



IRF5 ESD Review

Saurav De on behalf of IRF5 team

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Acknowledgments

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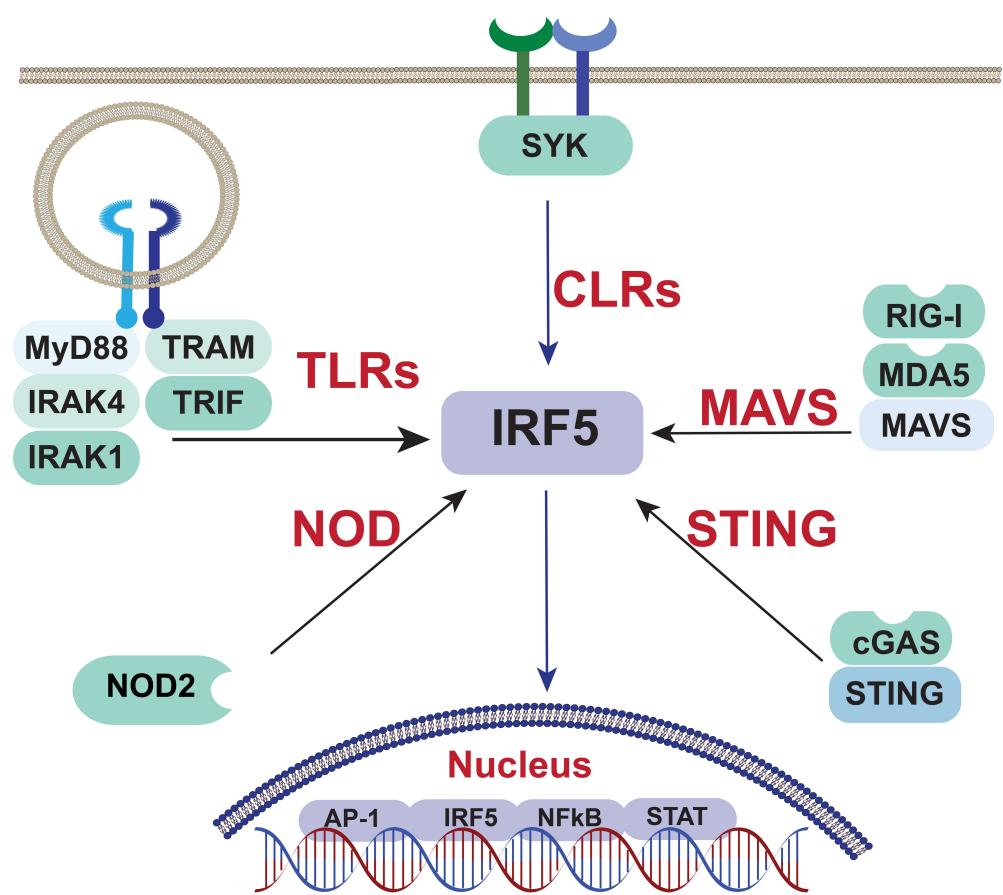
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IRF5 overview



- IRF5 drives inflammatory cytokine and chemokine expression downstream of multiple PRRs
- *IRF5*^{-/-} mice have reduced disease in animal models of IBD, RA, and SLE
- IRF5 expression upregulated in RA, SLE, UC
- In house data demonstrates *in vitro* IRF5 knockdown or knockout in primary human GM-CSF derived macrophages results in reduced inflammatory cytokine and chemokine expression downstream of multiple PRRs
- **Therapeutic Hypothesis:** Inhibition of IRF5 will impact inflammatory outcomes associated with multiple PRRs and therefore be beneficial in the treatment of RA

Current progress

- CiR

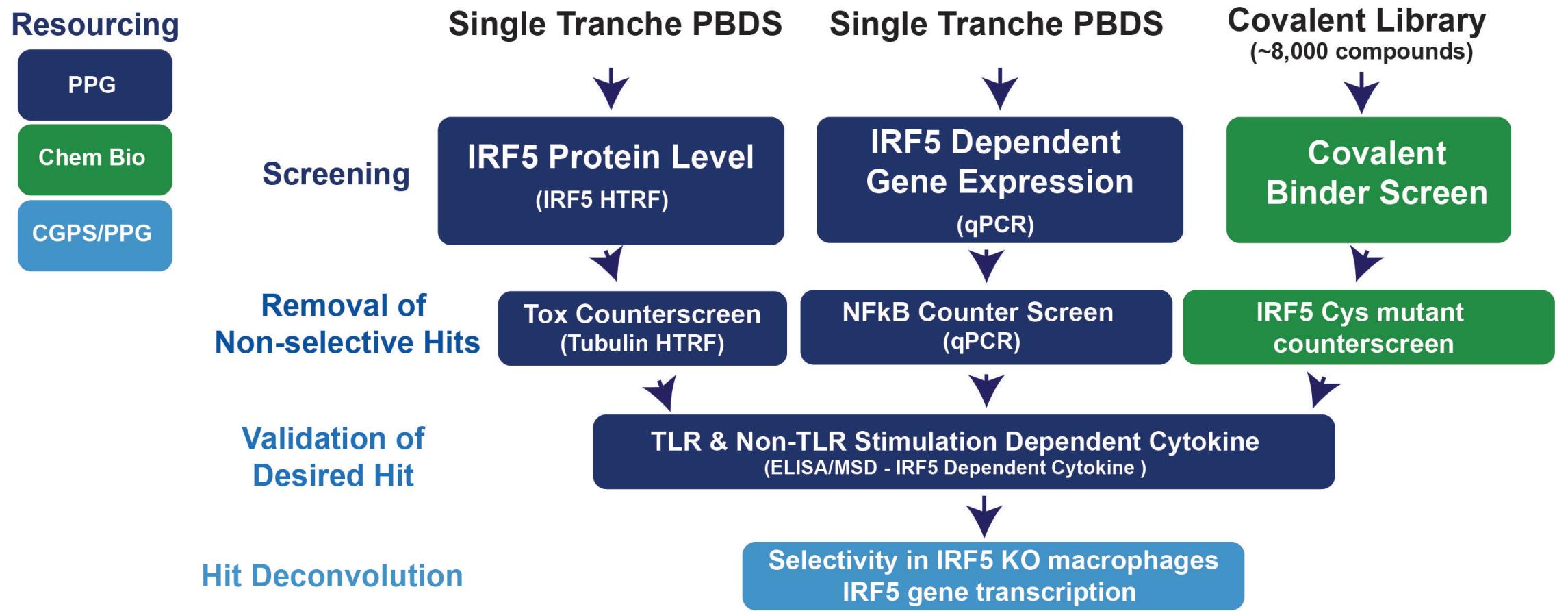
- ▶ *In vitro* reduction of IRF5 leads to decreased PRR driven cytokines when compared to IRAK4i – **COMPLETED**
 - Confirmed KD and KO in human macrophages cause significant reduction in IL6, TNF, IL-8, IP-10, MIP1 α & β , IL12p40 following TLR, STING, CLR, NOD stimulation
- ▶ *In vivo* CiR – **IN PROGRESS**
 - IRF5-dTag F0 generated N1 generation in progress
 - Characterization of founders ongoing
 - Plan established for generation of IRF5-dTag in DBA/1 background
 - CIA and STIA models to establish CiR of IRF5 for RA

- Screening Funnel

- ▶ Progressing orthogonal, complementary Hit ID methods in parallel
 - Human macrophage IRF5 protein level and transcriptional assays have been selected for HTS
 - Protein Level – Currently optimizing 1536 automation, subset screening to start August
 - qPCR – CRISPR protocol validation ongoing across donor set for RNA_Seq, completion expected in September
 - Covalent binder – ELISA development for screening ongoing, expected in Nov/Dec

Proposed screening funnel

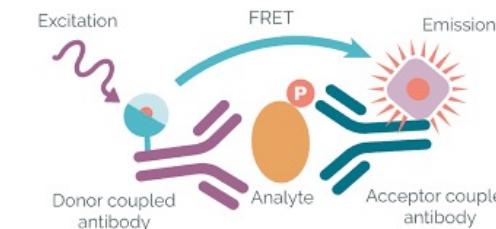
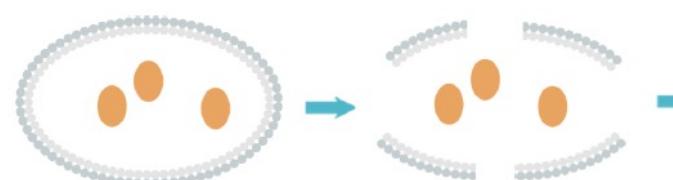
Plan is to operate orthogonal, complementary Hit ID methods in parallel using primary human monocyte derived GM-CSF macrophages



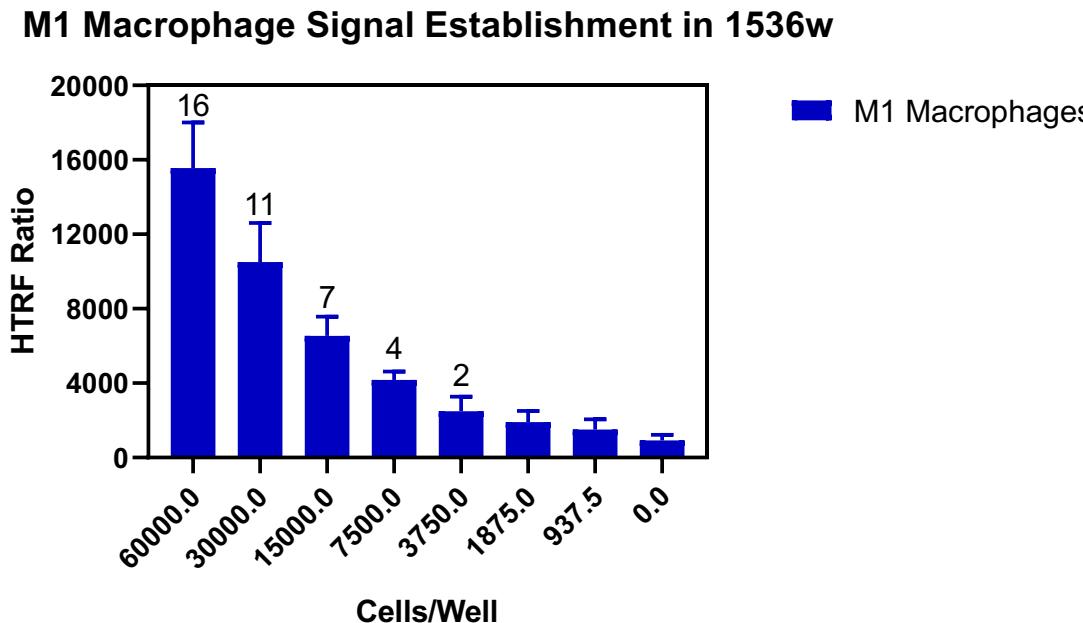
Desired hit will modulate IRF5 dependent cytokine and not modulate IRF5 independent cytokine (identified through RNA_Seq) without apparent toxicity

HTRF assay detects IRF5 levels and is scalable for HTS

HTRF captures IRF5 protein levels in cell types of interest and is scalable

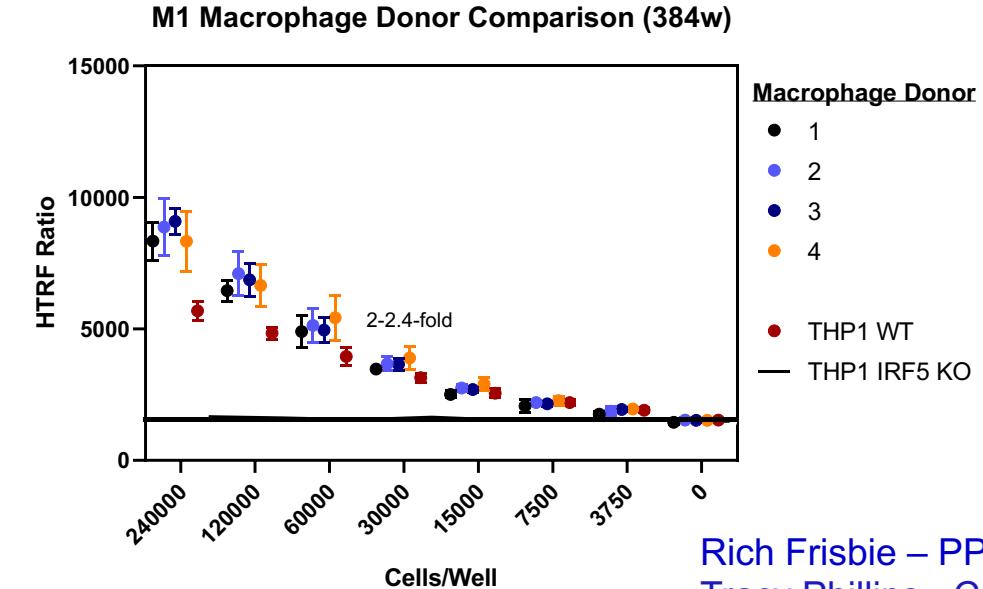


Successful scaling to 1536w format



6 billion macrophages banked for screening

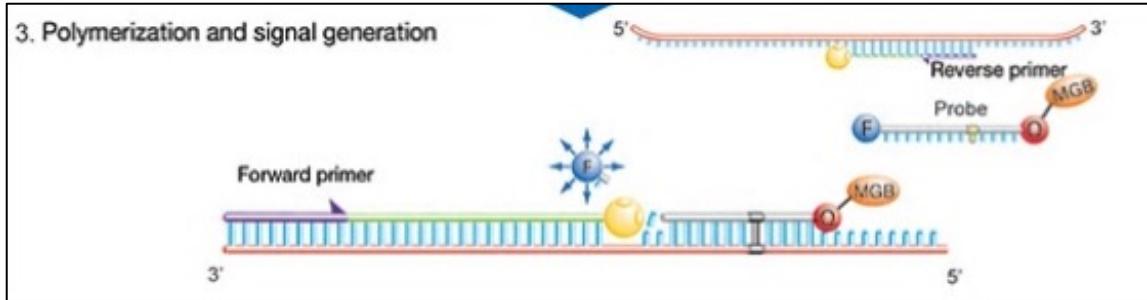
Consistent performance across donor sets



Rich Frisbie – PPG
Tracy Phillips - CGPS
Theresa Dickinson - CGPS

Development of in-house macrophage CRISPR capability

qPCR assay to capture IRF5 dependent transcriptional activity



Gene selection strategy
(expected completion in 2-3 months)

RNA_Seq Design

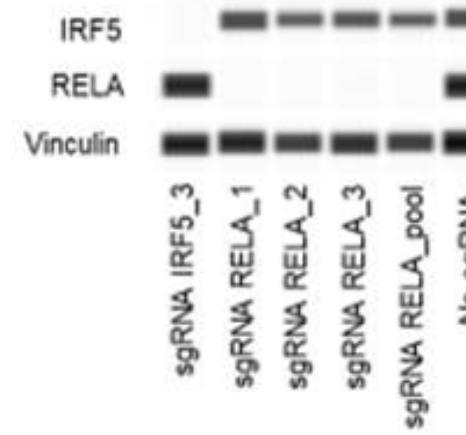
Macrophages (3 Donors)

NT	TLR7/8 (R848)	TLR4 (LPS)
Ctrl	Ctrl	Ctrl
Ctrl + IRAK4i	Ctrl + IRAK4i	Ctrl + IRAK4i
IRF5_KO	IRF5_KO	IRF5_KO
RELA_KO	RELA_KO	RELA_KO

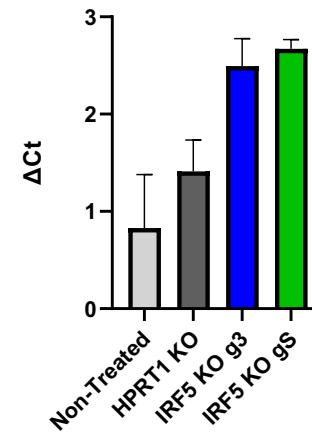
CRISPR completed in 3 donors
RNA submitted for RNA_Seq

Consistency between transcript and protein for IRF5 KO macrophages

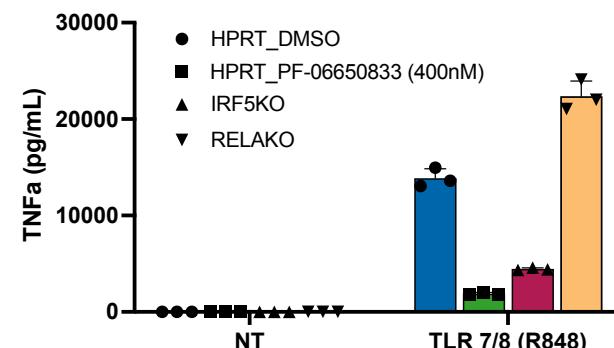
Western blot confirmation of KO macrophages



