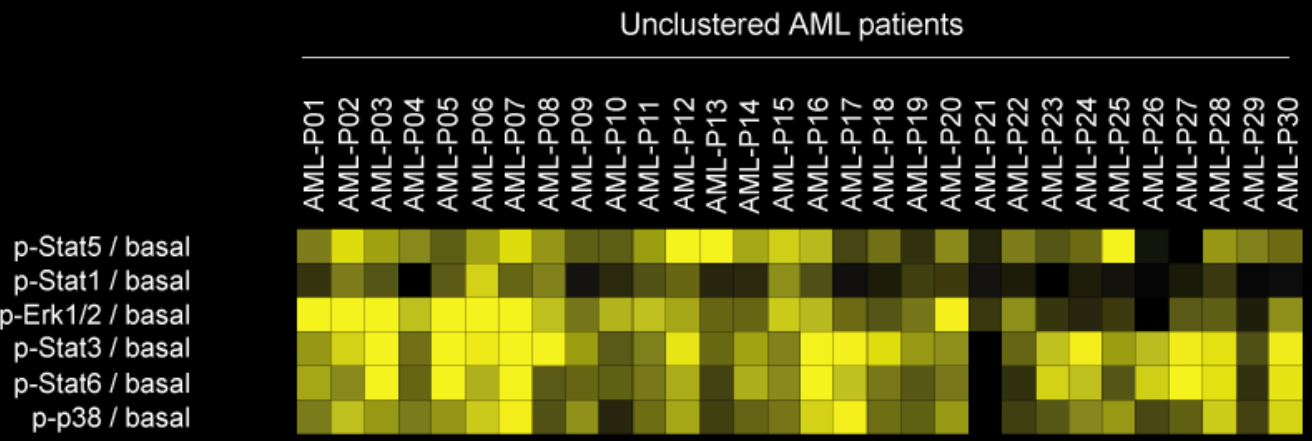
=> 'Rewired' signaling networks

= signaling varied significantly across AML patients
(more variation in AML than in healthy samples)

Att. Pot.