TNF transcript Ct values

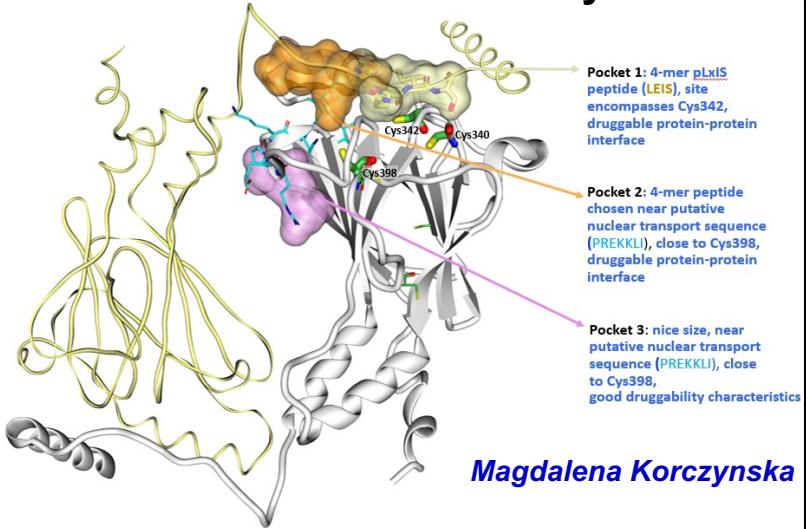


TNF cytokine



IRF5 Covalent First Strategy

Rationale in Covalency



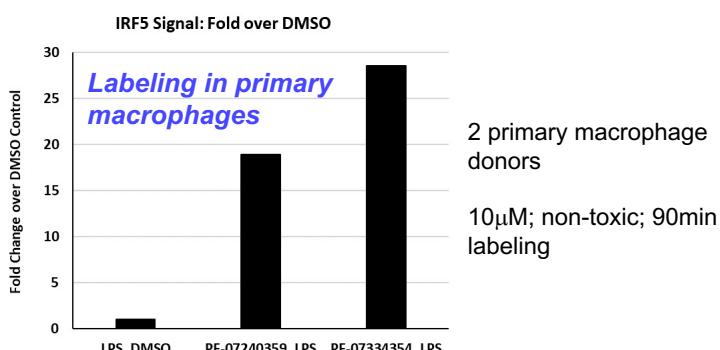
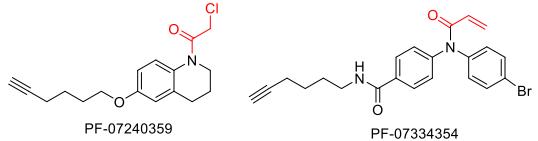
IRF5 has 5 druggable pockets and a druggable protein-protein interface

Evolutionarily Conserved: 5 IRF Cysteines

Cys340, Cys342: Near pLxIS site (potential druggable pocket – proximal to pocket # 1)

Cys398: Alkylation may disrupt nuclear transport, as well as ubiquitination (proximal to pocket # 2 & 3)

Early evidence of Cys ligandibility



Evidence of Cys Liganding from Expi293 overexpression system

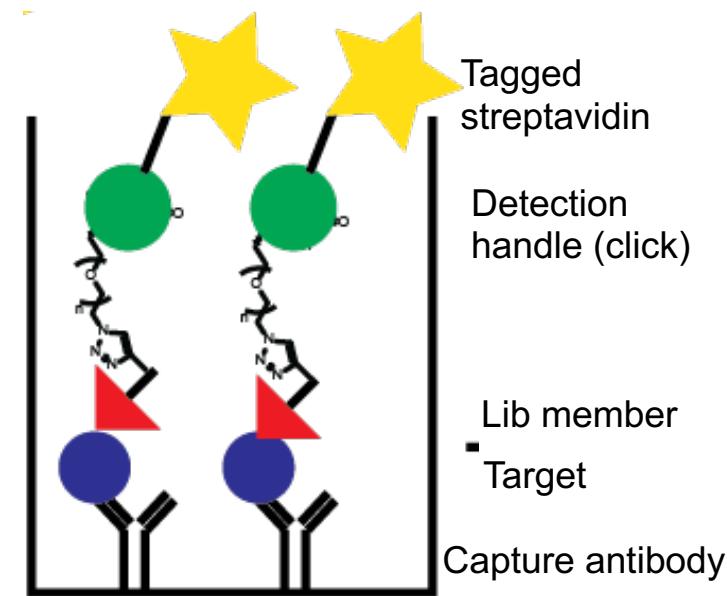
Strong	Medium	None
Cys350	Cys272	Cys121
Cys28	Cys92	Cys340*
Cys398*	Cys342*	Cys44
	Cys357	

* C→A & C→F mutants destabilize IRF5 in CETSA suggesting importance of cys residue for IRF5 stability & structure; 5/10 cysteines tested

Covalent assay concept

Developing high throughput covalent modification assay

Covalent ELISA concept



Assays to be developed (~6mo)

- Completion of covalent screening set
- Biotin-IRF5 ELISA
- High-throughput lysis & click
- Scout fragment identification

HTS Timeline

Q2 (2021)

Q3 (2021)

Q4 (2021)

Q1 (2022)

Q2 (2021)

May

Jun

Jul

Aug

Sep

Oct

Nov

Dec

Jan

Feb

Mar

Apr

May

June

Identify Gene For qPCR Based Screen

- Use IRF5 KO macrophage for RNA_Seq to ID genes
- Utilize union of ChIP_Seq and RNA_Seq data

Resourcing

PPG

Chem Bio

CGPS/PPG

Develop and Validate Screening Assays:

- Utilize known pathway inhibitors to define assay robustness
- Prepare macrophages and characterize donor variability for screening

HTRF HTS + hit confirmation and validation

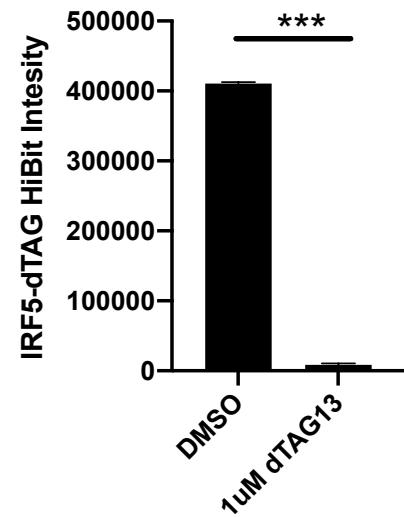
Covalent screening platform development + HTS + confirmation

qPCR HTS + hit confirmation

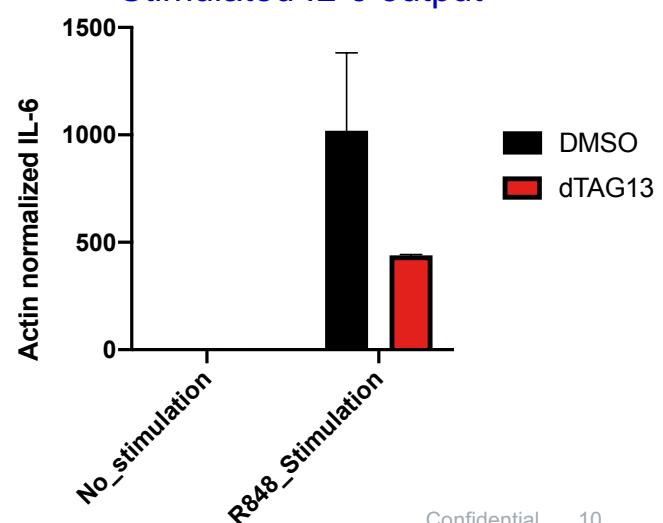
Overview of ex-vivo/in-vivo efforts

- IRF5-dTAG generation in C57BL/6j background for K/BxN serum transfer
 - F0 founders identified carrying C-term IRF5-dTAG knock-in allele
 - N1 Heterozygotes expected in July 2021
 - N-term IRF5-dTAG knock-in generation on-going
 - F0 expected July, N1 heterozygotes late August 2021
- IRF5-dTAG introduction into DBA/1 background
 - Only strain for which collagen induced arthritis model can be run
 - Backcrossing of IRF5-dTAG C57BL/6 strain to DBA/1 at Taconic will require no less than 6 generation
- Profiling of ex vivo BMDM will include:
 - Cytokine and RNA_seq readouts following PRR stimulation

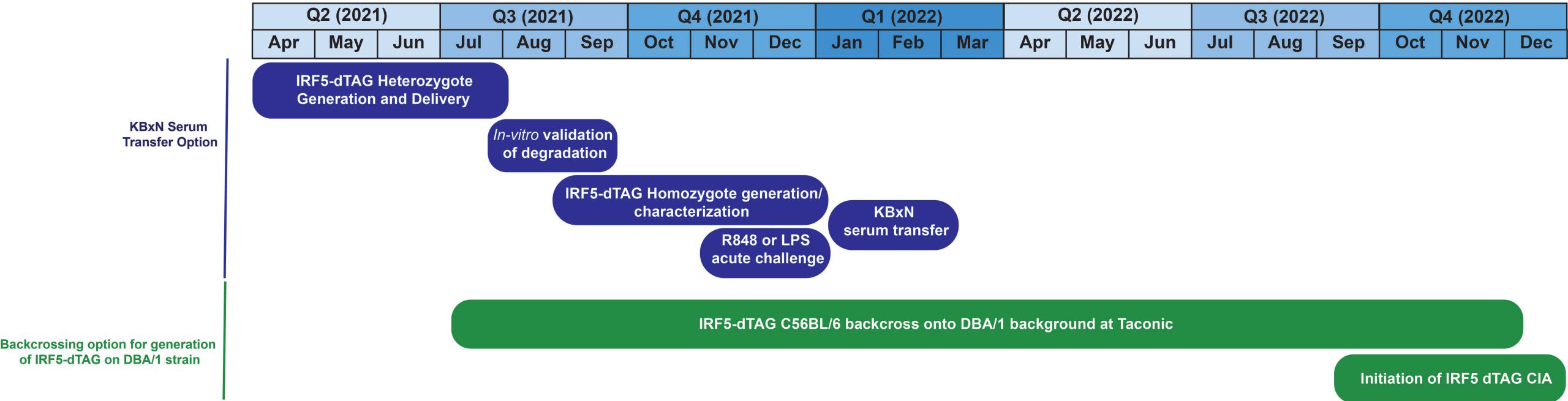
HiBit signal *IRF5* ^{+/dTAG(HiBit)}



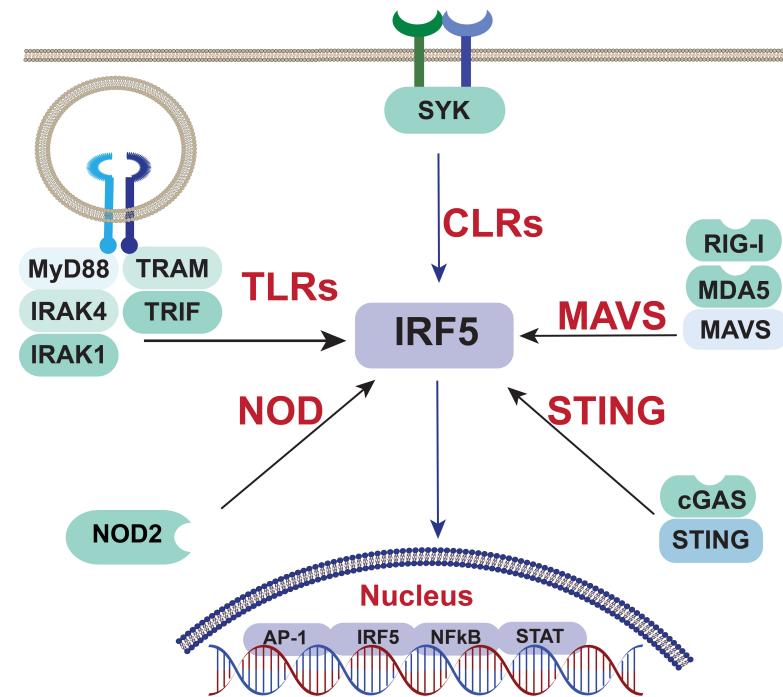
Reduction in R848 Stimulated IL-6 output



In-Vivo timeline



IRF5 Summary



Reasons to believe:

IRF5 impacts inflammatory cytokines and chemokines downstream of multiple PRRs and therefore inhibition of IRF5 should provide therapeutic benefit in the treatment of RA

Progress during previous quarter:

- Orthogonal complementary screening strategy has been agreed upon by team
- Significant progress in developing HTS capabilities for HTRF and qPCR screen

Timeline for progress ahead:

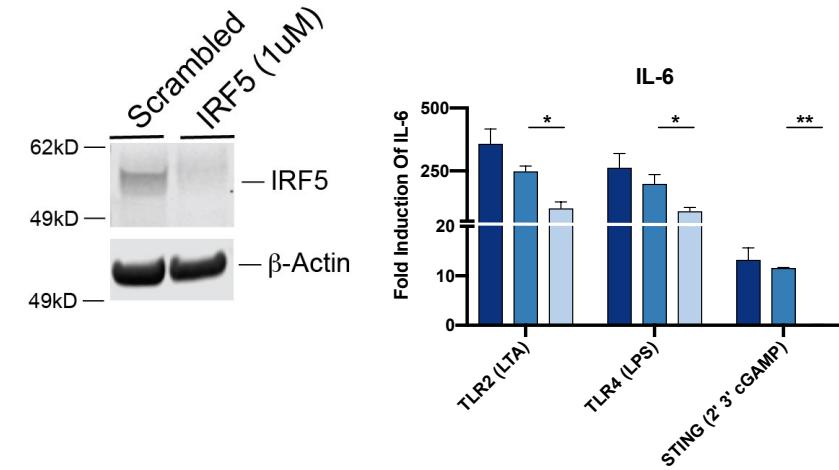
- Gene selection anticipated to be completed by **August/September**
- HTRF screening to be initiated in **August/September**
- qPCR screening to be initiated pending gene selection
- Covalent screen assay development completed by **October/November**

Backup Slides

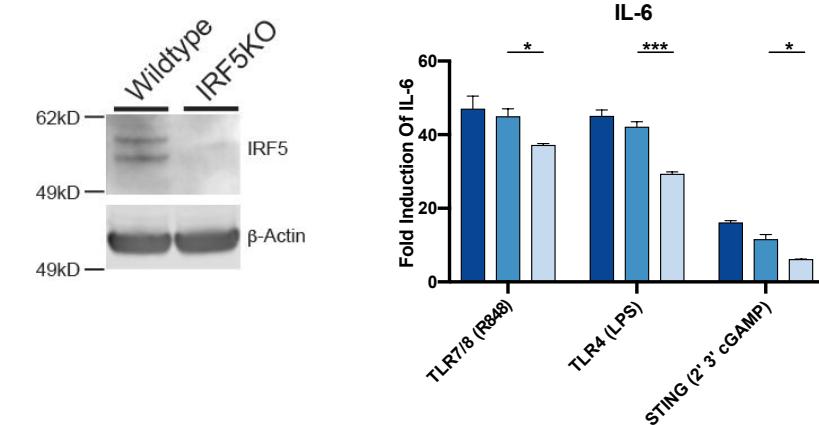
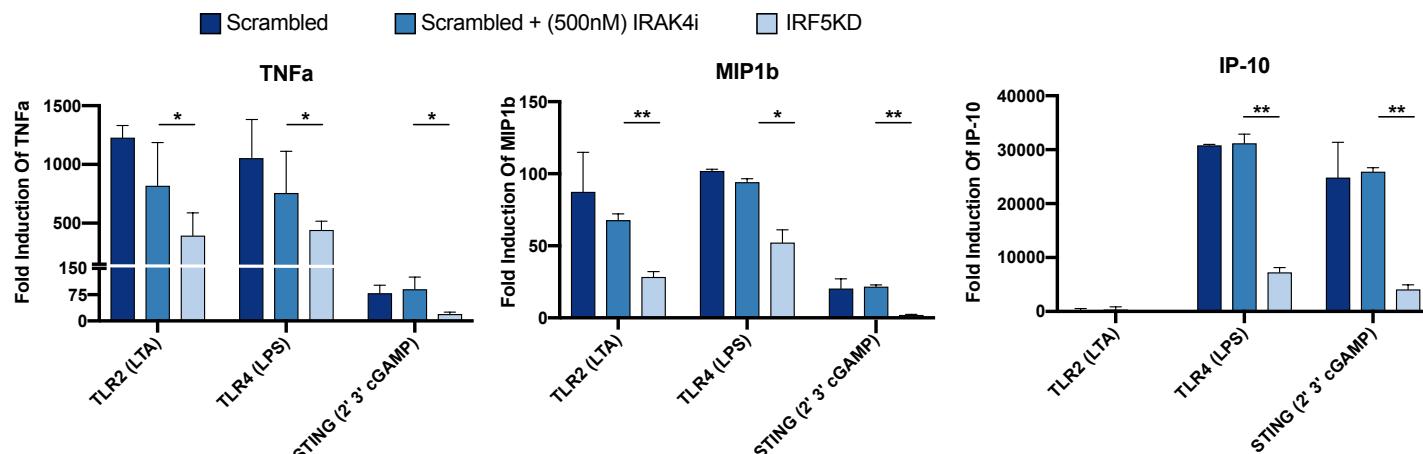
CiR Efforts