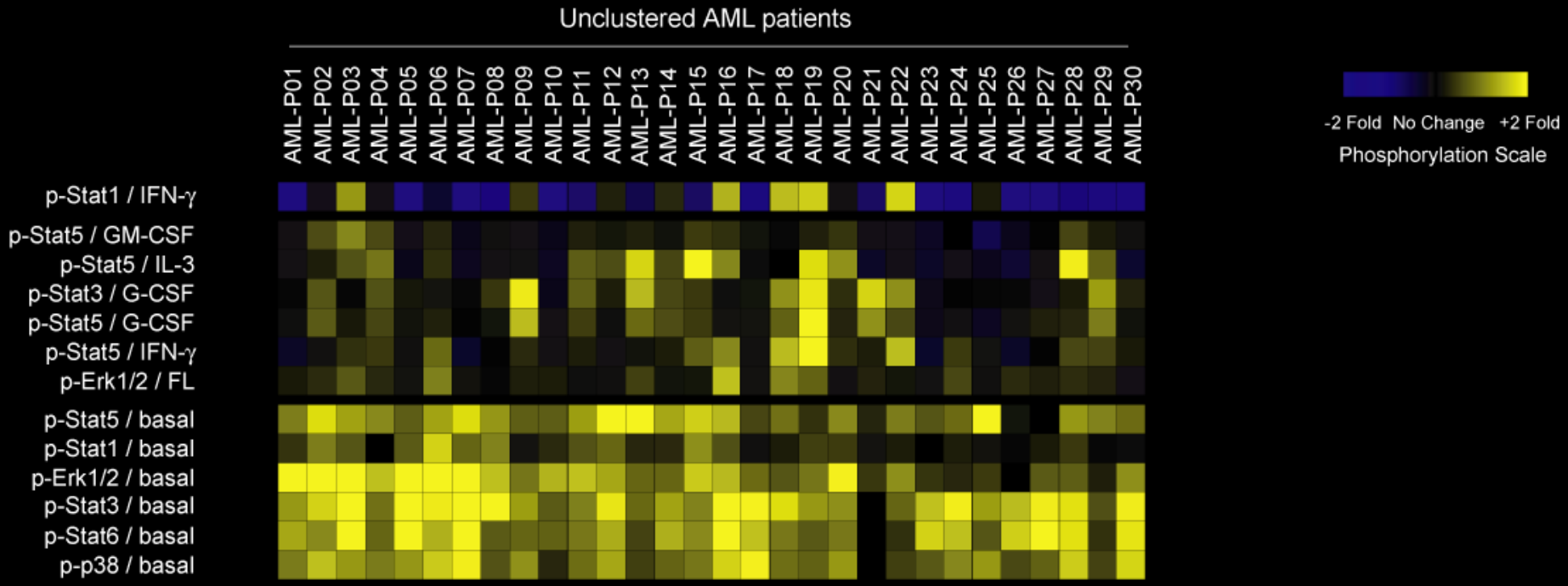
J. Irish

A Signaling Profile of AML Therapy Resistance



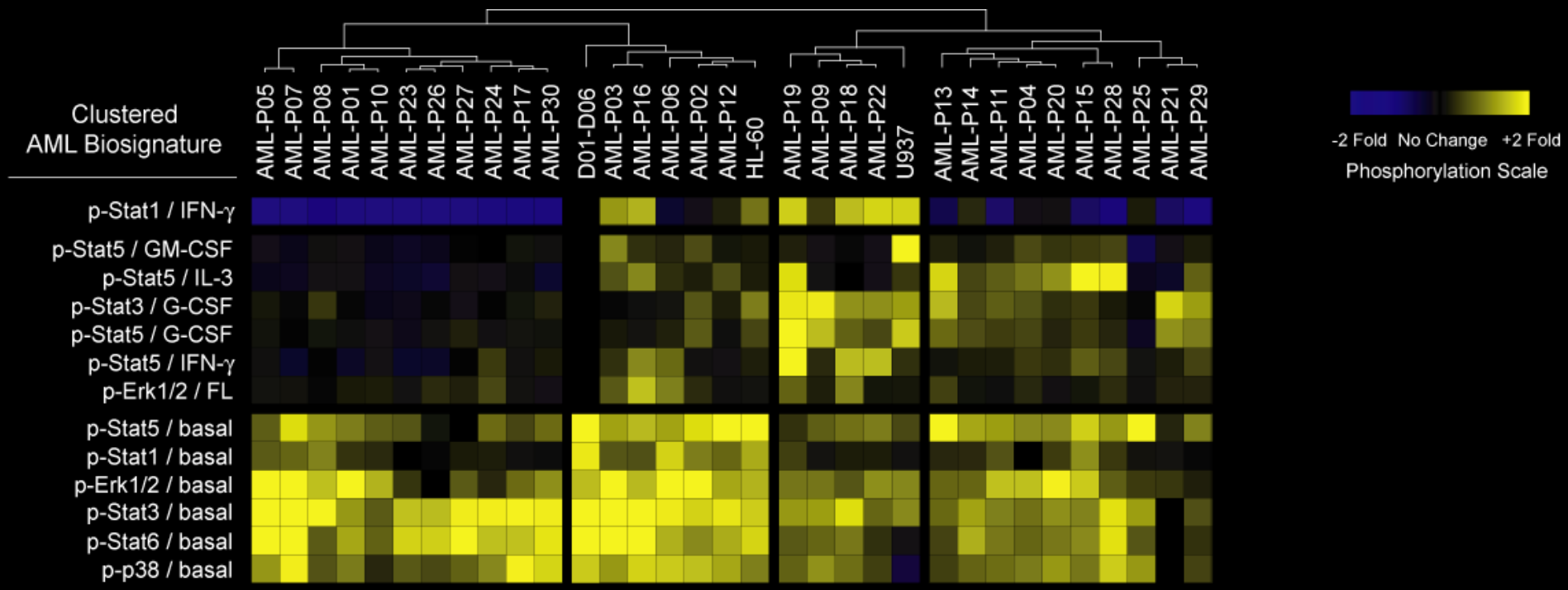
Irish et al., *Cell* 2004

A Signaling Profile of AML Therapy Resistance



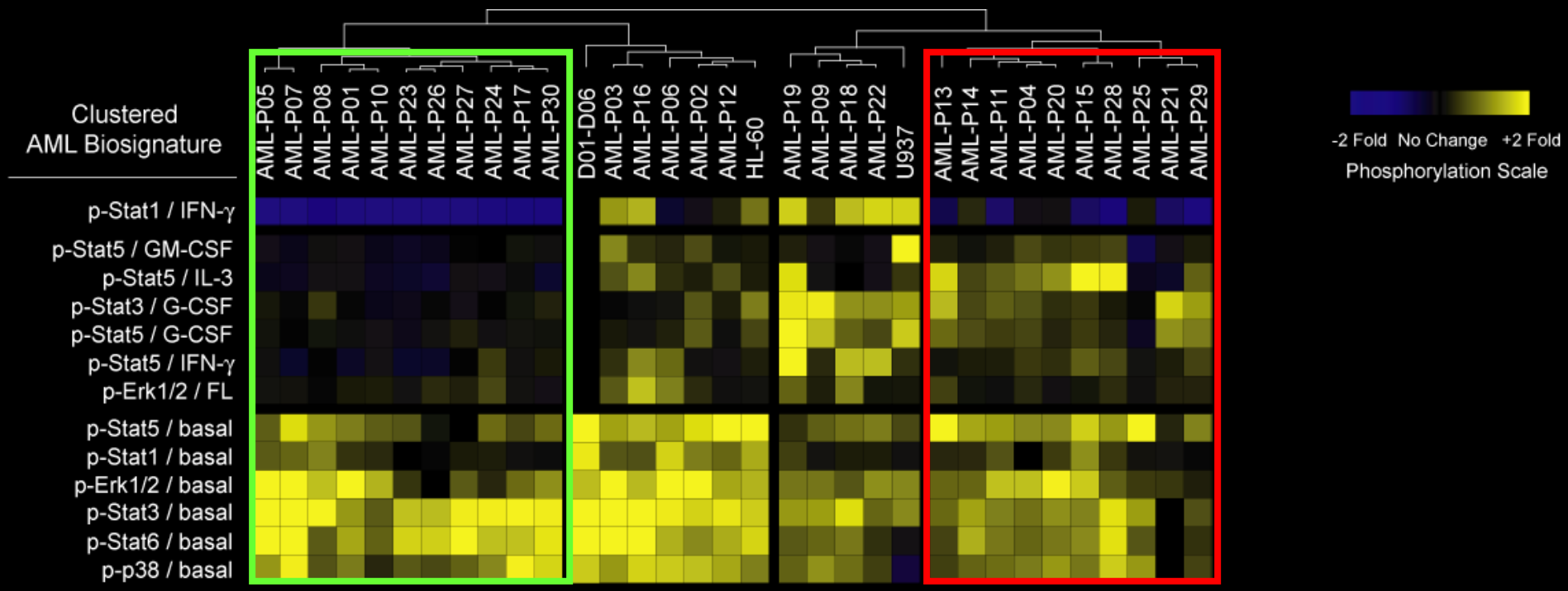
Irish et al., *Cell* 2004

A Signaling Profile of AML Therapy Resistance



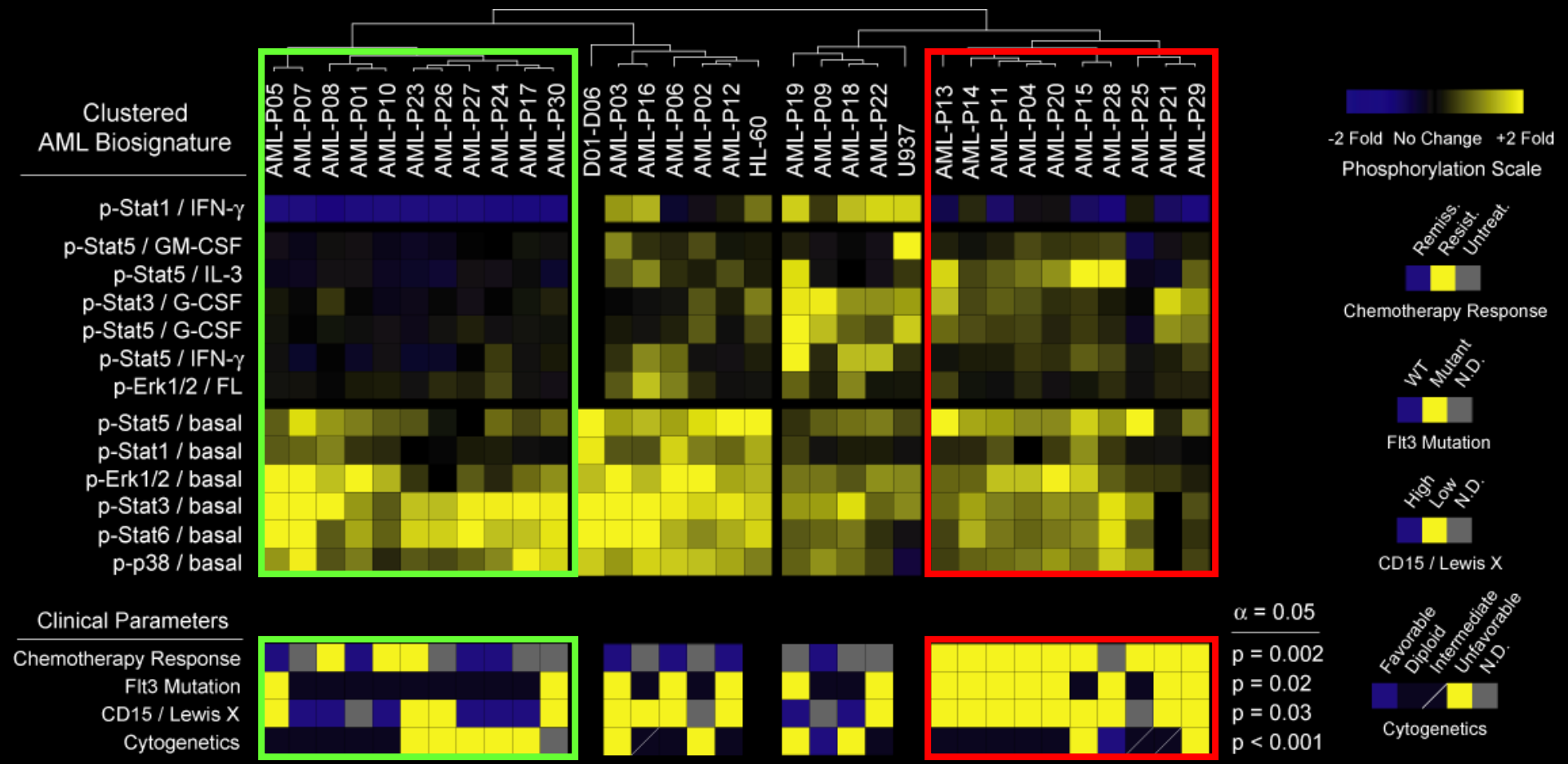
Irish et al., *Cell* 2004

A Signaling Profile of AML Therapy Resistance



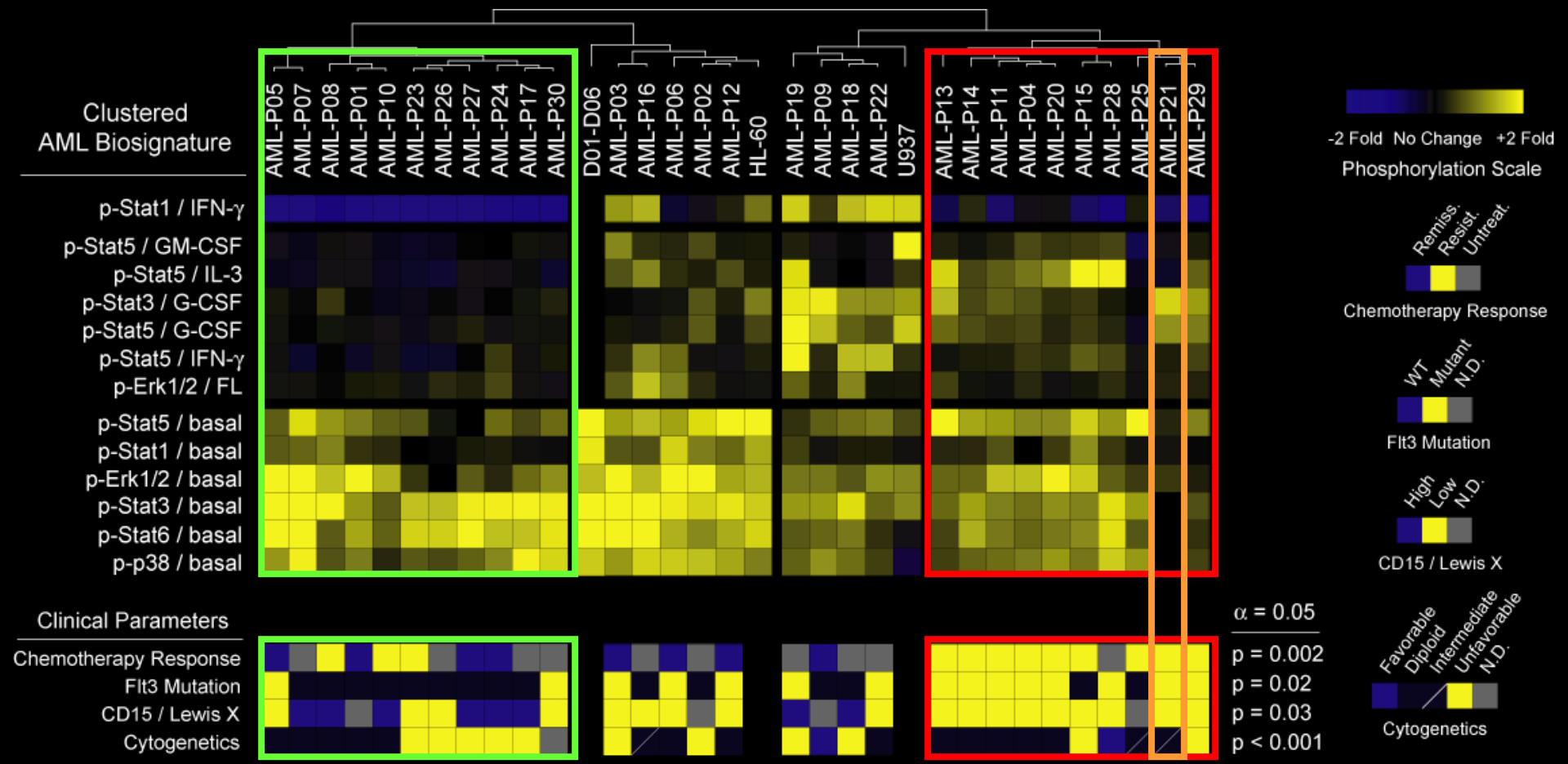
Irish et al., *Cell* 2004

A Signaling Profile of AML Therapy Resistance



Irish et al., *Cell* 2004

A Signaling Profile of AML Therapy Resistance



Irish et al., *Cell* 2004

Figure 4: Mutation of FLT3 (ITD) is associated with abnormal signaling

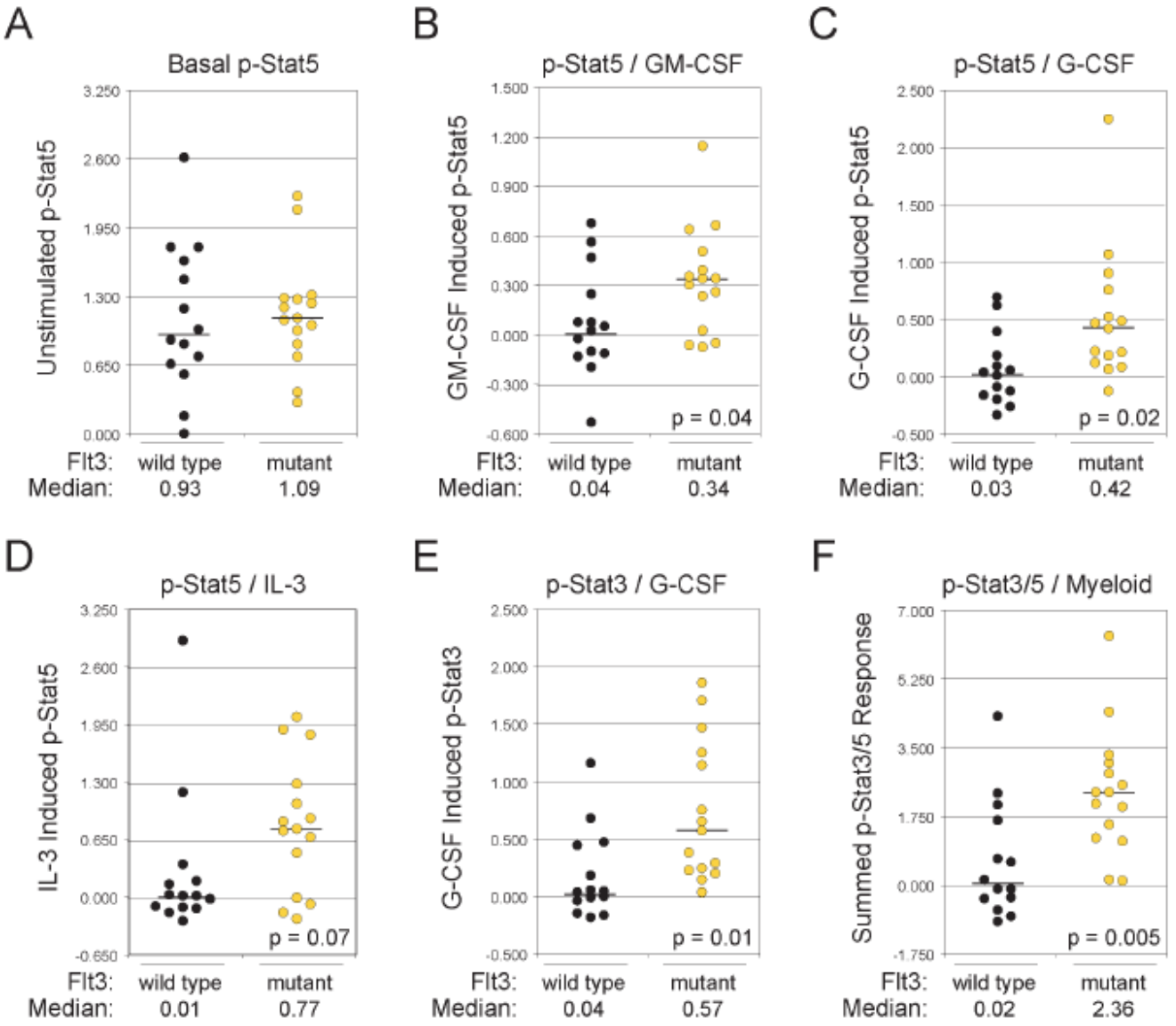
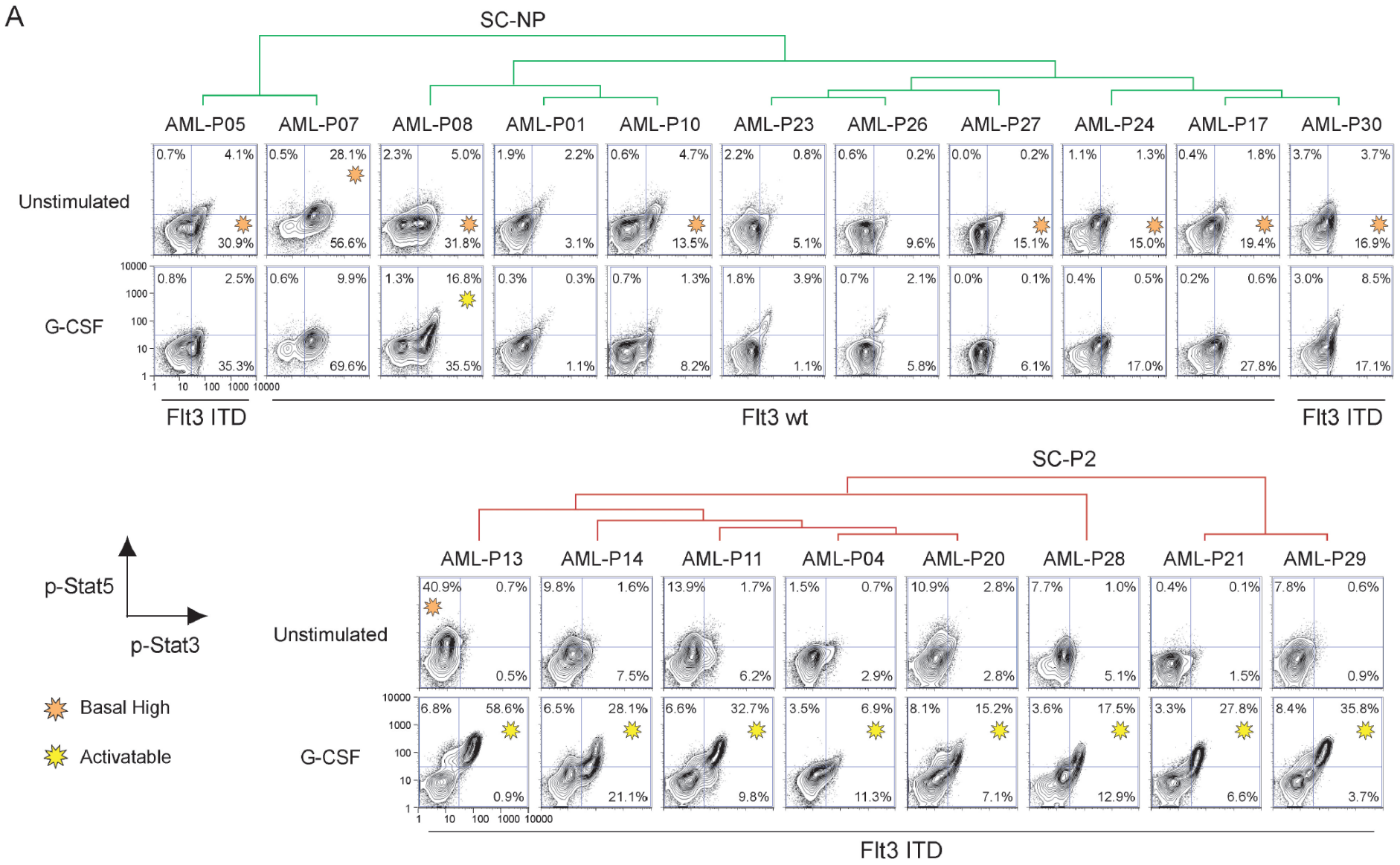


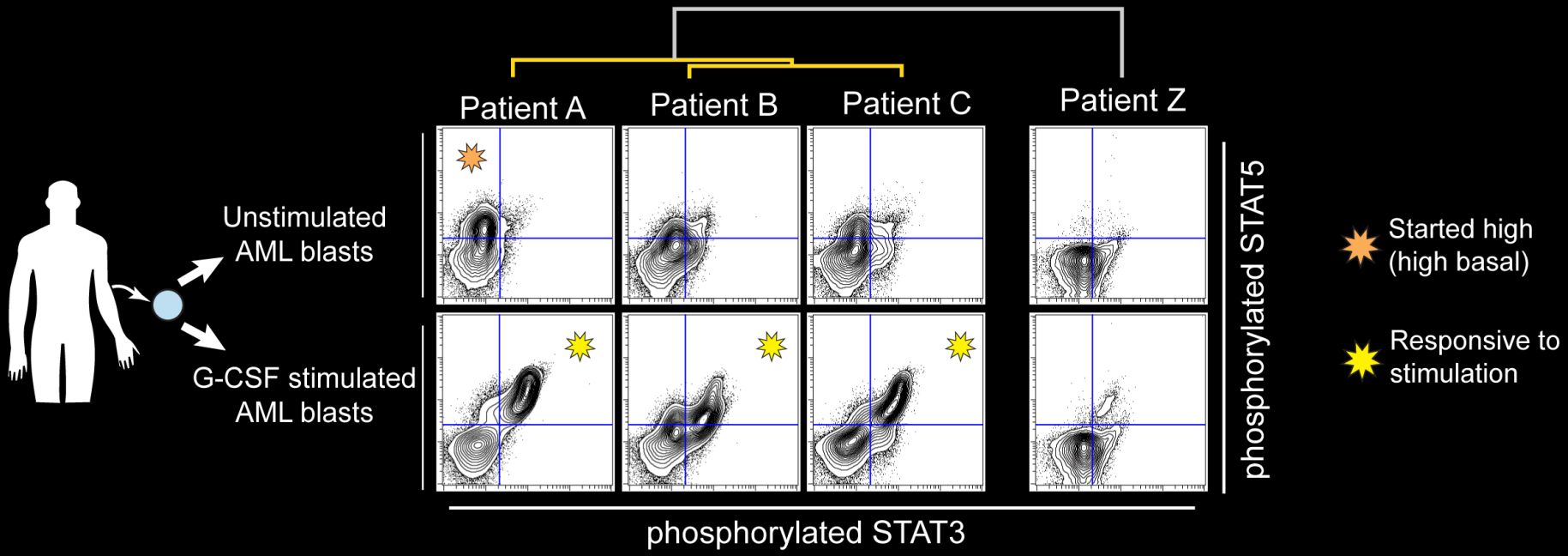
Figure 4. Flt3 Mutation in Primary AMLs Is Associated with Potentiated Myeloid Signal Transduction Nodes

Basal and cytokine response node states of patient samples with wild-type or mutant Flt3 are shown for the 30 AML samples assayed. Each circle represents the level of STAT phosphorylation detected in an individual AML patient sample, grouped according to wild-type Flt3 (black) or detected mutant Flt3 (yellow). (A) To assess basal phosphorylation, samples were compared to the minimum observed among cancers. (B–E) The phosphorylation of Stat5 detected following GM-CSF, G-CSF, and IL-3 and of Stat3 following G-CSF is shown as a fold increase above basal. (F) Cumulative myeloid cytokine responses, calculated by summing individual responses (B–E), were compared in patients with and without Flt3 mutations.