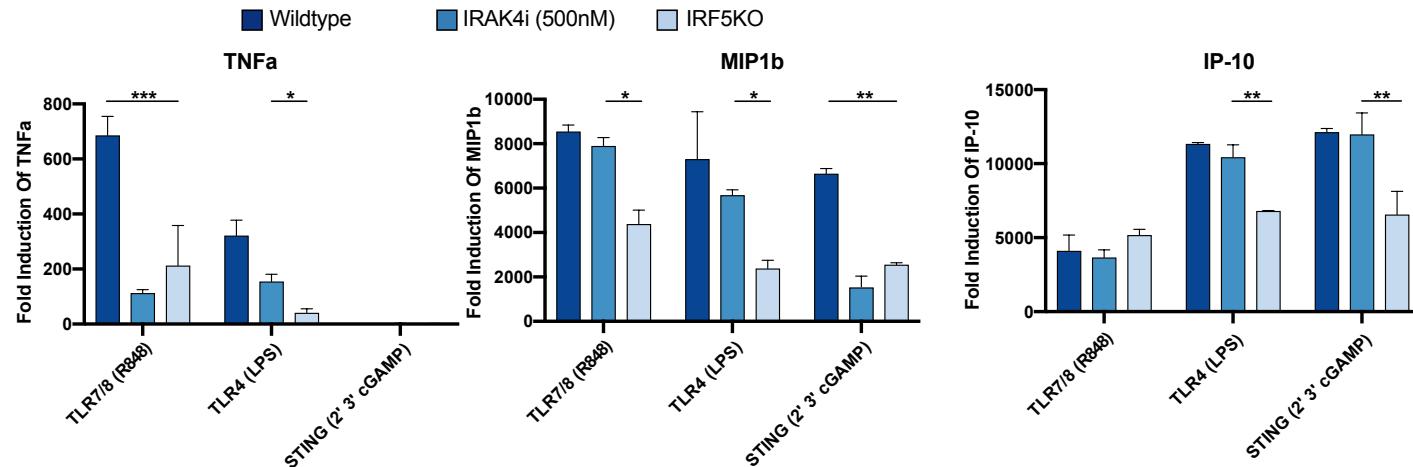
Reduction of IRF5 Levels Dampens PRR Inflammatory Responses



siRNA In Primary (GM)Macs

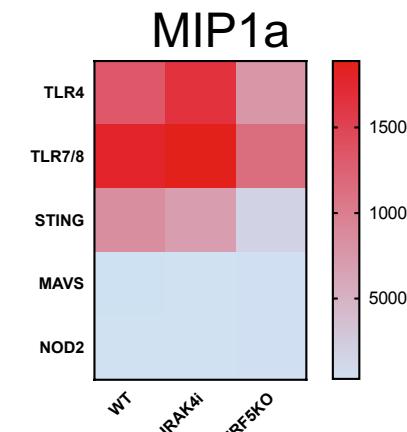
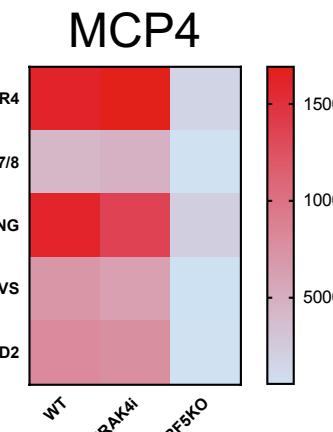
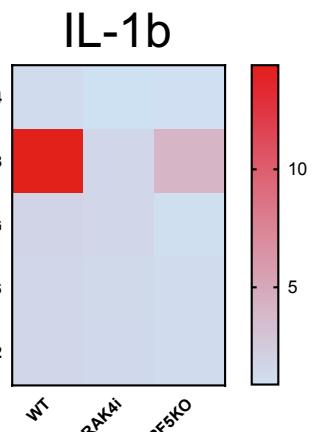
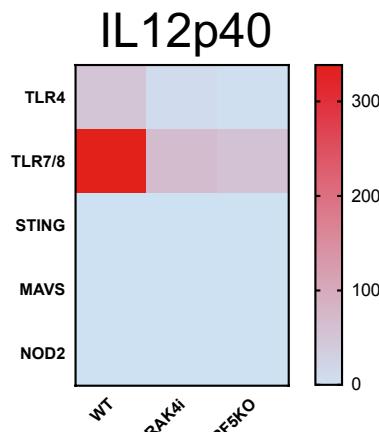
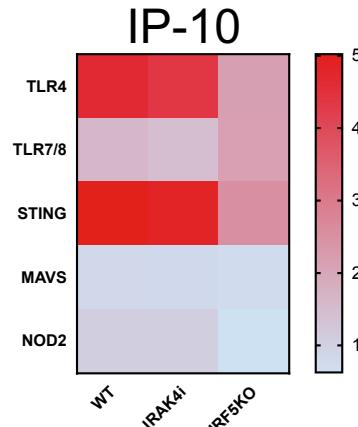
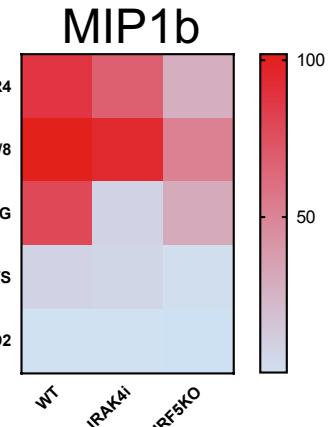
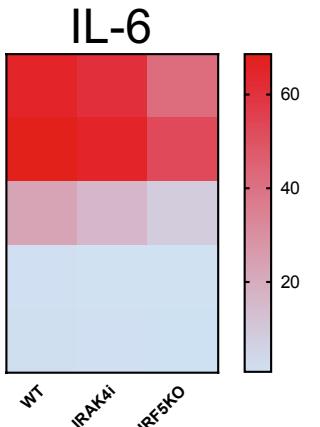
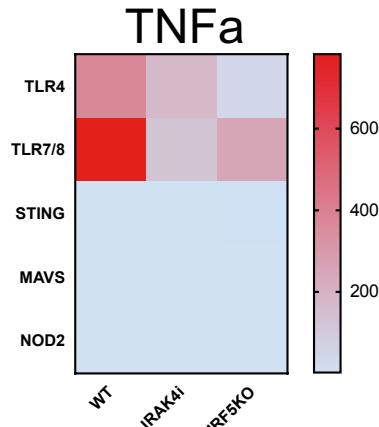


CRISPR In iPSC Derived Macs



Overview of CENSO iPSC Data

Data Plotted As Fold Change Over Unstimulated WT

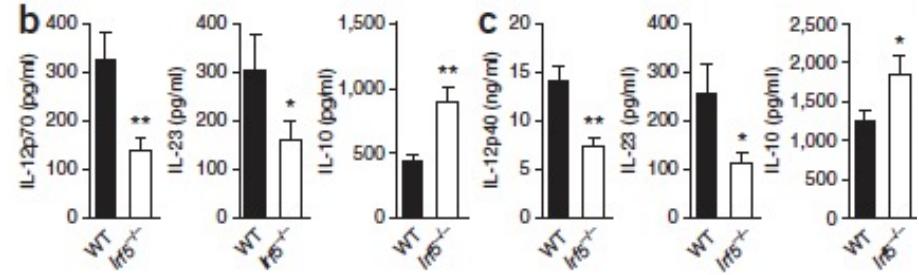


Loss Of IRF5 Impacts More PRRs And Achieves A Greater Reduction Of Inflammatory Outcomes Than IRAK4i

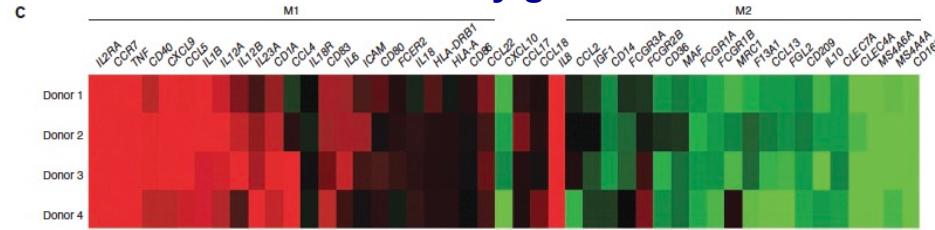
Summary of literature regarding IRF5

- *IRF5^{-/-}* BMDMs have reduced IL6, TNF, IL12p40, IL23 (PMID: 15665823)
- IRF5 is necessary for TNF secretion in macrophages/ monocytes (PMID: 15695821)
 - Late phase TNF production in MDDCs require IRF5 (PMID: 20237317)
- Decrease in IRF5 activation demonstrated to be primary MoA of IRAK4i (PMID: 28924041)
- Age associated B cells (Tbet⁺ B cells linked to RA) require IRF5 for antibody production – (PMID: 29483597)
 - Over-expression of IRF5 results in increased production of inflammatory macrophage polarization and cytokine production (PMID: 21240265)
- IRF5 activation seen *in vitro* downstream of IKK β , MAVs, and TBK1 (PMID: 25326420, 25326418)
- IRF5 expression shown to increase in CD3/CD28 activated T cells (PMID: 32610123)
 - Shown to regulate IFNg, IL2, IL17, and chemokine release in T cells
- Strong association with IRF5 in RA GWAS – miniCredit

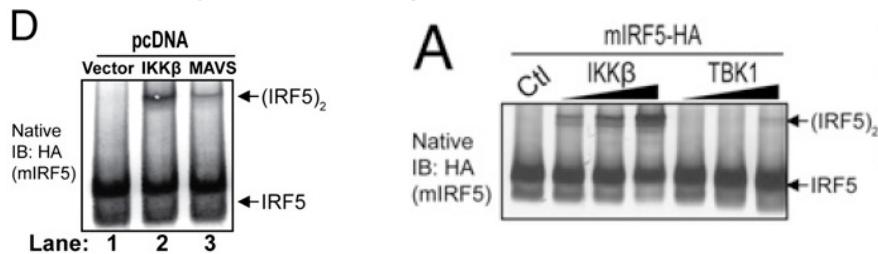
IRF5 driven inflammatory responses in BMDMs



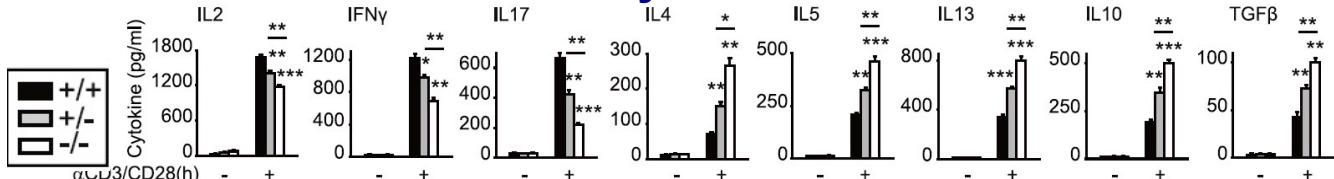
Induced inflammatory genes with IRF5 OE



Pathways leading to IRF5 activation



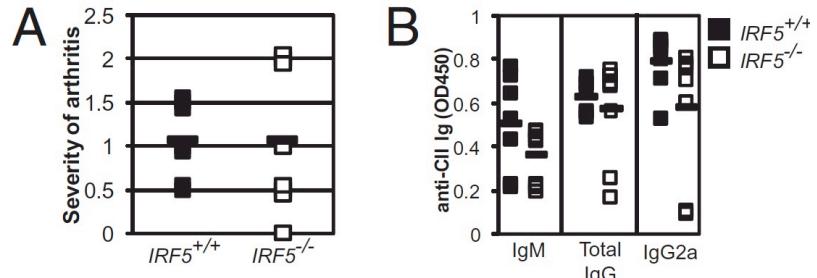
Decreased cytokines in *IRF5^{-/-}* T cells



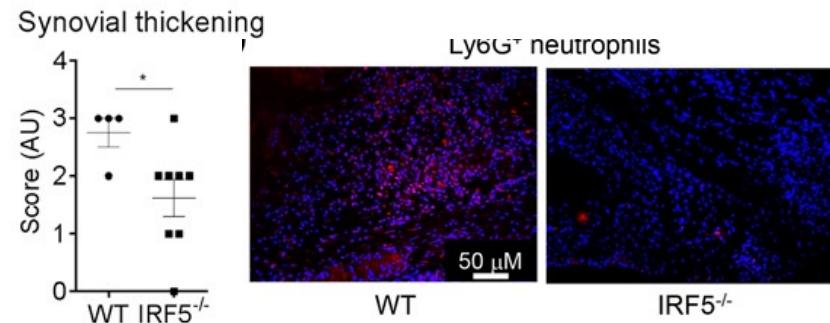
Summary of literature regarding IRF5 for RA

- *IRF5^{-/-}* mice develop normally, are protected from LPS or CpG driven lethal shock, have reduced IL6, TNF, IL12p40, IL23 (PMID: 15665823)
- *IRF5^{-/-}* mice have reduced arthritis in AIA (PMID: 26283380)
 - Significant decrease in synovial thickening at day 7 in *IRF5^{-/-}*
 - Significantly reduced IL-6, IL12p40, IFNg, IL17a, IL-1b, IL23a transcripts seen in joint extract
 - Decreased neutrophil infiltration into joint
- *IRF5^{-/-}* mice have reduced arthritis in K/BxN serum transfer model (PMID: 26315890)
 - Significant decrease in ankle thickness and arthritis score at day 7 in *IRF5^{-/-}*
 - Decreased neutrophil infiltration into joint
- *IRF5^{f/f} x Lyz2^{Cre}* mice have reduced arthritis in AIA model (PMID: 32743529)
 - Decreased knee swelling seen at day 2
 - Correlation seen with IRF5 reactivity of sera
- *IRF5^{-/-}* mice show no impact on arthritis score in CIA model (PMID: 20479222)

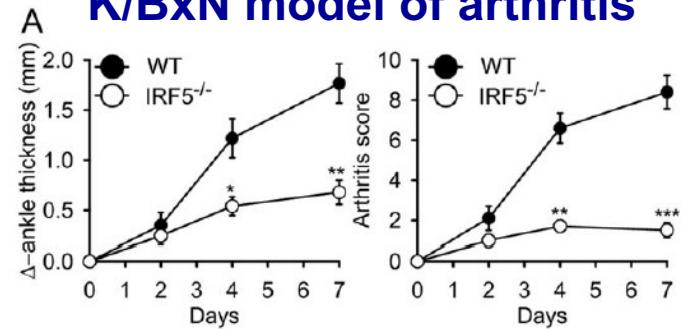
IRF5^{-/-} CIA model of arthritis



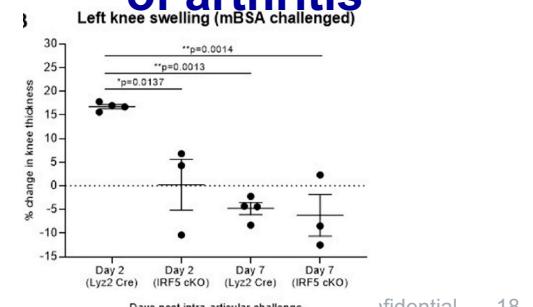
AIA model of arthritis



K/BxN model of arthritis



IRF5^{f/f} x Lyz2^{Cre} in AIA model of arthritis



Differentiation From IRAK4i And Relationship To RA

IRF5 will impact wider range of PRRs and therefore provide greater efficacy than IRAK4i

- **STING (RIGI/MAVs)**

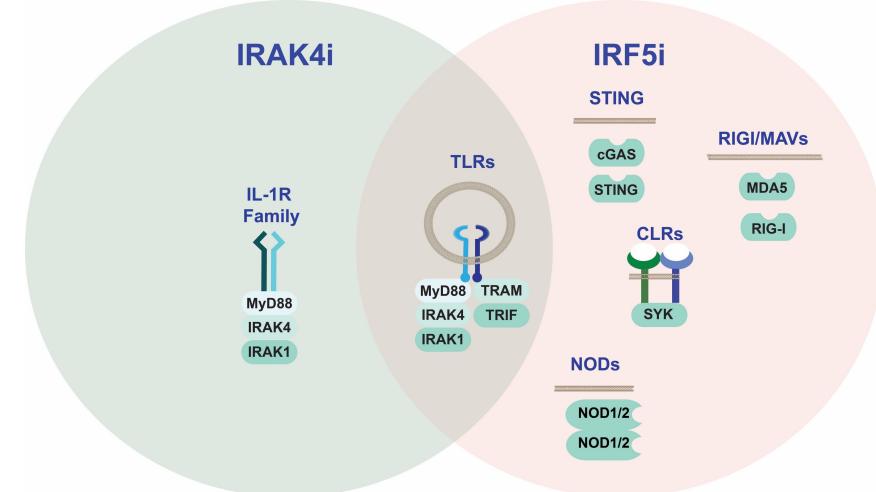
- DNAse II KO mice present with polyarthritis - not rescued by *IFNAR*^{-/-} (PMID: 17066036)
- TMEM173 KO rescues polyarthritis seen in DNAse II KO mice (PMID: 23132945)
- IRF5 Is Activated Downstream of STING (PMID: 24198409, 25326420, 25326418)
 - Mixed bag on pDCs – (PMID: 27125983, 32471881)

- **NODs**

- MDP IV administration drives acute arthritis (PMID: 25717000)
- Patient with NOD2 activating mutation present with inflammatory pediatric arthritis (PMID: 17968944)
- NOD2 acts on IRF5 to initiate inflammatory cytokine (also glycolysis) (PMID: 27545875, 19578435)
- NOD2 expression and NOD2 ligand (MDP) observed in synovial tissue of RA patients (PMID: 18574154)
- NOD2^{-/-} mice have reduced arthritis score in SCW model