Figure 5: Subsets of cells exist within SC-NP cases and explain the SC-P2 phenotype



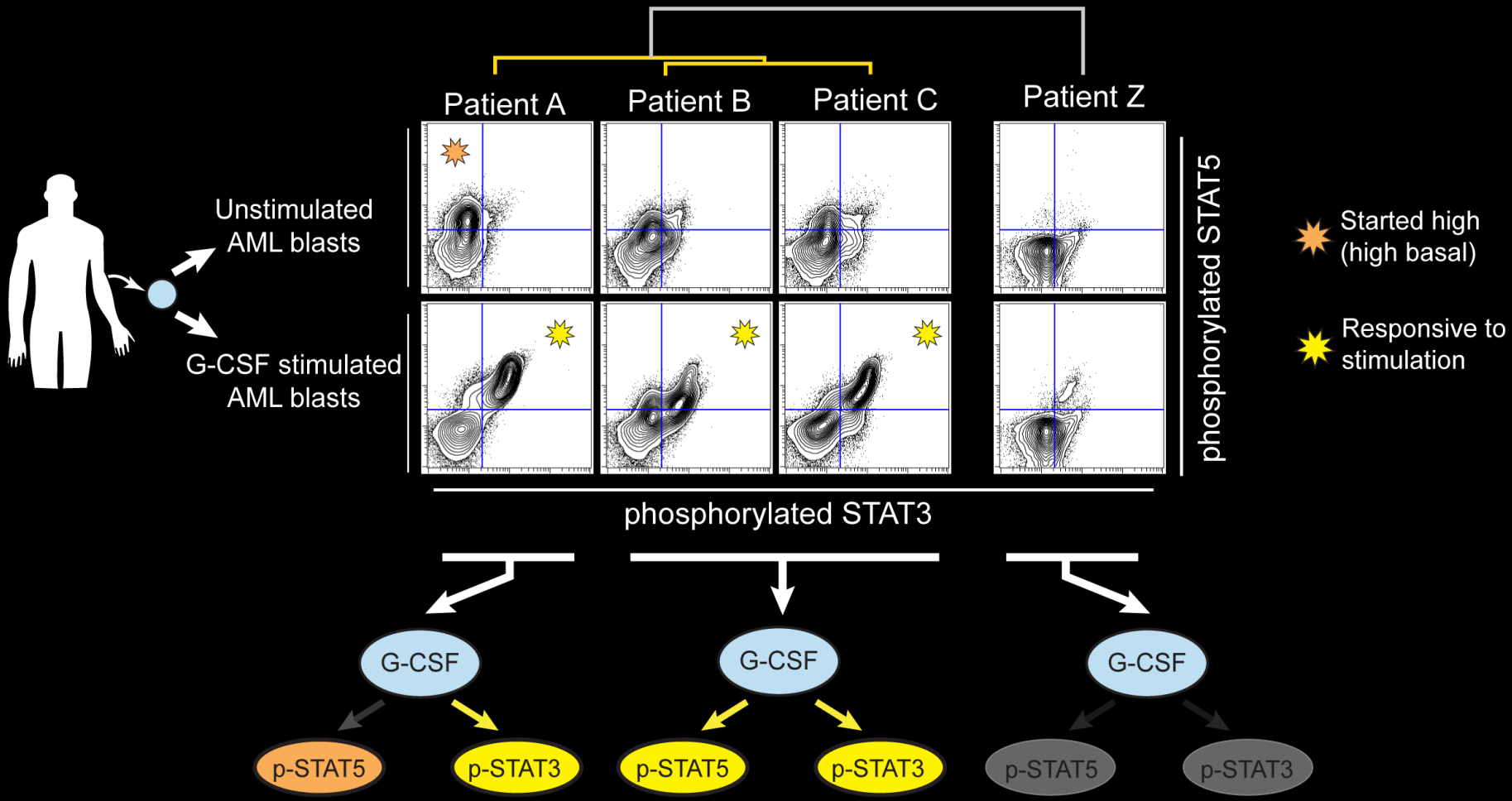
Group Patients by Signaling → Describe Key Signaling Features → Compare Outcomes



Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006

Irish et al., *Cell* 2004

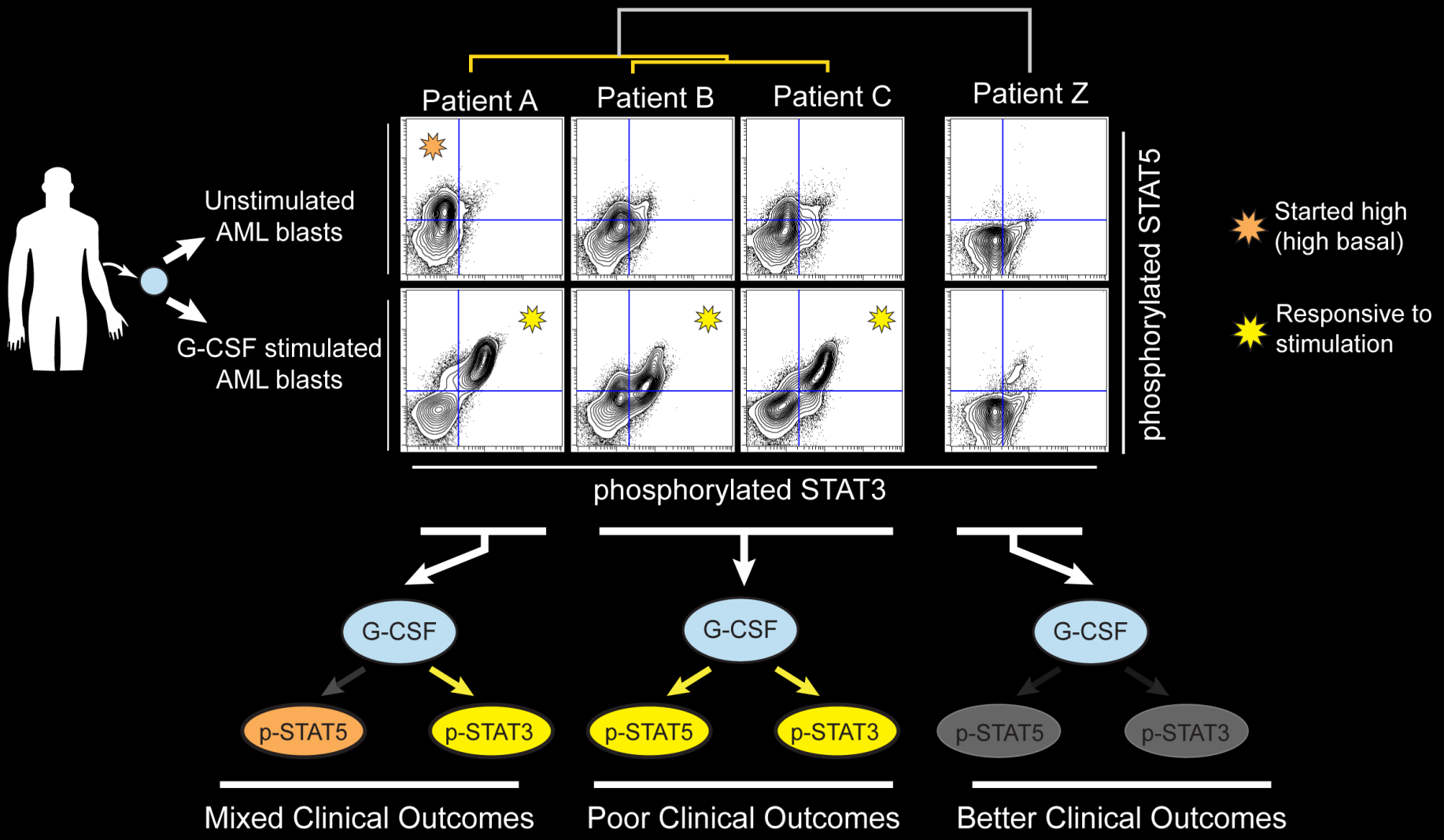
Group Patients by Signaling → Describe Key Signaling Features → Compare Outcomes



Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006

Irish et al., *Cell* 2004

Group Patients by Signaling → Describe Key Signaling Features → Compare Outcomes

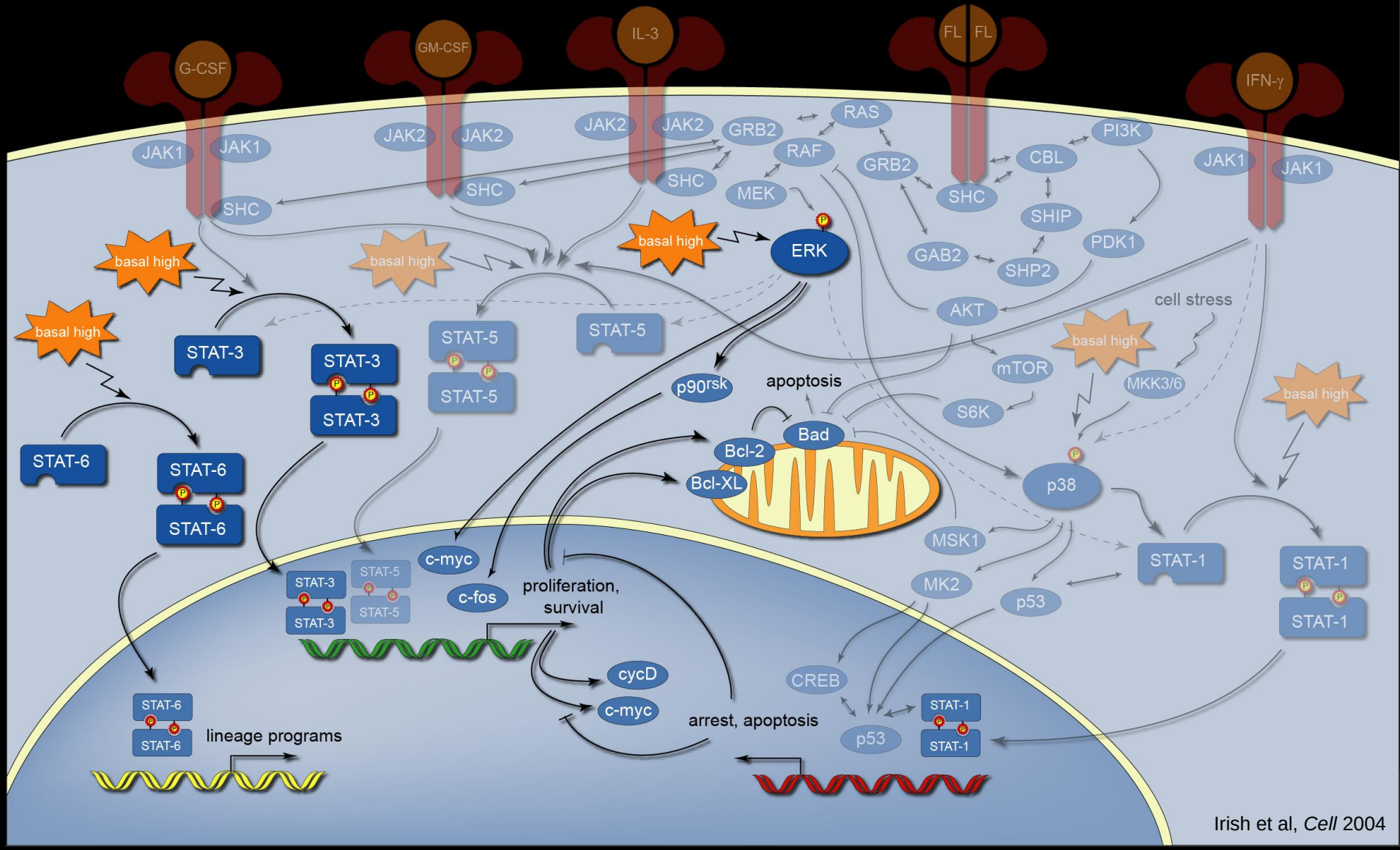


Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006

Irish et al., *Cell* 2004

Figure 6B: Signaling Profile of Patients with Better Clinical Outcomes

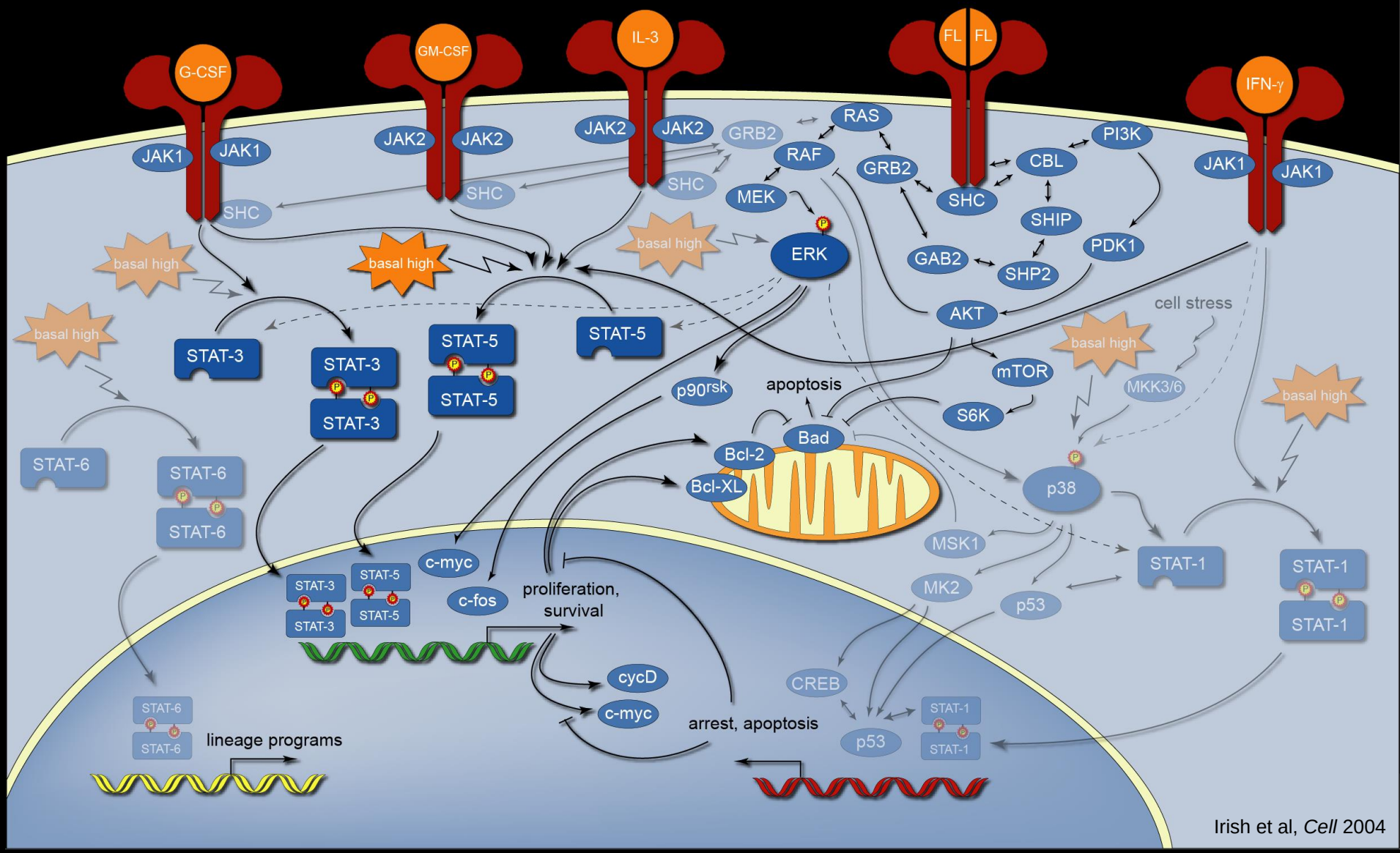
SC-NP Composite Profile



Irish et al, *Cell* 2004

Figure 6B: Signaling Profile of Patients that Resisted Course 1 Chemotherapy

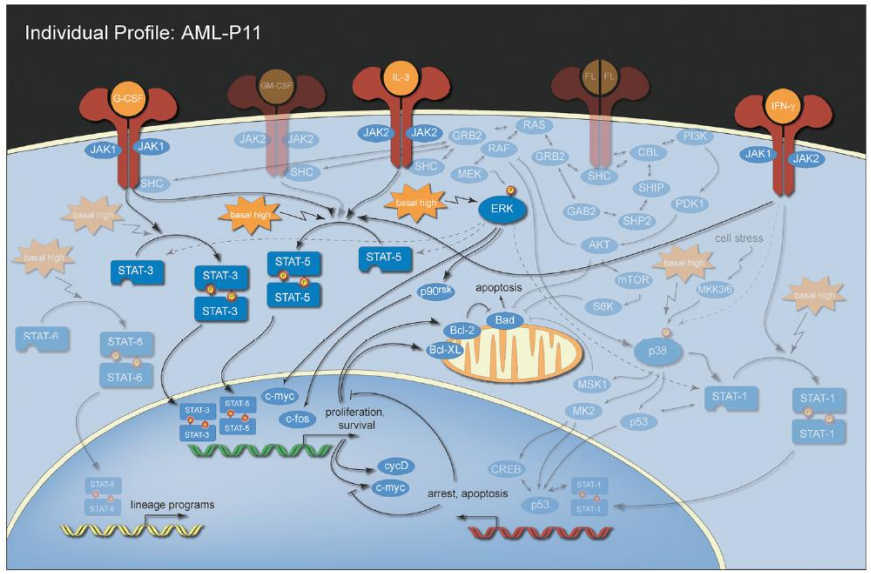
SC-P2 Composite Profile



Irish et al, *Cell* 2004

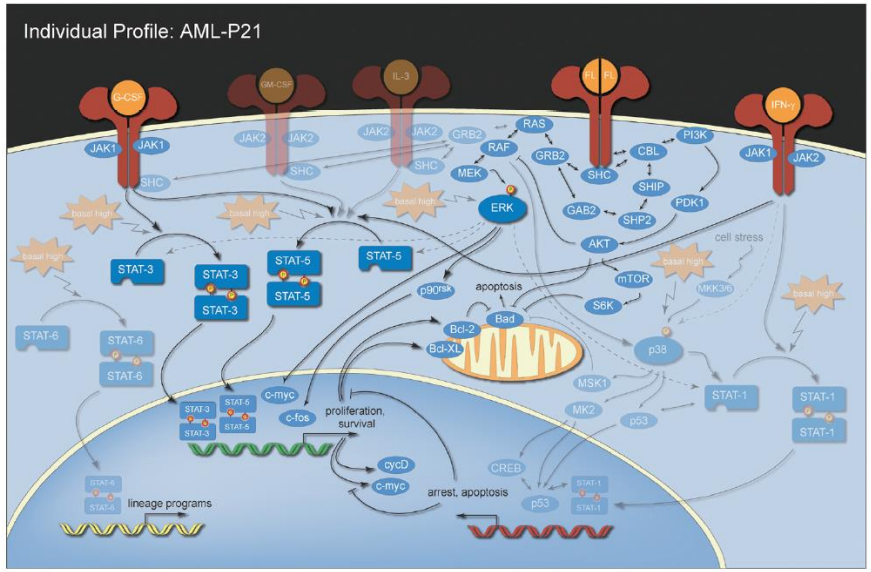
Figure 7: Personalizing therapy based on signaling network profile