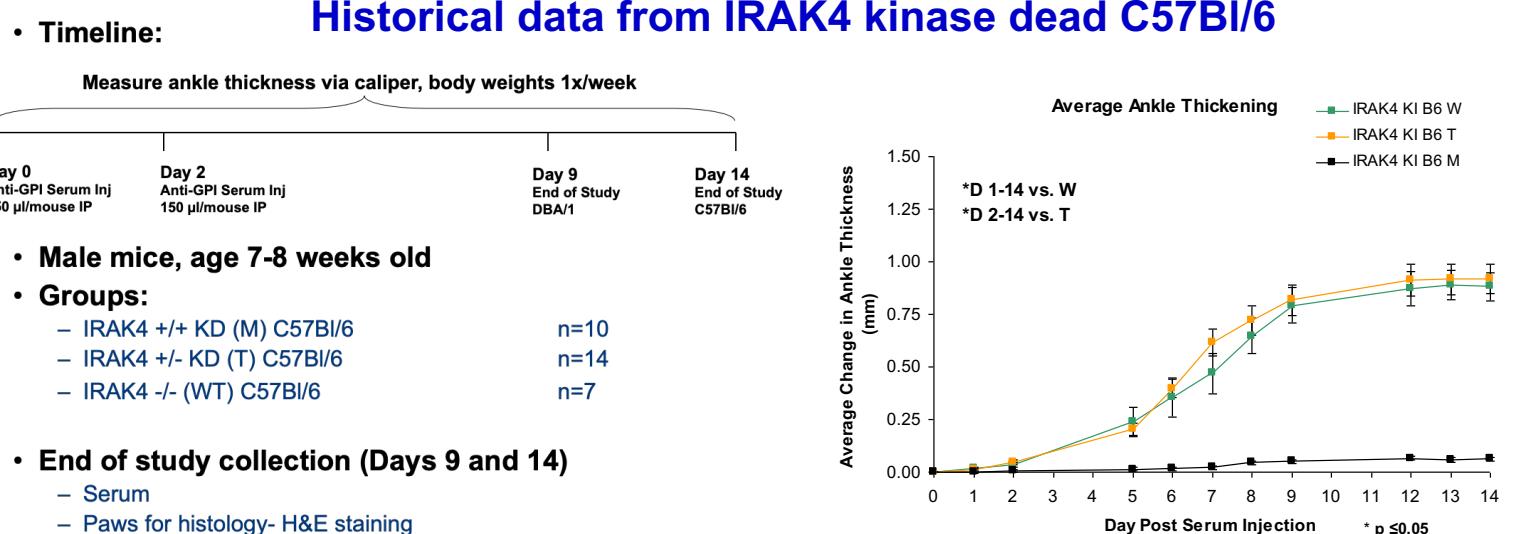
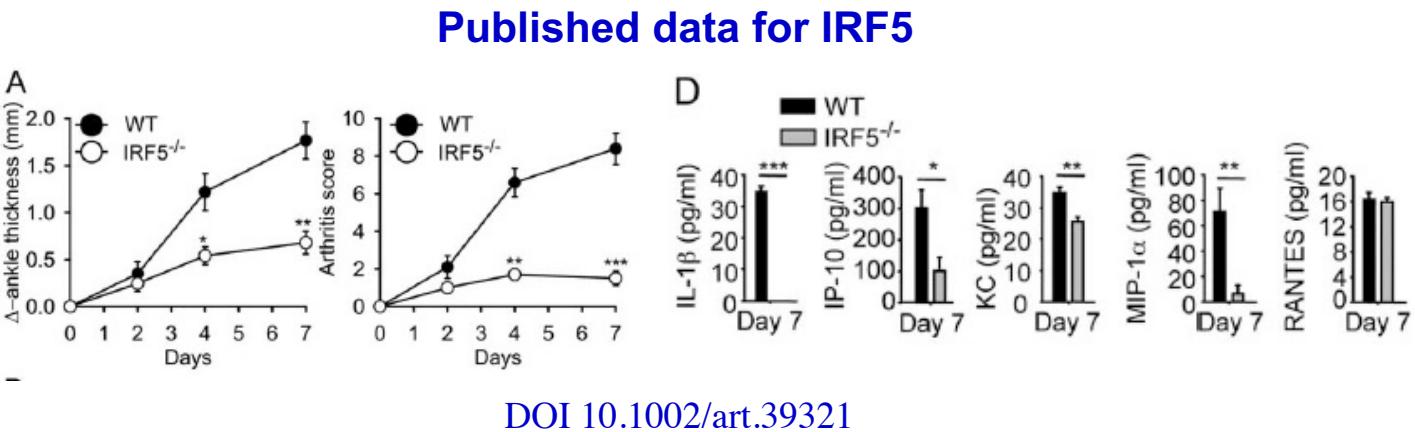
- **CLRs**

- Report of SKG mice requiring Dectin1 for arthritis onset (PMID: 15781585)
- GWAS correlation with C-type lectin receptors in RA (PMID: 17665455)
- IRF5 activated downstream of Dectin 1 via SYK (PMID: 23770228)

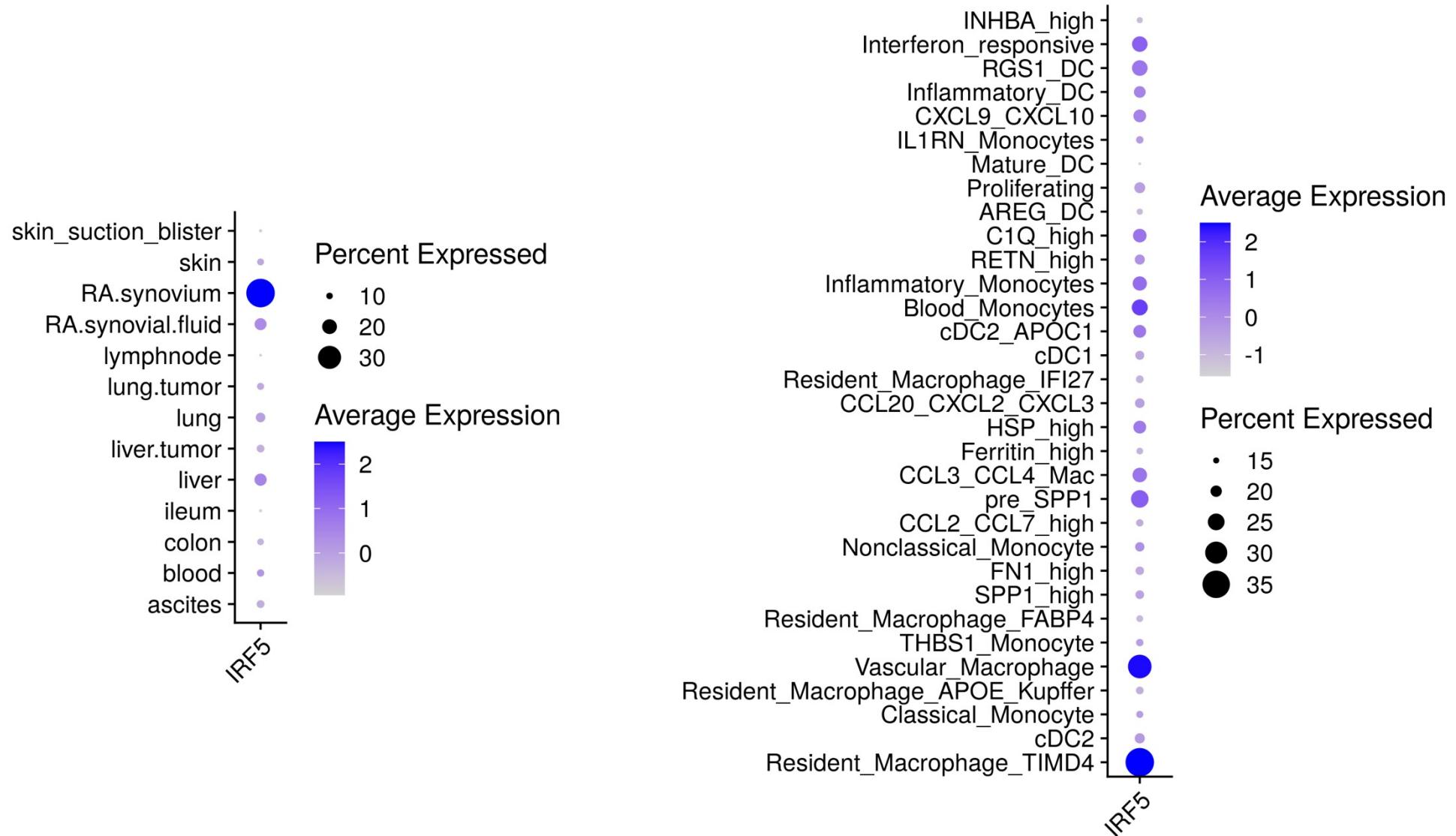


Pros/Cons for KBxN serum transfer model

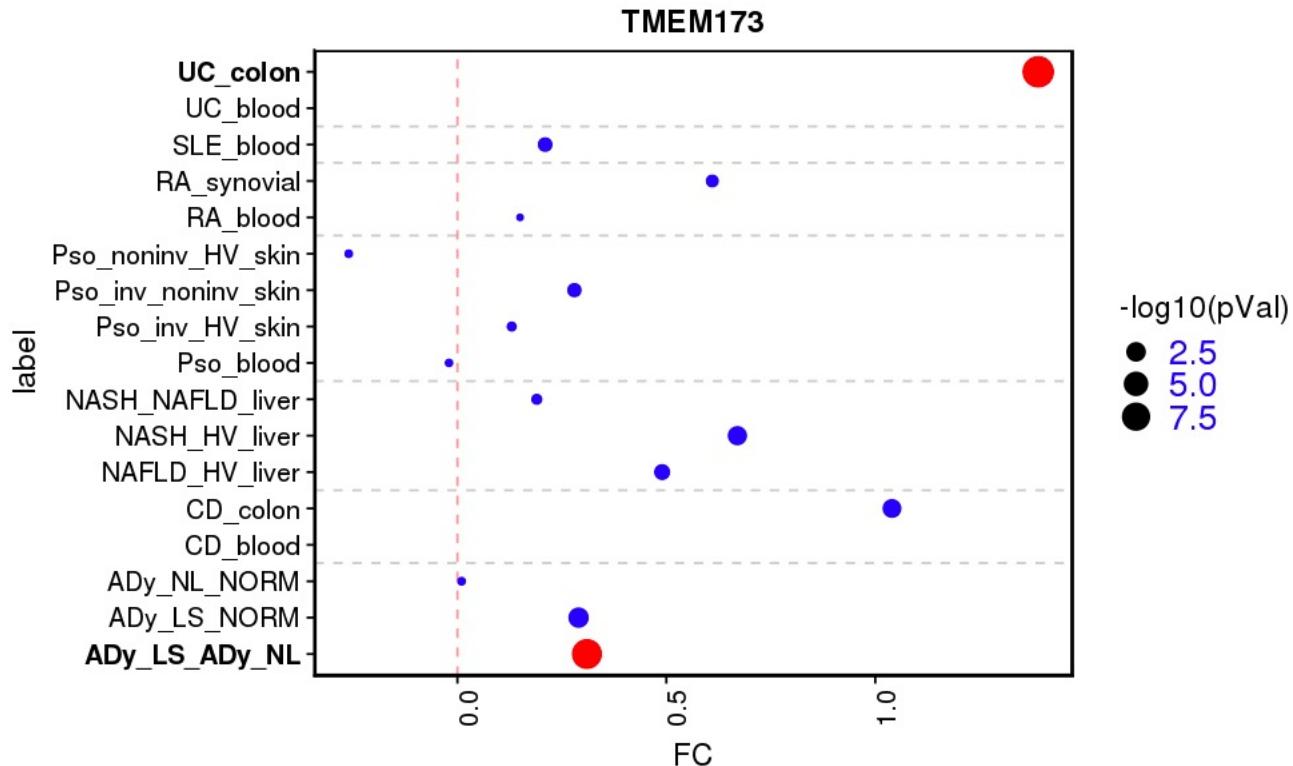
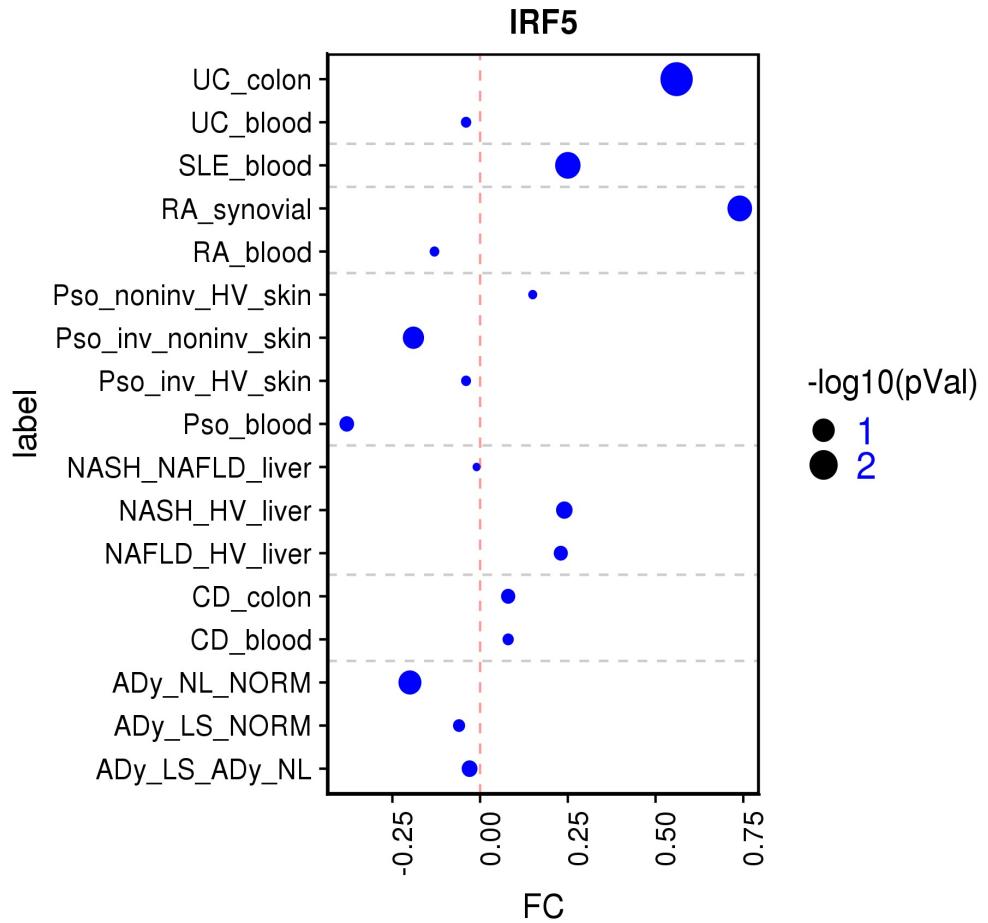
- Pros
 - Existing data regarding IRAK4
 - Earlier availability than DBA/1
 - Existing published data suggesting impact of *IRF5^{-/-}*
- Cons
 - May not fully represent contributions of TLRs/PRRs relevant to IRF5
- Readouts for study
 - Serum, histology,
- Defined positive outcome
 - Matching published data?
 - Decreases in serum cytokine



High expression of IRF5 in RA synovium, resident macrophage populations

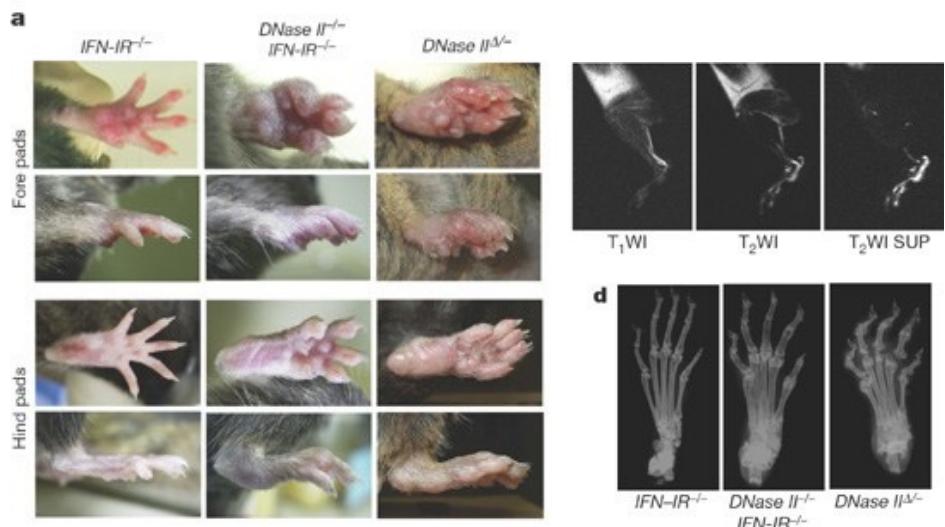


IRF5 And STING Expression Are Increased In RA



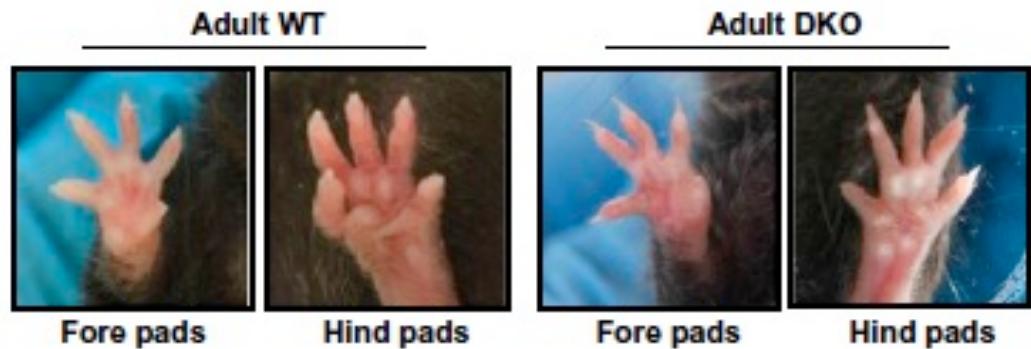
STING Contributes To Arthritis Seen in DNase II KO Mice

DNase II KO mice present with polyarthritis which is not rescued by *IFNAR*^{-/-}



PMID: 17066036

TMEM173 KO rescues polyarthritis seen in DNase II KO mice

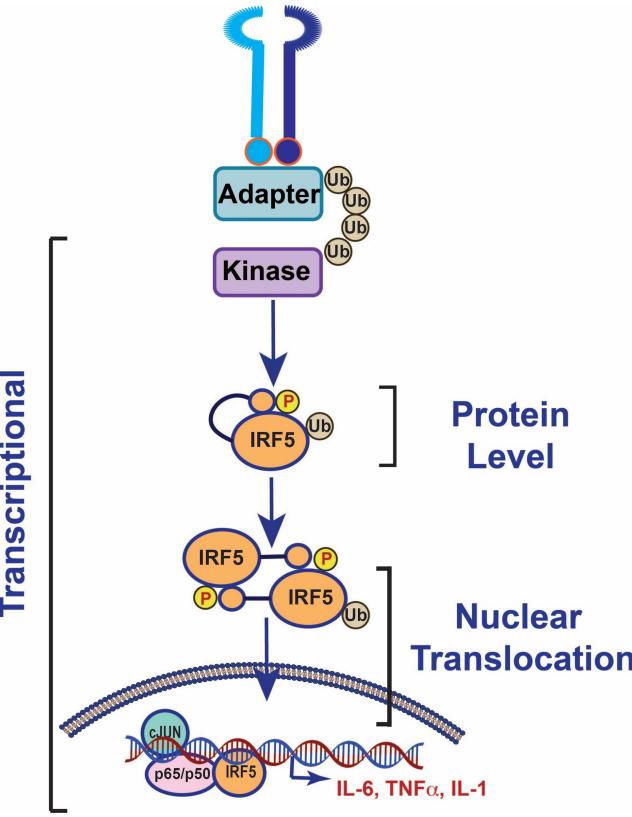


PMID: 23132945

Additional Screening Capabilities



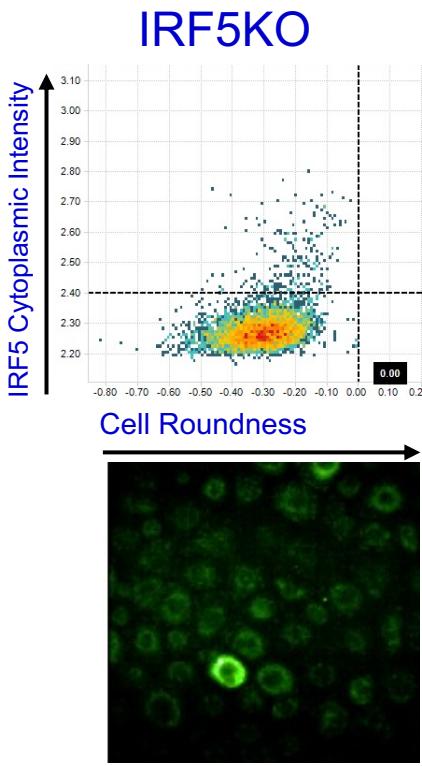
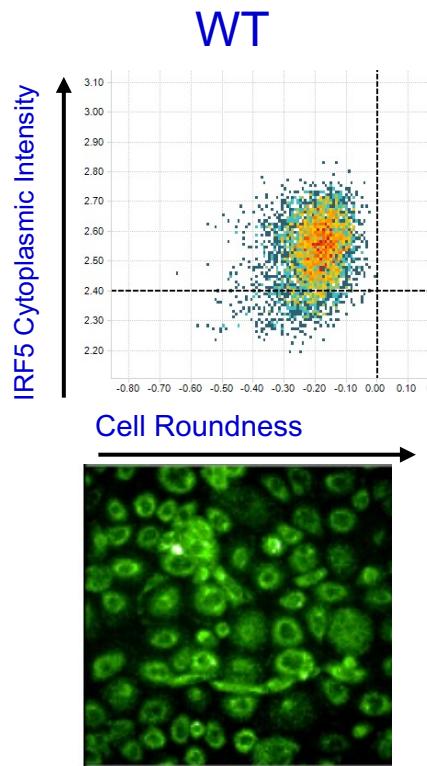
IRF5 - cellular assay options for screening



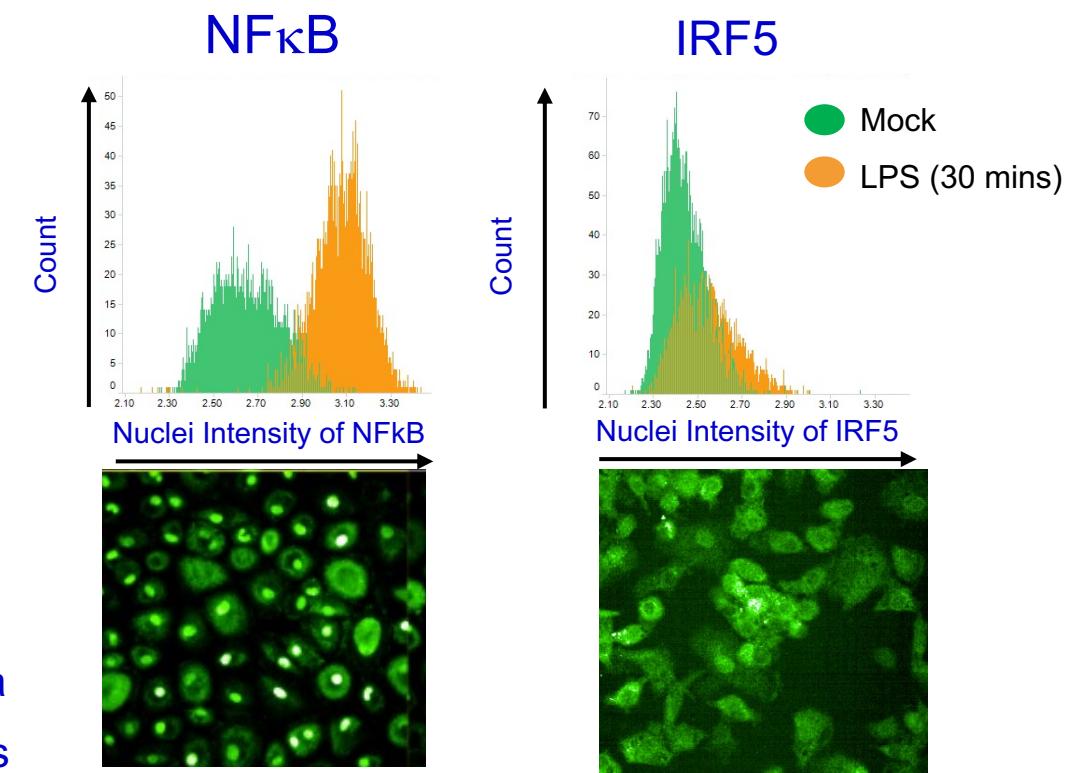
	Protein Levels (HTRF)	qPCR	Covalent Screen
Cell system	Primary cells (macrophages)	Primary cells (macrophages)	Primary cells (macrophages)
Stimulus	No stimulation	LPS	No stim / LPS
Readout	IRF5 protein levels	IRF5-mediated gene transcription	IRF5 covalent ELISA
MoA coverage	Restricted to levels	Comprehensive	IRF5 Binding mediated
Throughput	High	Medium / High	10k compound set
Key concern(s)	MoA coverage: proportion of valid hits impacting IRF5 levels	Gene Selection	ELISA assay development

Imaging of IRF5 nuclear translocation is not of sufficient resolution to allow for HTS

Antibody recognizes IRF5



IRF5 nuclear translocation is not sufficiently detected



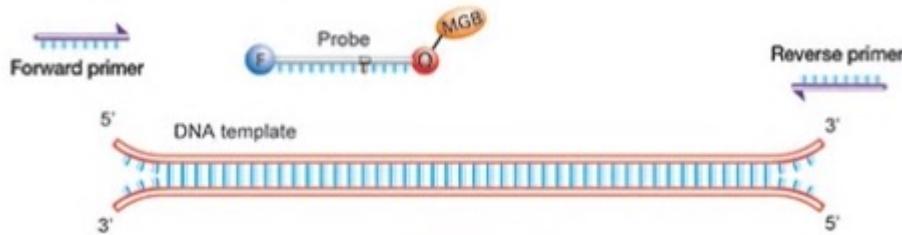
Leena Kuruvilla
Regis Doyonnas

Multiple conditions were explored, however, robust nuclear translocation was not observed

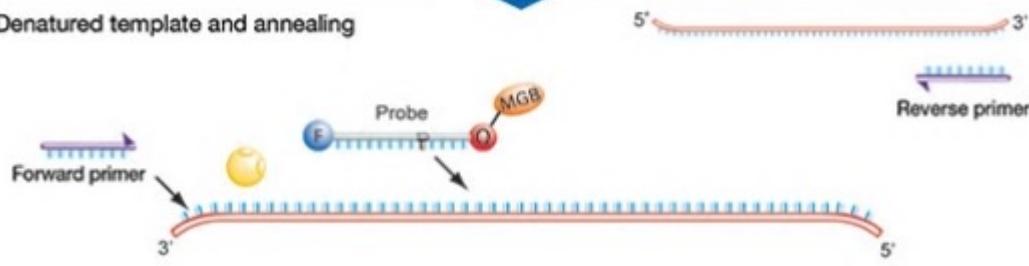
Nuclear translocation is not a feasible path forward for HTS

RT-qPCR TaqMan Overview

1. Assay components and DNA template



2. Denatured template and annealing



3. Polymerization and signal generation



Legend

- F Applied Biosystems™ FAM™ or VIC™ dye
- O Nonfluorescent quencher (NFQ)
- MGB Minor groove binder
- AmpliTaq Gold™ DNA Polymerase
- Probe
- Primer
- Template
- Newly synthesized DNA

Assay Strengths:

- Multiplexing with multiple targets
- Highly sensitive
- 384w format

Potential Limitations:

- High cost/well
- Throughput limitations:
 - RNA stability at RT
 - Long Read Time

qPCR Throughput and Cost Analysis (Rough Estimates)

Throughput:

Plates/Day	Confidence Level
5	Very High
10	High
15	Medium
>20	Lower

Cost Breakdown (Includes Some Bulk Discounts):

Product	Price/plate	Price/well
Labcyte TC Echo Plate	6.53	0.017
Armadillo PCR Plate	7.68	0.02
Lysis Buffer (25%) Roche	34.94	0.091
Roche Mastermix (5ul rxn)	111.36	0.29
Sigma RNase Protect	107.52	0.28
Life Technologies Primer/probe 60X (2 genes)	80.64	0.21
Total Cost:	348.67	0.91

- Note, analysis does not include cost of cells
- Cost *may* be further reduced through assay optimization and bulk purchases

CONFIDENCE ↑

50k subset screen (~140 plates); plus validation and IC50 (~280 plates)

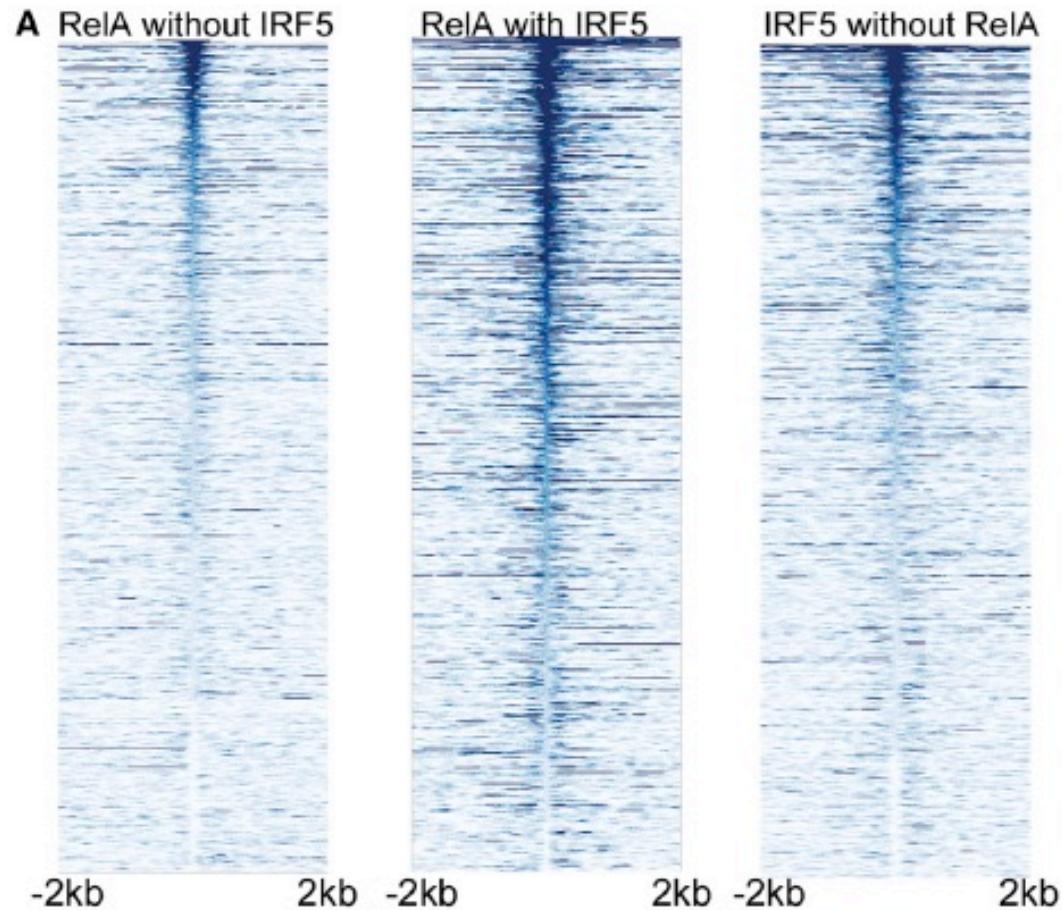
PBDS Compressed 450K compound Screen (~189 plates); plus validation and IC50 (~378 plates)

GDRSIII 150k compound screen (~420 plates); plus validation and IC50 (~840 plates)

PBDS Singleton 450K compound Screen (~1200 plates); plus validation and IC50 (~2400 plates)

Alternative strategy for gene selection

Public dataset utilized BMDMs to identify IRF5 and NF-κB cooperative and independent gene regulation