A

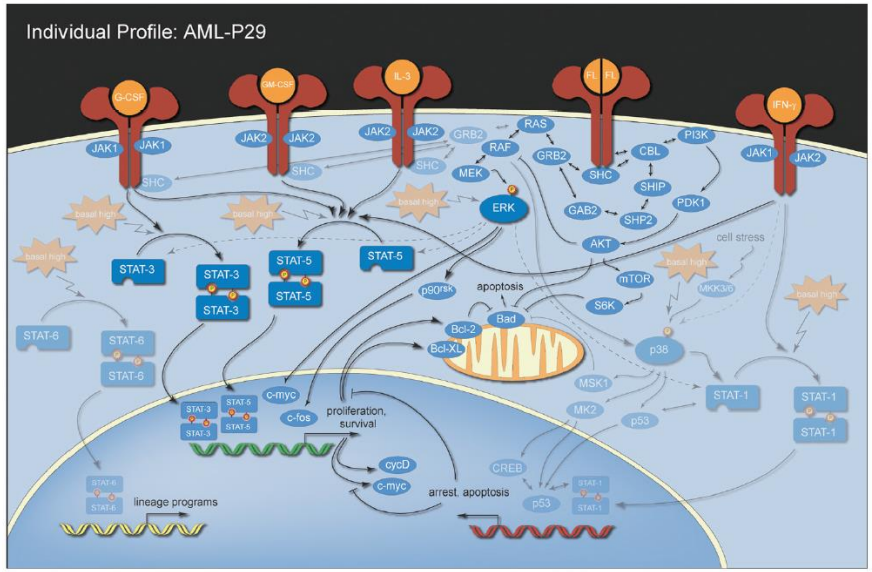


Signaling profile summaries for individual AML patients profiled as SC-P2. Each had detectable Flt3-ITD and resisted course 1 chemotherapy.