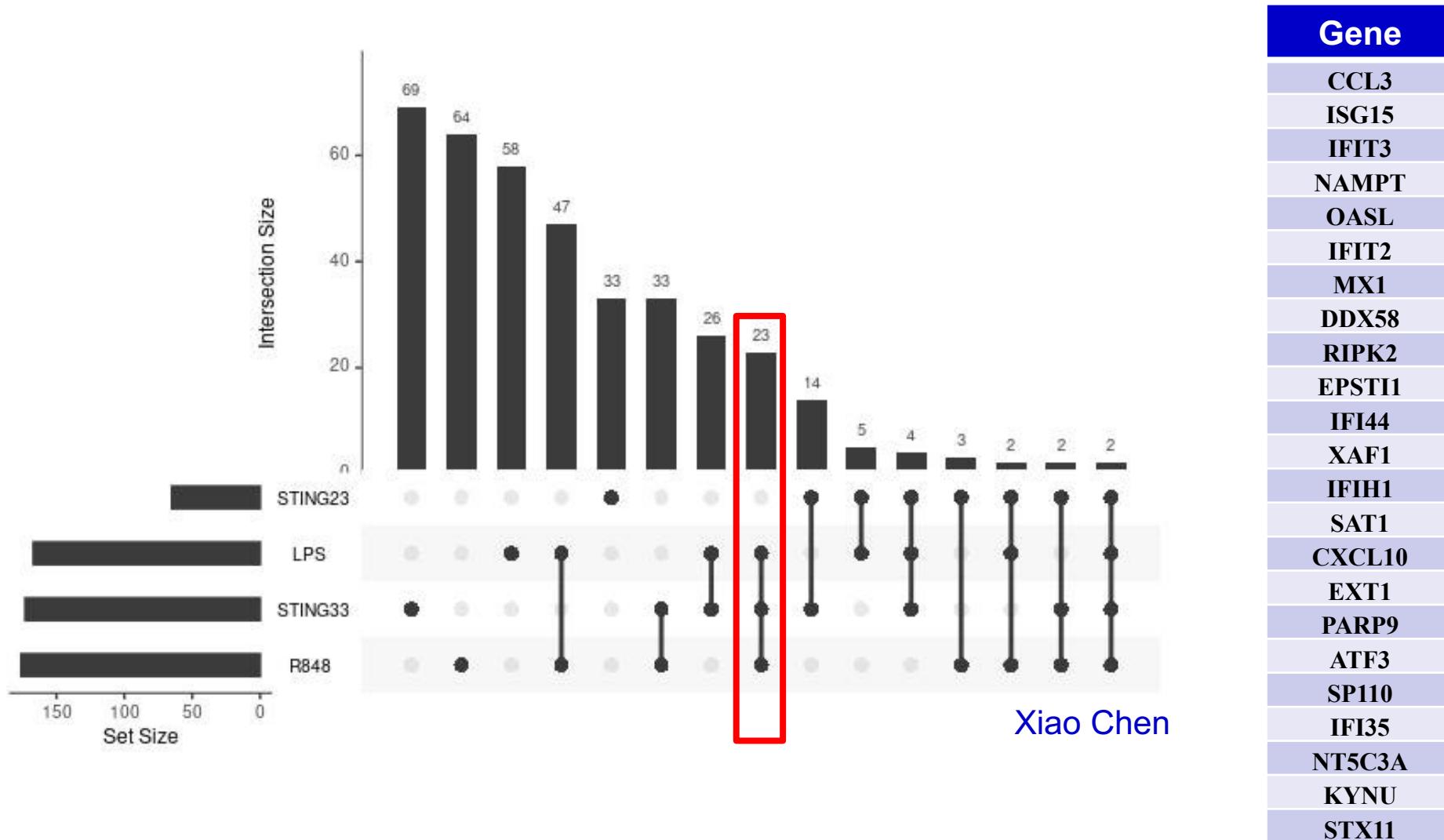


Reported uniquely IRF5 dependent genes

Category 3		
	down	up
<i>Nfe2l2</i>		<i>Mgll</i>
<i>Acsl1</i>		<i>Etv4</i>
<i>Ets2</i>		<i>Atp2b1</i>
<i>Rasgrp1</i>		<i>Zfp36l1</i>
<i>Gpr176</i>		<i>Ltb</i>
<i>Cpd</i>		<i>Rhof</i>
<i>Ly6i</i>		<i>Eef2</i>
<i>Lass6</i>		<i>Rftn1</i>
<i>Apbb2</i>		<i>E130012A19Rik</i>
<i>Rbpms</i>		<i>F2r</i>
<i>Htra4</i>		<i>Tmem65</i>
<i>BC028528</i>		
<i>Fndc7</i>		
<i>k</i>	<i>Tshz1</i>	
		<i>Ckap2l</i>
		<i>Gmfg</i>
		<i>Nos2</i>

Will proceed through comparative qPCR between IRF5 KO and RELA KO macrophages

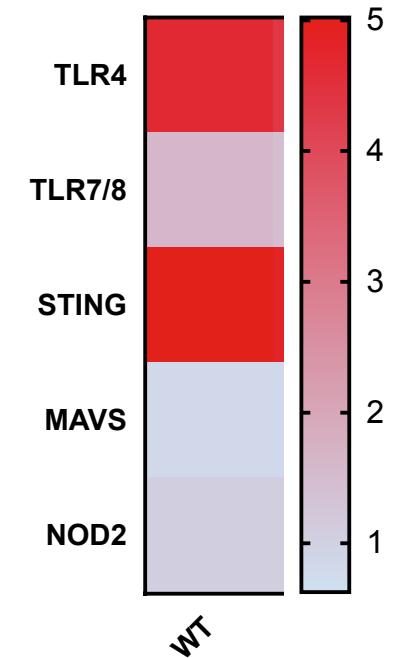
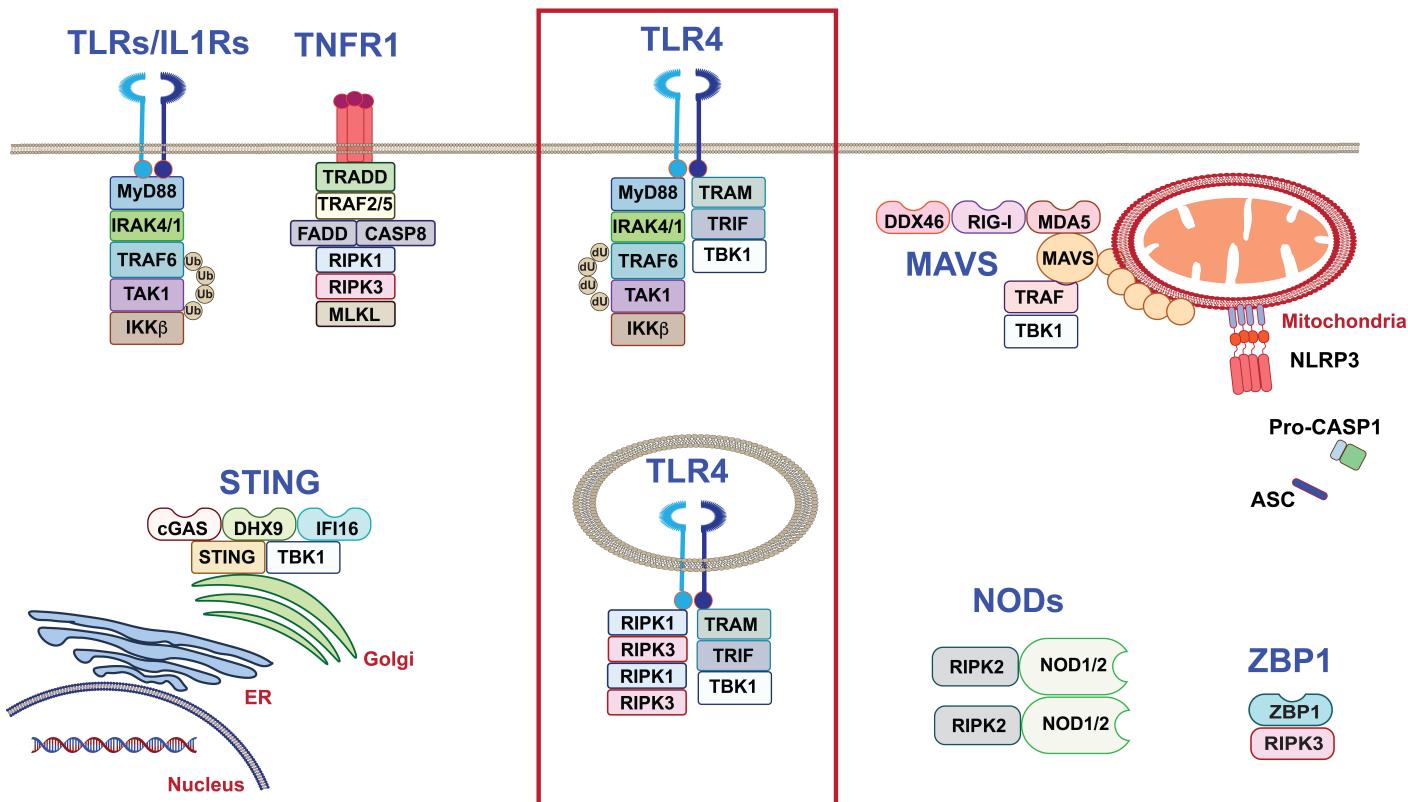
Gene Selection Data From PRR Signature Work



- Comparative readout of IRF5 and RELA KO macrophages could allow for gene identification

Why TLR4 For Screening May Be Ideal Stimuli

TLR4 Stimulation Captures Activation Of Components Involved in Multiple Pathways – Exemplified By Unique Upregulation of IP-10 in STING and TLR4 Conditions



- Kinetics of TLR4 stimulation are better understood than non-TLR ligands currently
- IRF5 knockdown/knockout has demonstrated reduction of LPS driven cytokine as compared to IRAK4i

IRF5 HTRF Cisbio Kit

- HTRF assay developed with CISBIO able to detect endogenous IRF5 levels in THP1s using an IRF5 knockout line developed by Jeff Stock and Heather Wheeler
- Kit works by having a donor and acceptor antibody that recognize different epitopes of IRF5
- These epitopes are to regions outside of the phosphorylation sites on IRF5 that regulate dimerization as well as the tier 1 cysteines (340, 342, 398).

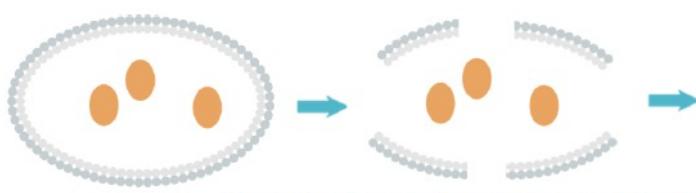
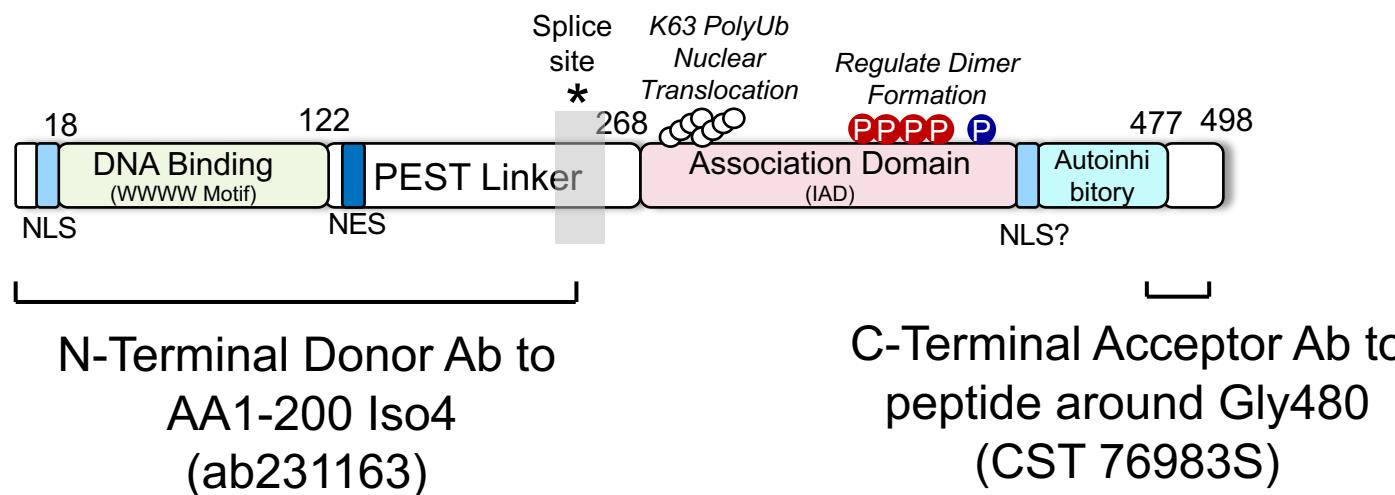
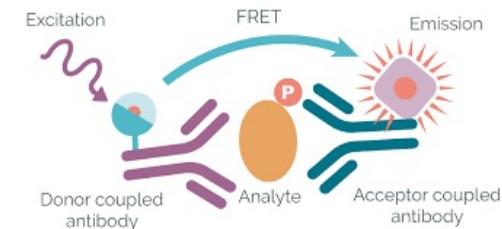


Figure 1: Schematic of HTRF IRF5 protein assay



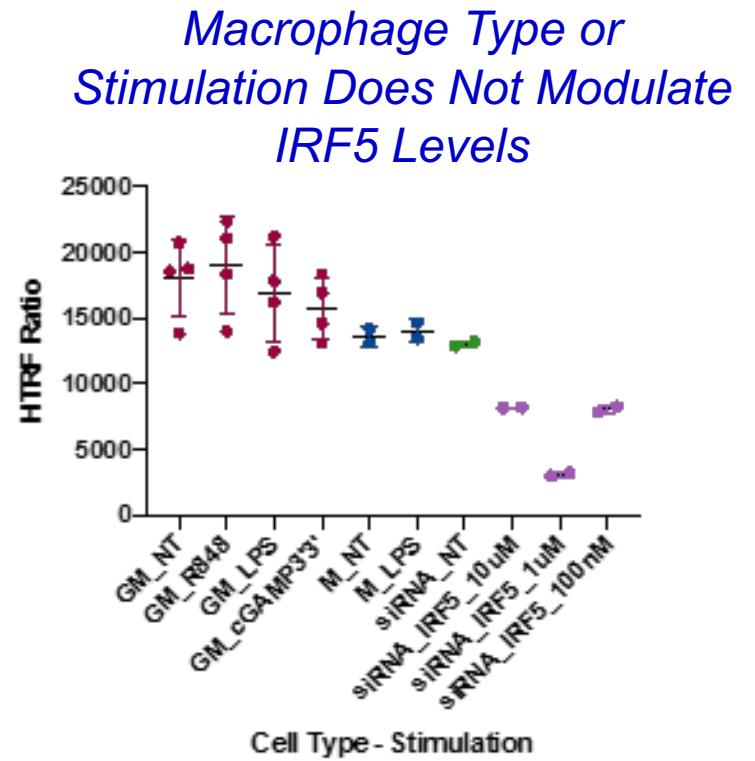
Pros and Cons of HTRF Approach

Pros:

- Direct measure of IRF5
- Loss of IRF5 well aligned with CIR mechanism
- Could capture stabilization in addition to destabilization
- HTRF assays in place for counterscreening against technology
- No stimulus needed

Cons:

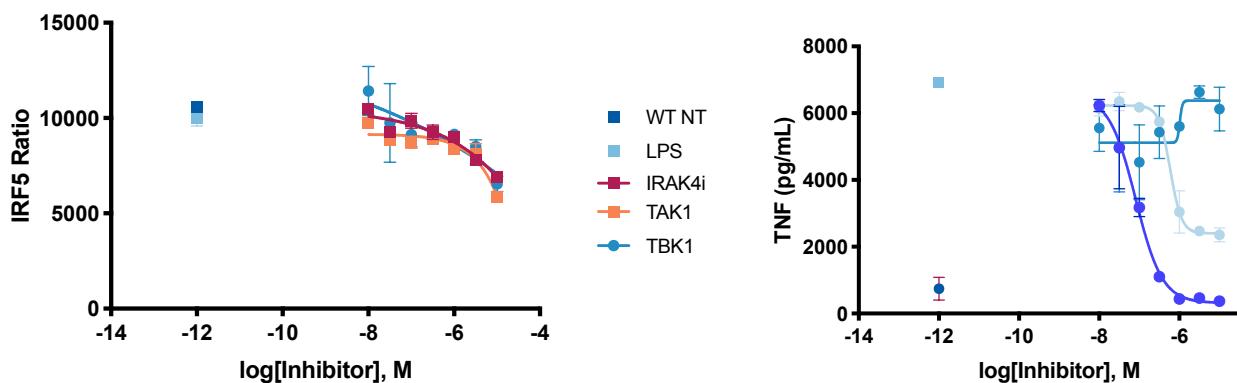
- Does not capture all MoA's (i.e. modulators of the IRF5 pathway)
- No control compound for modulating IRF5 levels in primary macrophages – monitor fold over background
- Assay likely more sensitive to donor variability in IRF5 levels – optimization for each donor batch with cell #'s/siRNA?