B



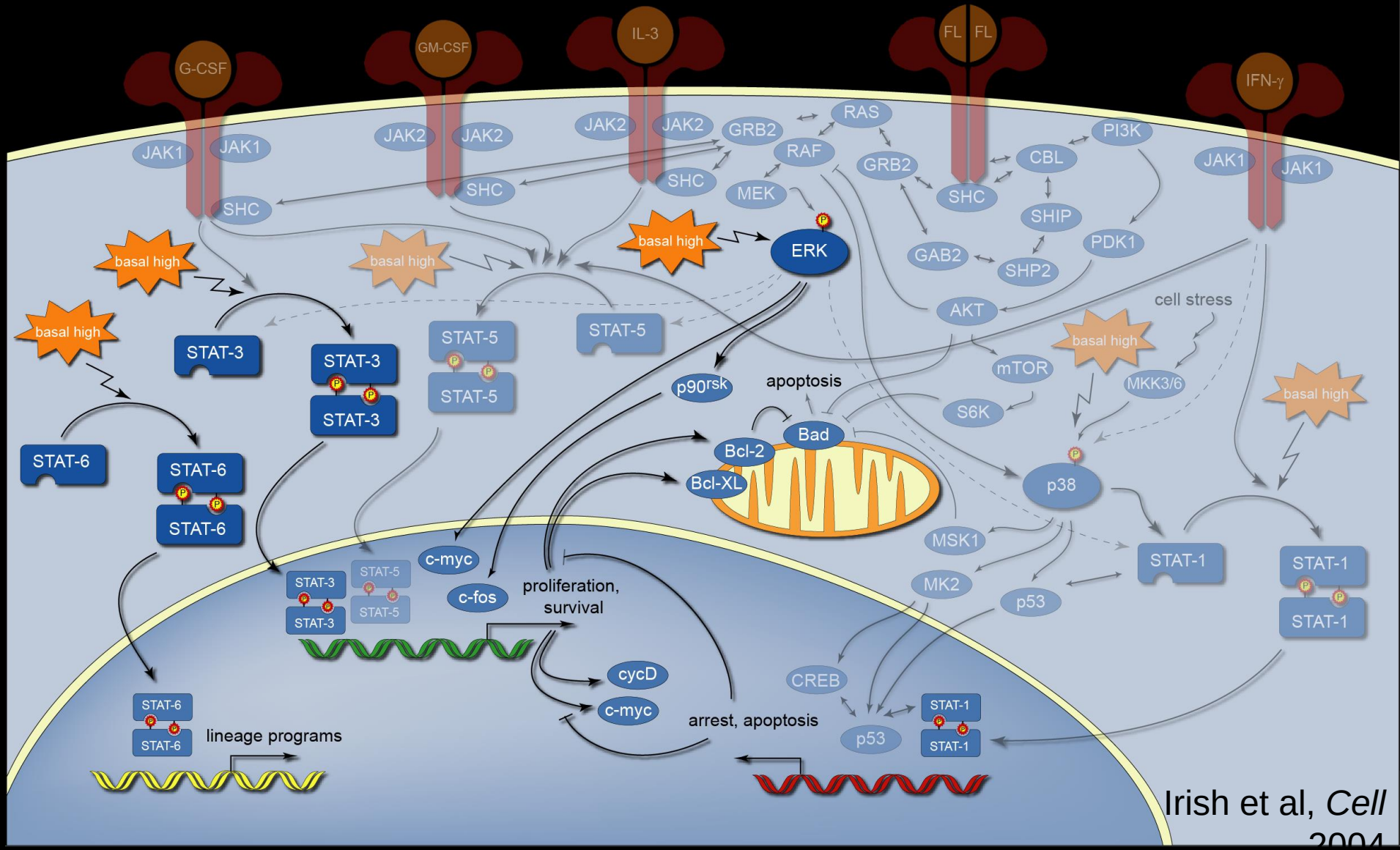
C



Individual Variation in Signaling Mechanisms

Signaling Profile of Patients with Better Clinical Outcome

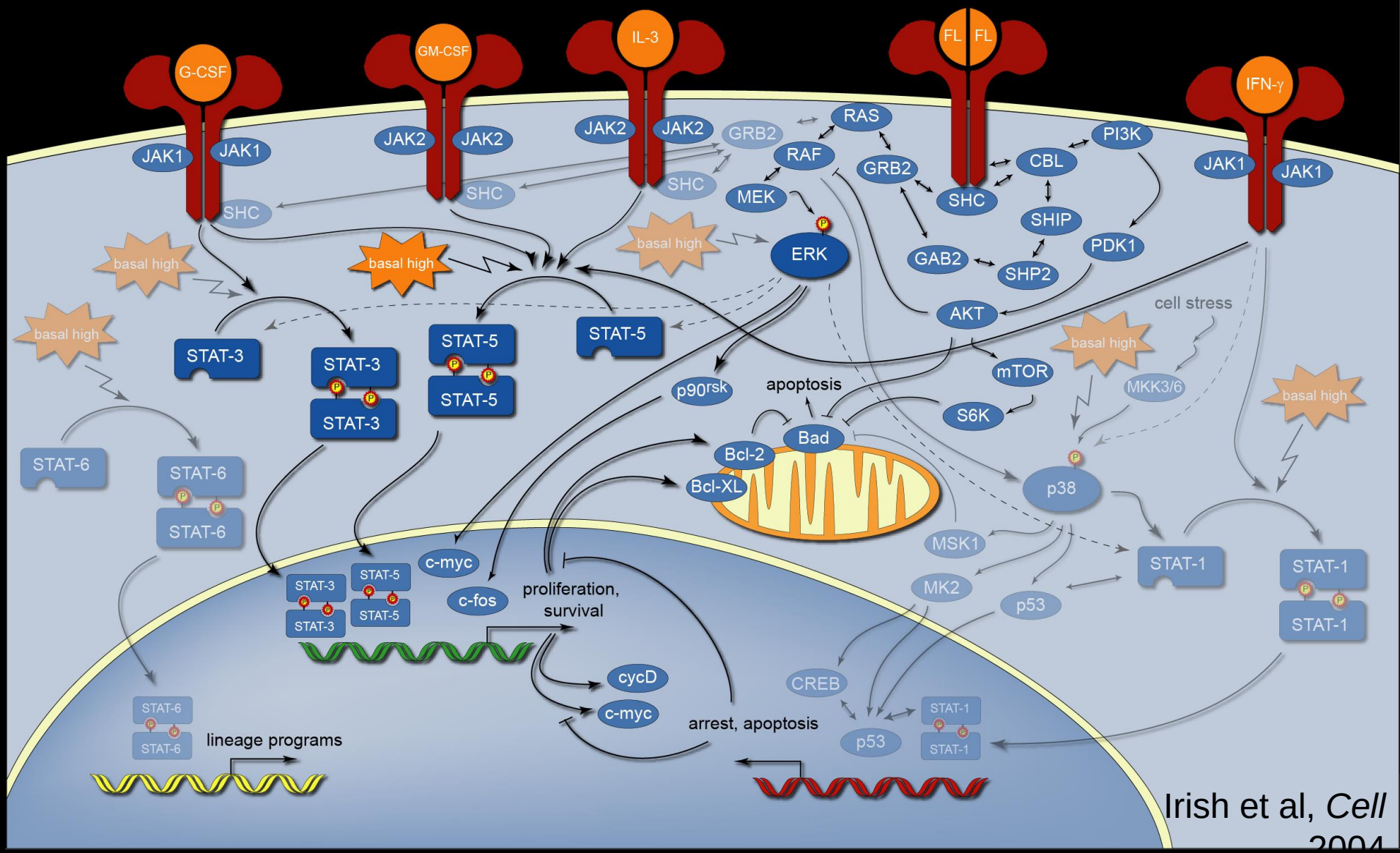
SC-NP Composite Profile



Irish et al, *Cell* 2004

Signaling Profile of Patients that Resisted Therapy

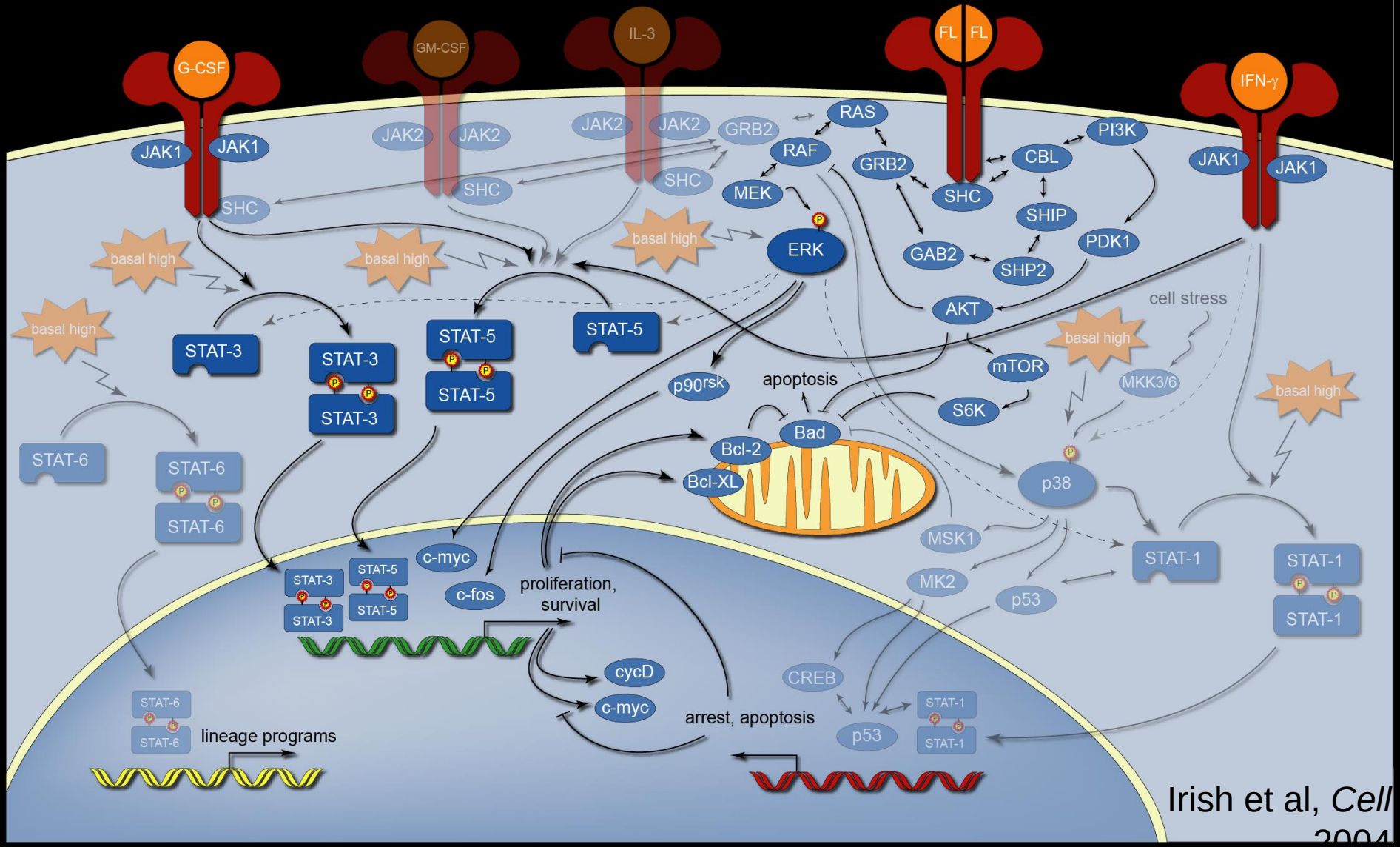
SC-P2 Composite Profile



Irish et al, *Cell* 2004

Map for AML Patient 21 (Flt3-LM, Resisted Chemotherapy)

Individual Profile: AML-P21



Irish et al, *Cell* 2004

Summary: Tumor Signal Transduction Profiling

- Summary:
  - Mapped signaling mechanisms across tumors and constructed a signaling taxonomy of AML.
  - Characterized the state of phospho-protein signaling nodes within the tumor cell network at rest and following exposure to environmental cues.
- Conclusions:
  - 1) Heritable changes to tumors linked to modified signaling networks.
  - 2) Patients whose tumors shared mechanisms of proliferative signaling responded similarly to tumor cell killing (course 1 chemotherapy).
  - 3) The absolute level of phospho-proteins in cells is not as important to tumor survival as the signaling potential of the tumor cell network.
  - 4) Cell by cell enumeration of signaling mechanisms reveals tumor heterogeneity and distinguishes tumor cell subsets.

What's Next for AML?

- Turn the panel into a clinical diagnostic for AML:
  - Prune the non-biosignature nodes.
  - Retest the model in more samples. (BTG has 30-60 new patients w/ extremely detailed Flt3 mutational analysis).
  - Follow up on cytogenetics in different (cytogenetically defined) patient pools.
- Expand understanding of AML signaling:
  - 1) Do signaling profiles change during therapy?
  - 2) Does inhibition of Flt3 signaling affect (kill) cells with the Flt3 signaling profile?

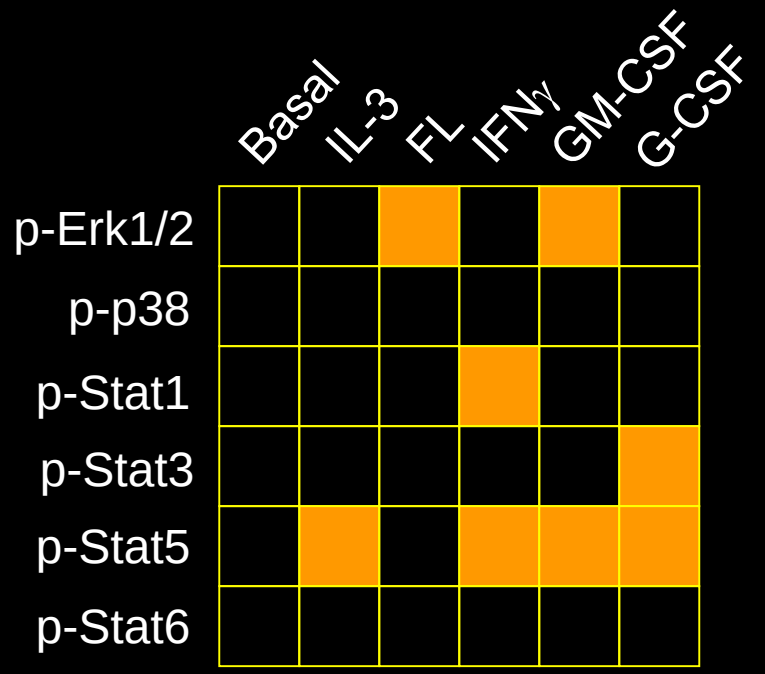
Signaling Profiles of Lymphoma

- Specific Aim I: Create *in vitro* flow cytometry assays for cell signaling functions in lymphoma cell lines and primary tissues.

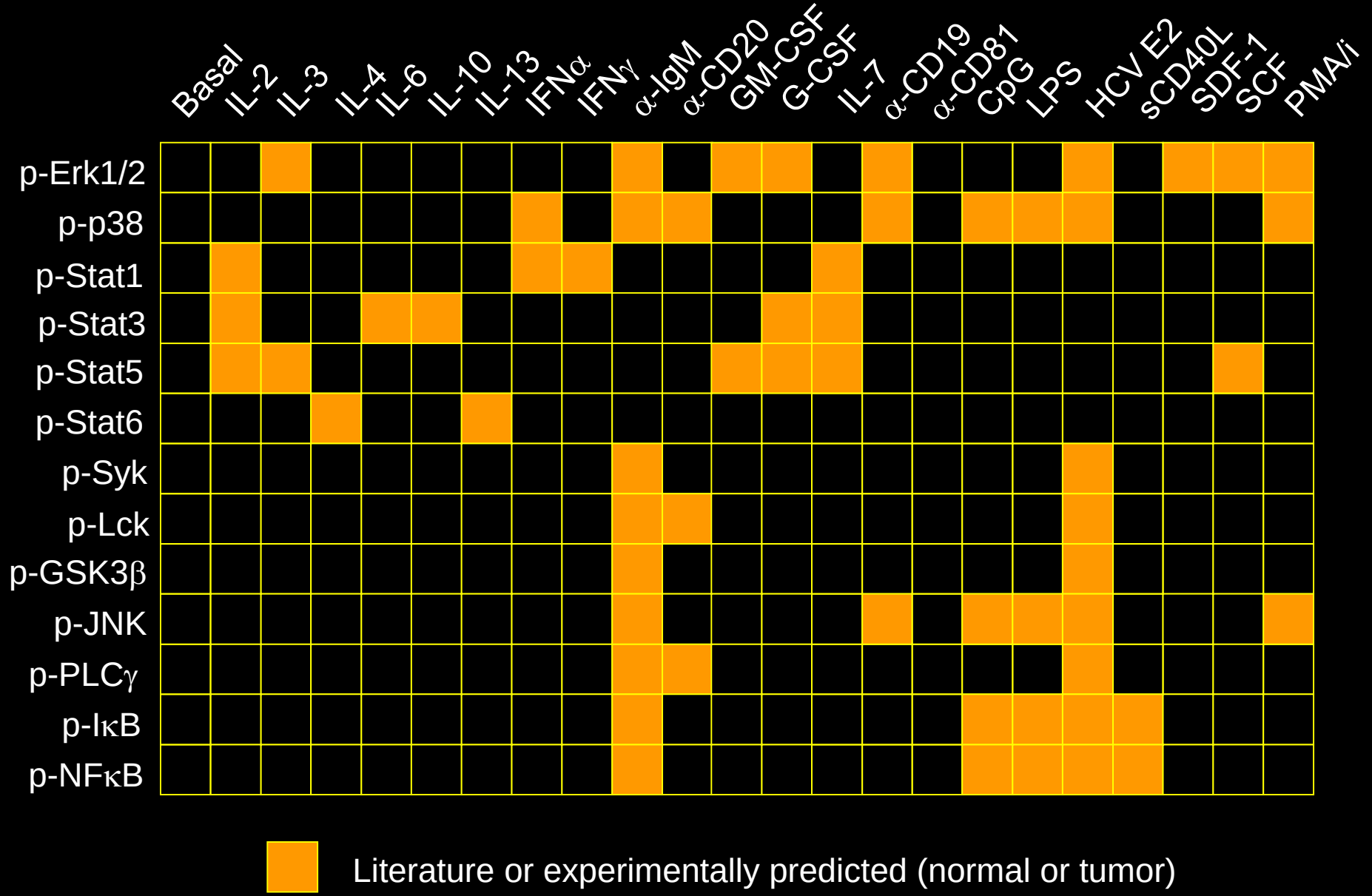
- Specific Aim II: Classify lymphomas (FL) based on signal transduction mechanisms.