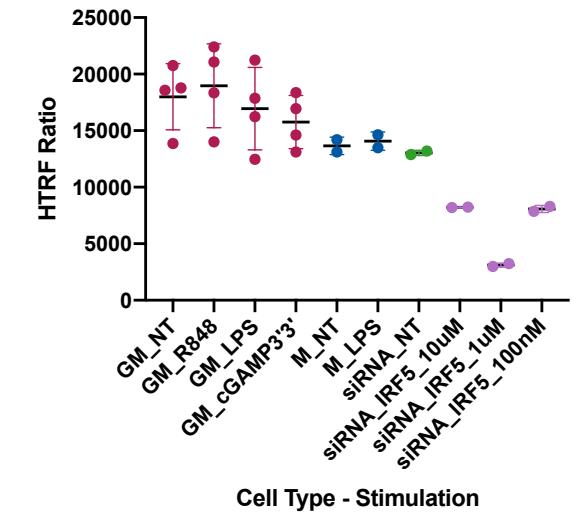
As expected, no change in IRF5 levels were detected under different stimuli or time points in primary macrophages (as well as THP1s)

Expected Limitations of HTRF

Pathway Inhibitors Do Not Impact IRF5 Levels While Impacting Outcome



Macrophage Type or Stimulation Does Not Impact IRF5 Levels



Both Of These Are Expected Outcomes That Does Not Impact CiR Established Via Reduction In IRF5 Levels

HTRF PBDS Screen Cost

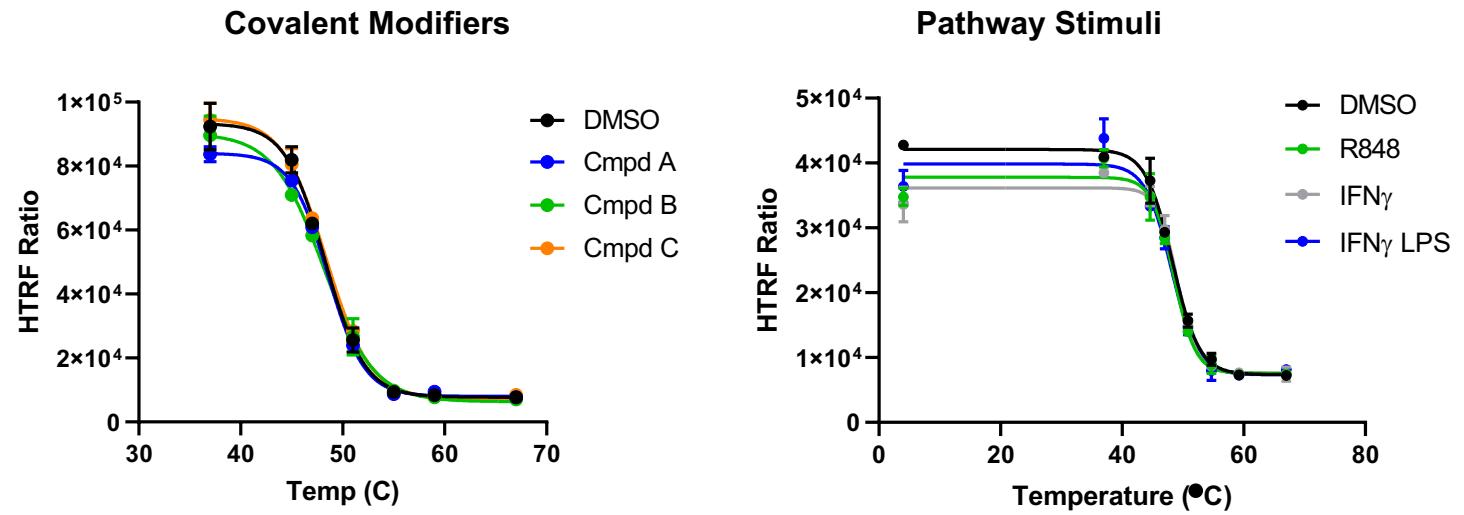
- Costs based on HTS for CDK4 that used similar Cisbio HTRF technology
- Assumptions:
 - Kit reagent usage and costs similar
 - 1536 compatible – TBD with primary macrophages
 - Screening done at HDB
- Cost: \$260-300k for screen + \$50-70k for the primary cells
 - \$100k – Cisbio kit reagent
 - Potentially lower depending on bulk discount for IRF5 kits
 - \$140k – Primary screen of PBDS Tranche 2 Singleton (~475k compounds)
 - \$16-65k – Hit confirmation and dose response for 333 primary, 75 conf, and 110 DRC plates
 - Either 384-well (\$65k) or 1536 (\$16k)

CETSA: Uncertainty Around of MoA Coverage



CETSA	
Stimulus	No stim / LPS
Readout	Stability Changes
MoA coverage	Unknown but potentially large
Throughput	High
Status	Not selected for screening: Lack of shifts in response to stimuli/modulations

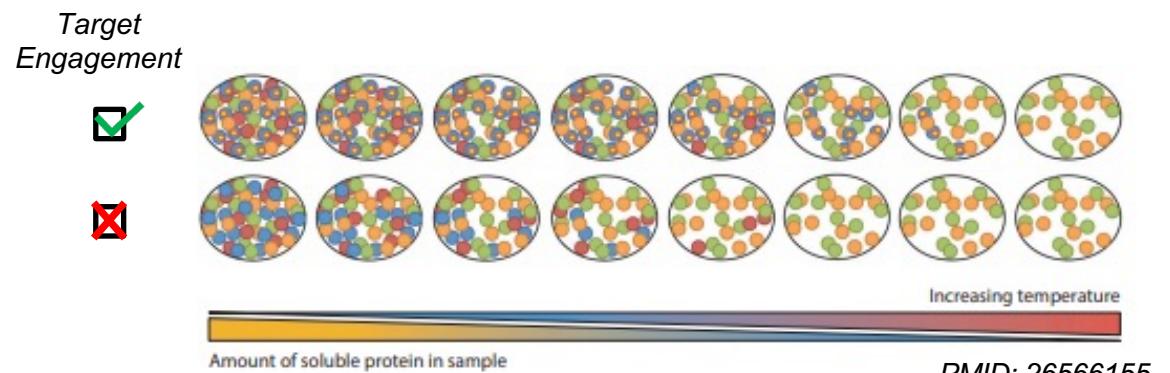
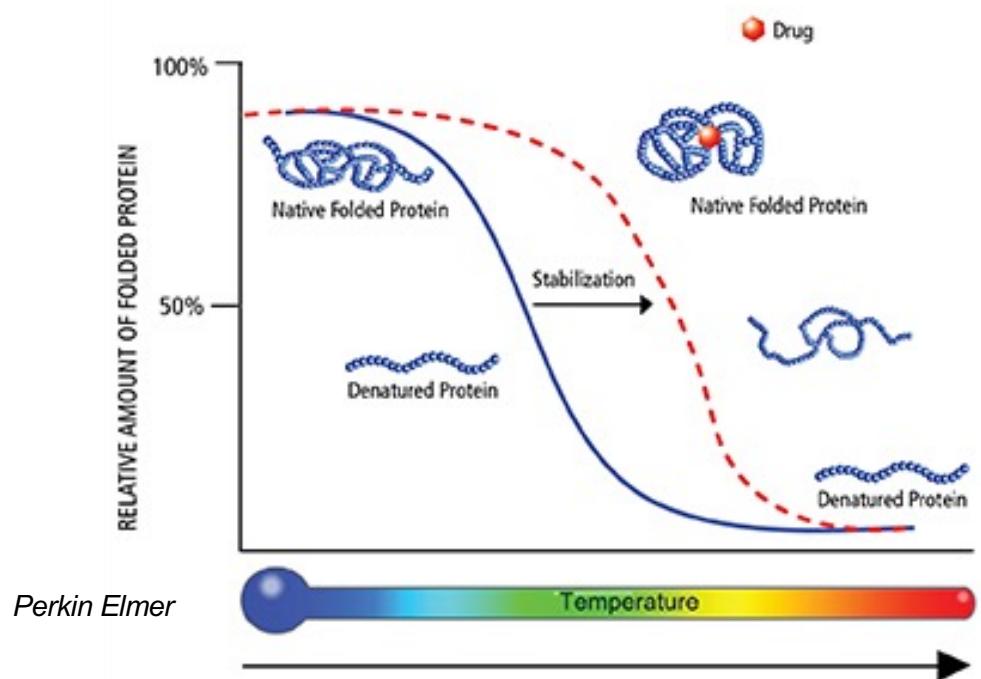
No Modulation Detected with IRF5 Pathway Modulators



Ruth Sommese (CGPS)

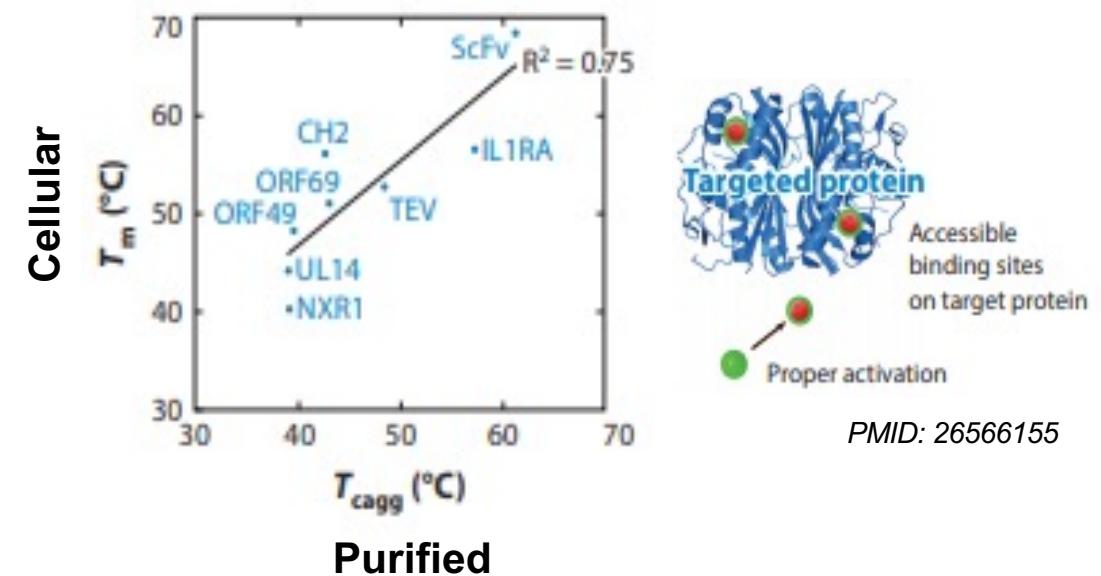
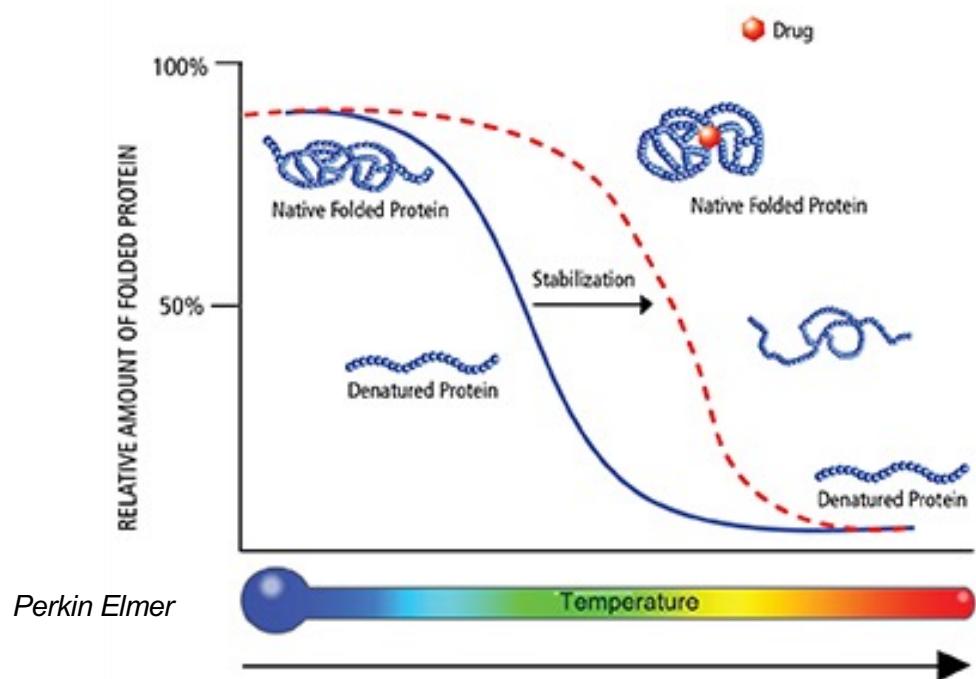
- Endogenous IRF5 demonstrated good thermal profile in lysate and intact cells
- Variables explored:
 - Different PRR stimuli measured at variety of timepoints 0.5-2 hr
 - Treatment with covalent compounds confirmed to promiscuously modify IRF5

Cellular Thermal Shift Assay (CETSA)



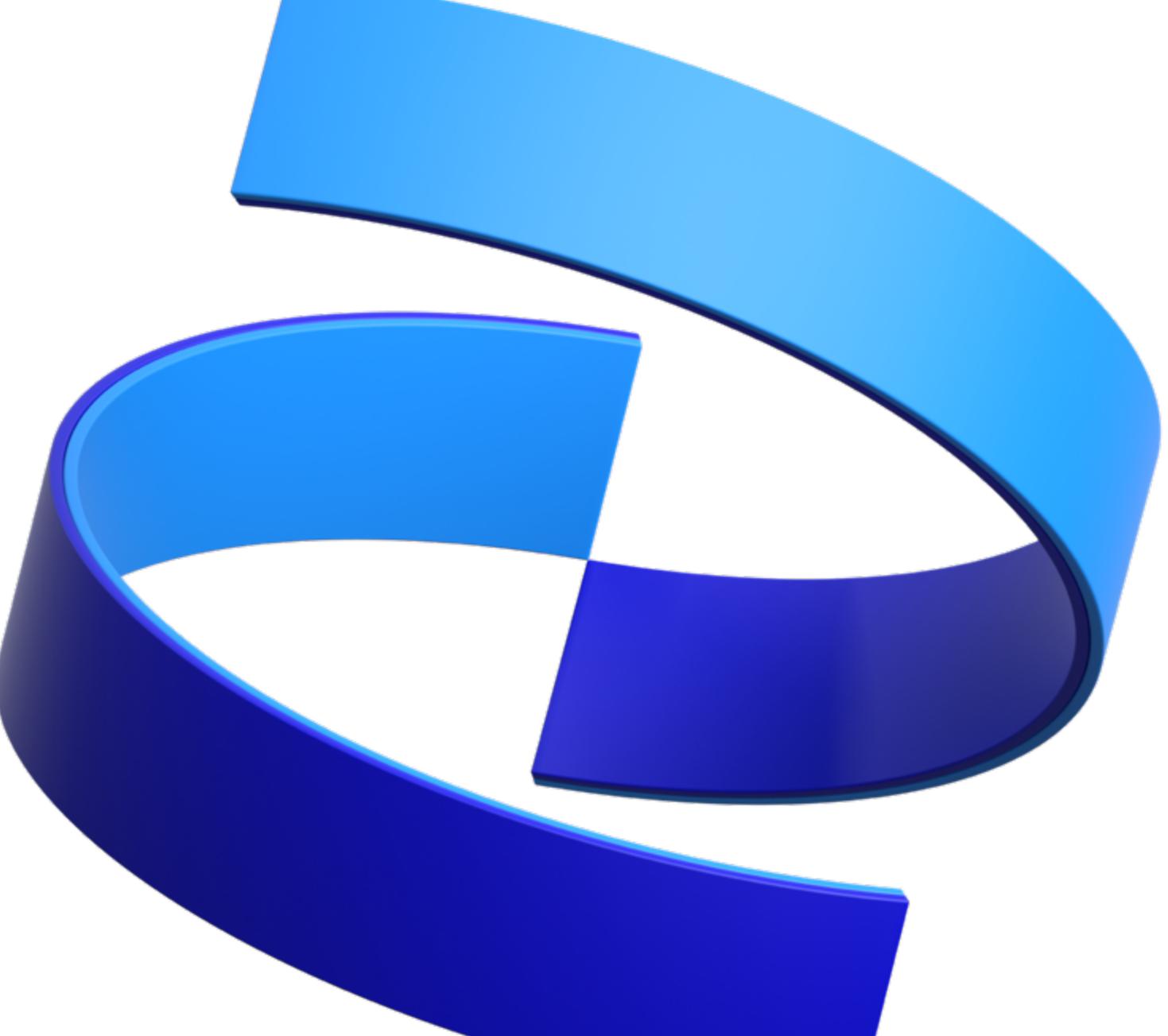
- CETSA is a biophysical measurement of protein stability in a cellular context
- Applies to any complex mixture (lysates, cells, and tissue samples) as opposed to Tagg/Tm measurements often made with purified proteins.

Cellular Thermal Shift Assay (CETSA)



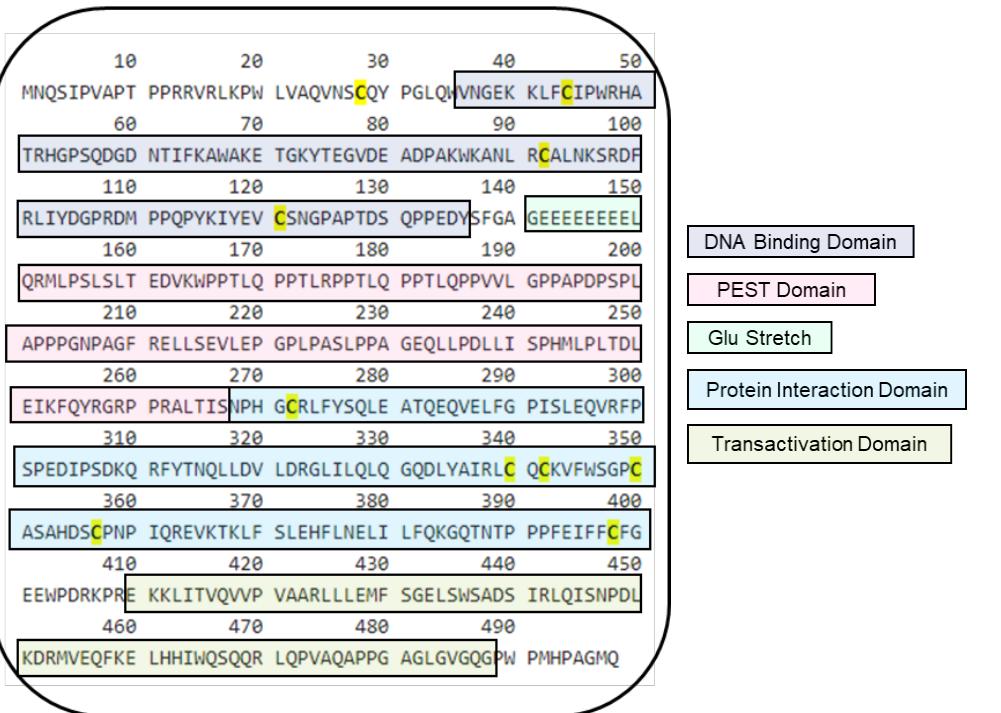
- When heated, many proteins within cells will unfold/precipitate in a similar manner to purified protein
- A reasonable correlation between CETSA and purified protein T_m s have been observed.
- As with purified proteins, not all proteins will show a clear transition or large shifts upon ligand binding. Larger and more complex proteins also tend to show smaller shifts upon ligand binding.