- Specific Aim III: Develop and test a predictive model of lymphoma clinical outcome based on profiles of cancer cell signaling.

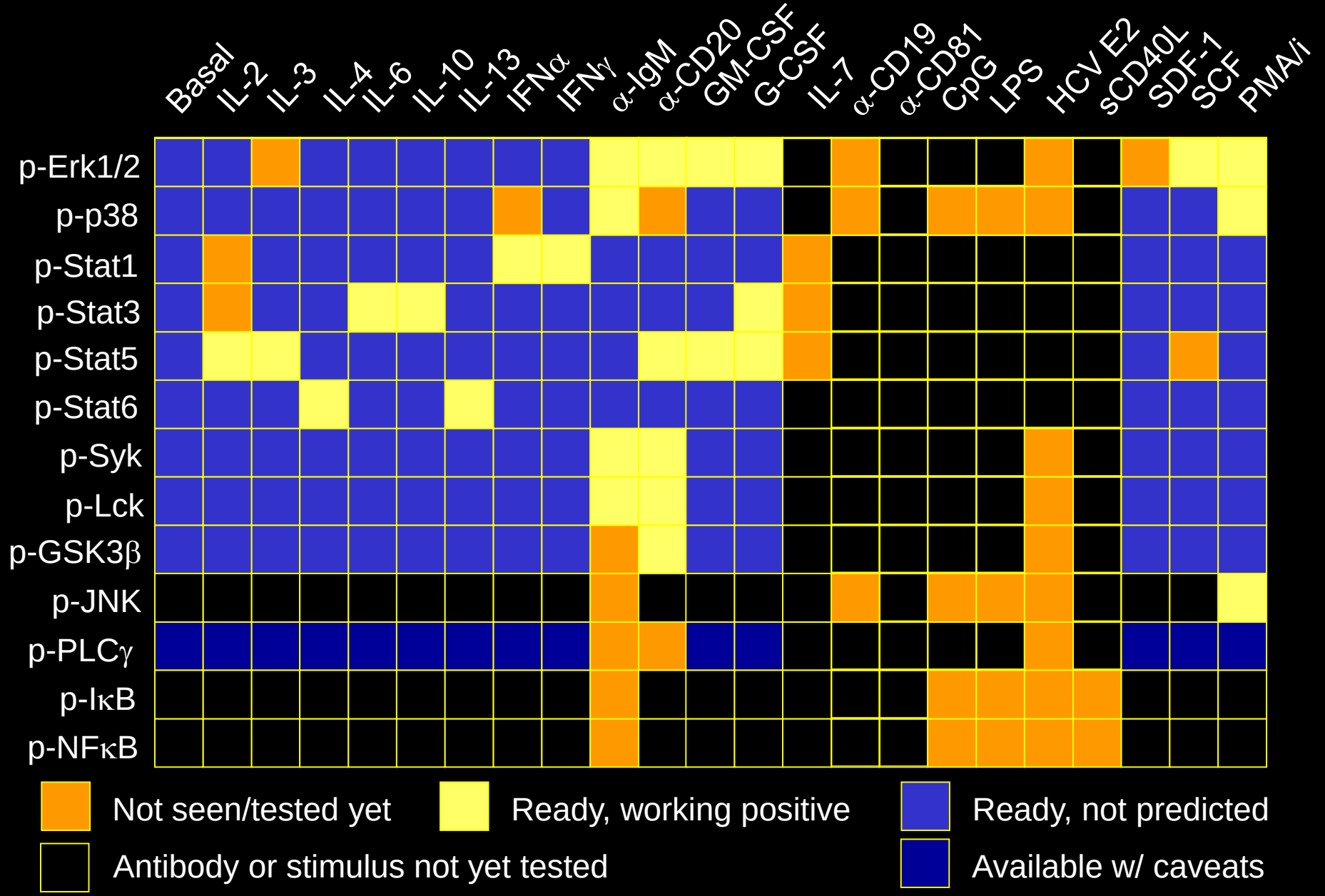
AML Response Panel



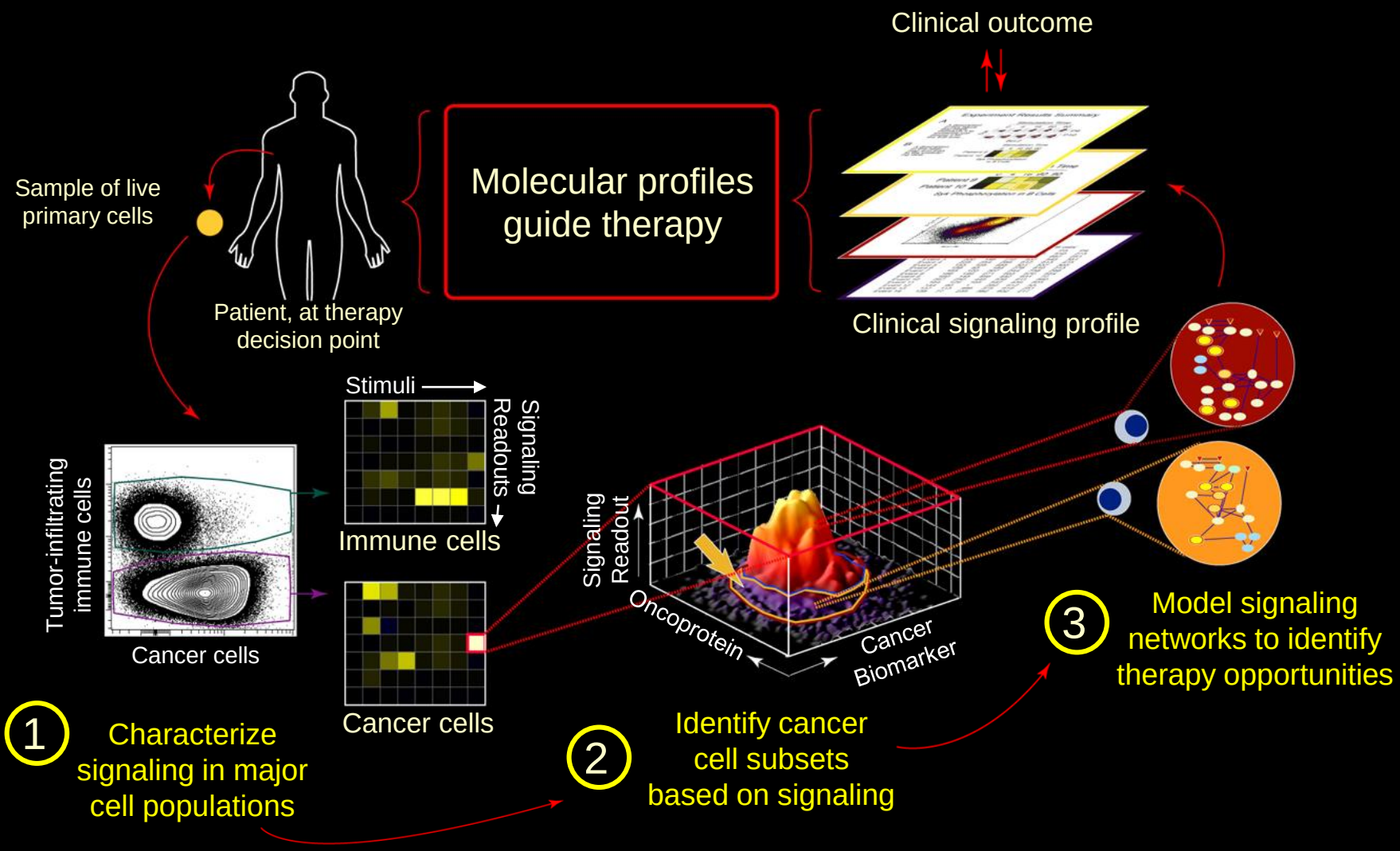
Expansion » Lymphoma Response Panel



Status of Lymphoma Response Panel



Overall Goal: Use Signaling Biology to Improve Therapies



J. Irish

Developing a Clinical Signaling Profile Begins with Choosing Stimuli and Readouts