Covalent Binder Screen



Rationale in Covalency for IRF5

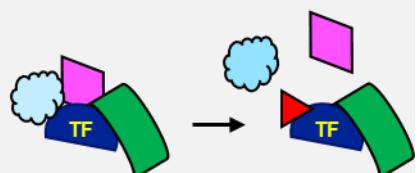
10 Cysteines within IRF5



IRF5 cysteines

DNA binding Domain

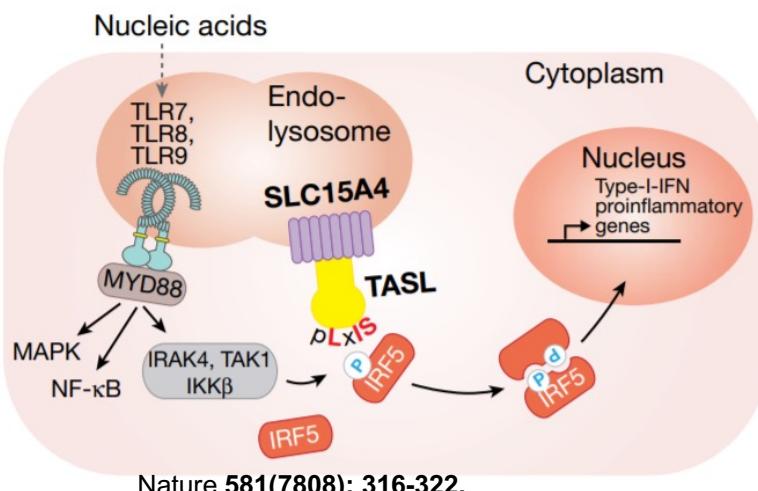
Protein Interaction Domain



Inflammation & Immunology

5 Cysteines conserved across species

Cysteine	Human	Chimpanzee (99% Identity)	Mouse (85% Identity)	Zebrafish (50% Identity)	Chicken (51% Identity)	Tortoise (58% Identity)
Cys28				Gly	Gly	Asn
Cys44			Tyr	Arg	Val	
Cys92						
Cys121						Tyr
Cys272						
Cys340						
Cys342						
Cys350				Gly		
Cys357				Pro	Arg	Gly
Cys398						



The pLxIS sequence is a known motif to modulate binding of adaptor proteins STING, MAVS, etc to IRF3 as well as dimerization.

— IRF5 Cellular Covalent Screen

What will we screen? Revamped Covalent Libraries

Covalent Subset	Available Now	Expansion set (by July 2021)
Legacy CA/Acrylamides	429	429 (+0)
Internal Butynamides/Vinylsulfones/sulfonamides	109	109 (+0)
Fully Functionalized subset (electrophile + alkyne)	124	600 (+476)
Enantiomeric Pair Diversity Subset	356	356 (+0)
PMC work (Wuxi)- complex acryl/butynamides	0	2500 (+2500)
External Buy-up (Enamine SOD or purchase) - acryl/other	0	3350/1000 (+4350)
TOTAL	1018	8344

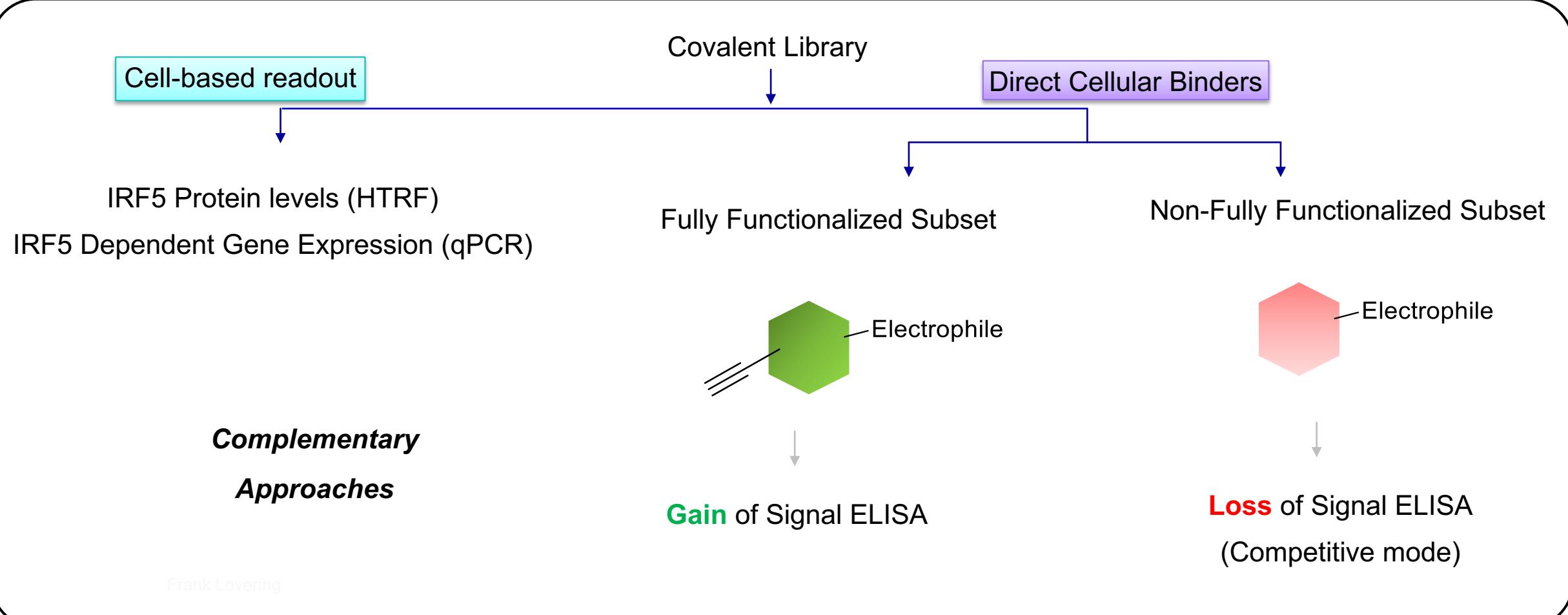
Libraries in progress, ETA from vendors/synthesis by June

Frank Lovering

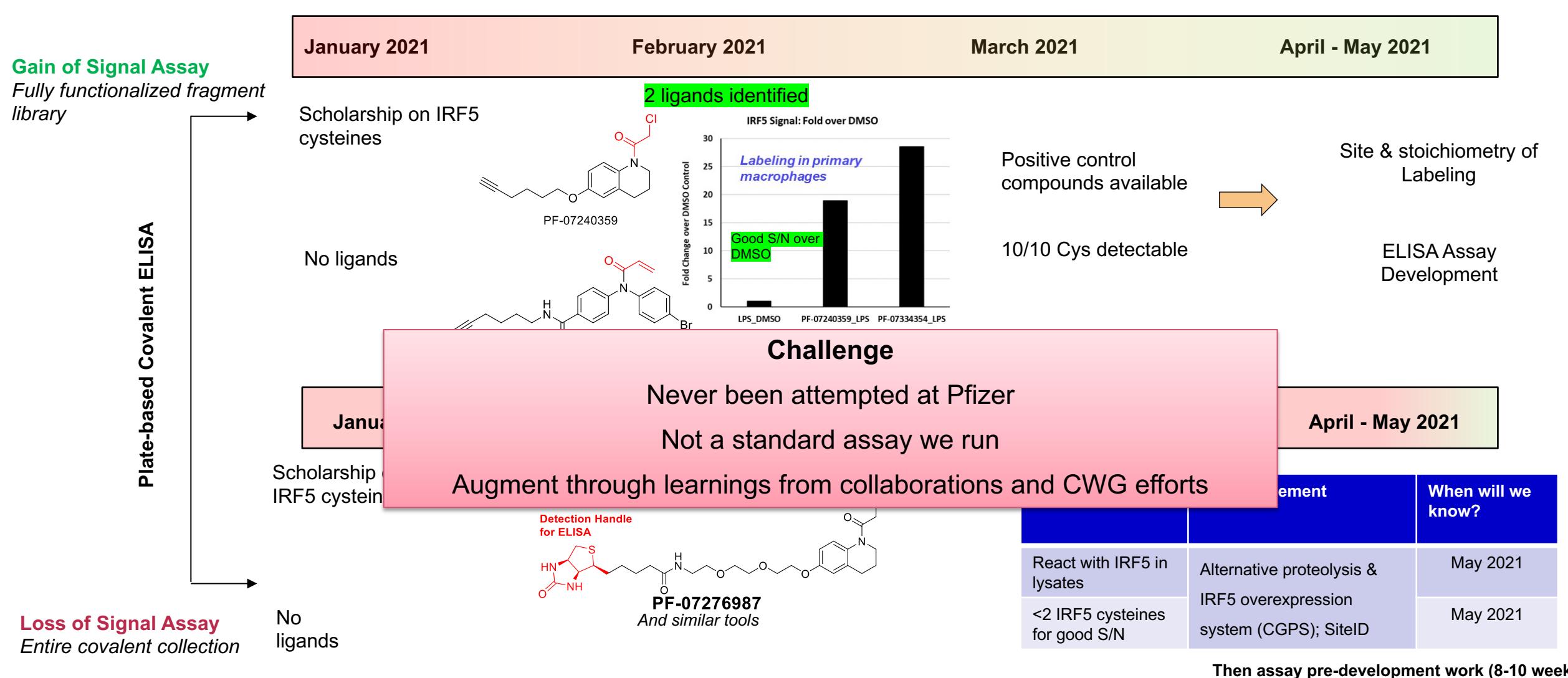
Covalent Working Group in communication with Compound Management for “assay ready plates”

— IRF5 Cellular Covalent Screen

How will we screen?
Direct Binding (CovELISA) + HTRF & qPCR Assays

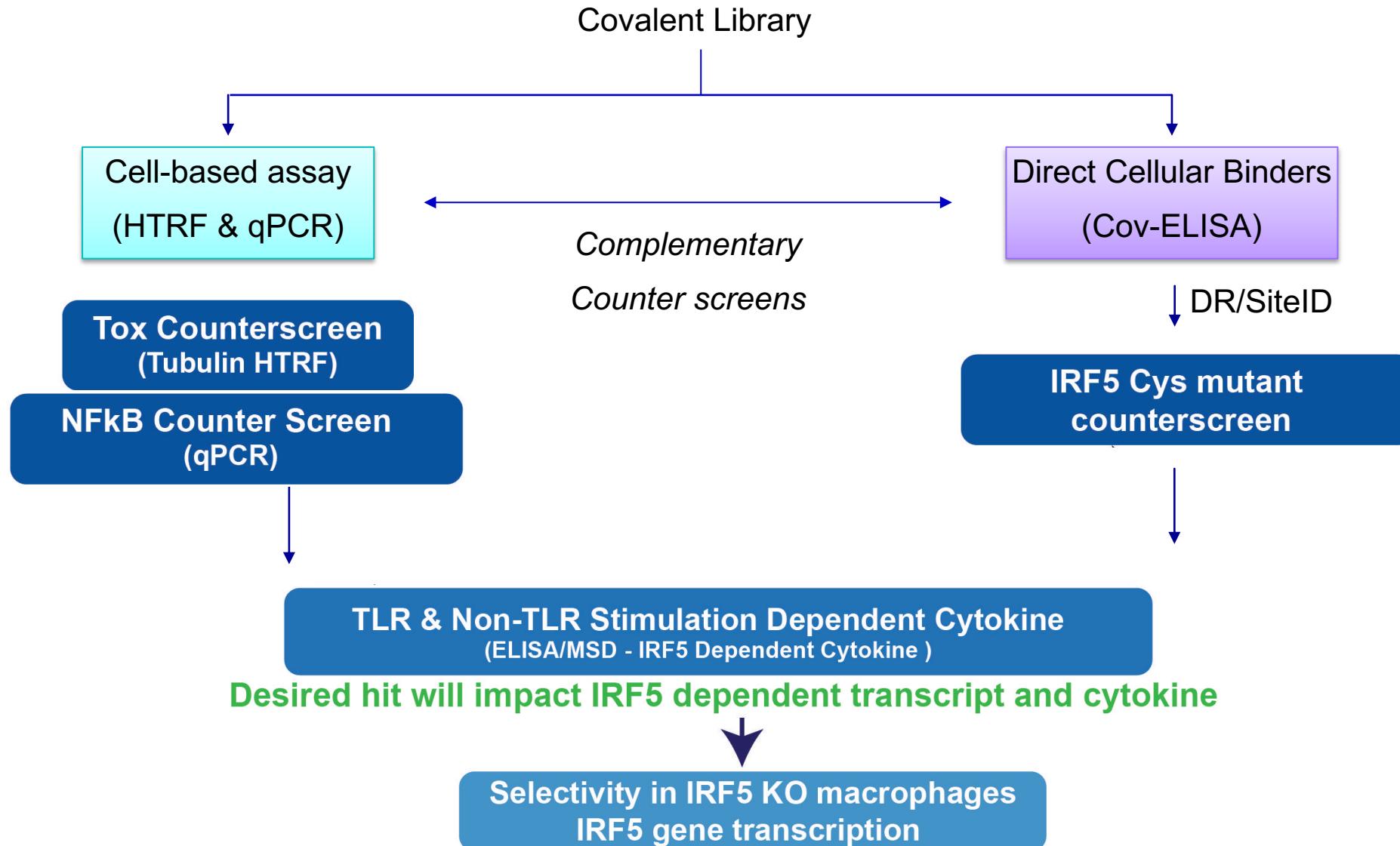


Progress towards Covalent ELISA platform



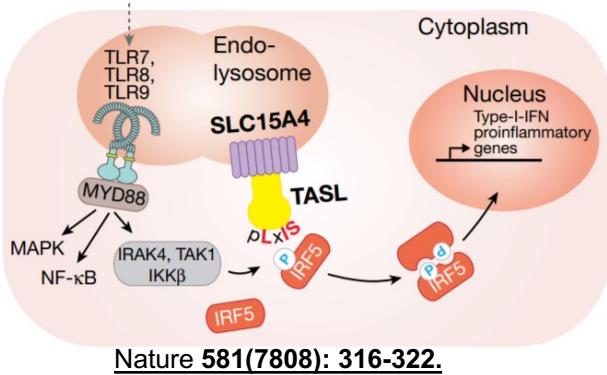
— Deliverables and Covalent Screening Funnel

Expectation: Identification of cellular IRF5 binders & functional hits in parallel

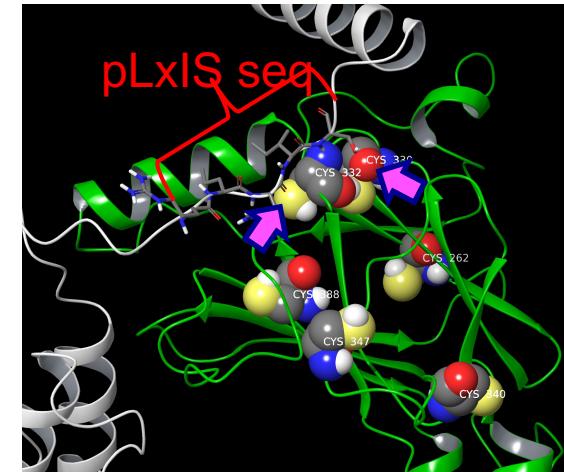
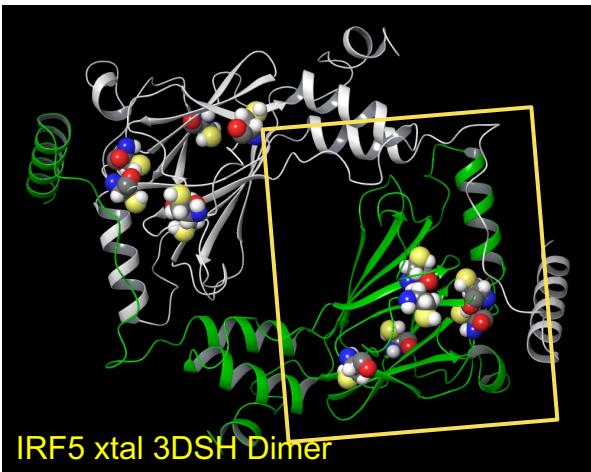


Building Confidence in Covalency for IRF5 - II

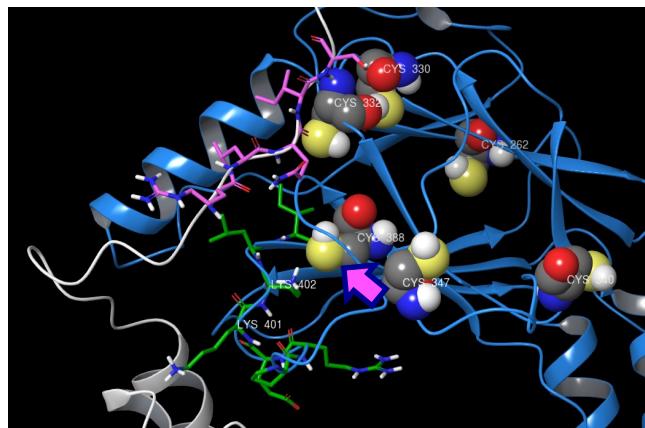
Nucleic acids



The pLxIS sequence is a known motif to modulate binding of adaptor proteins STING, MAVS, etc to IRF3 as well as dimerization.



Cys 340 and Cys342 offer an opportunity to disrupt binding to adaptor proteins



Alkylation of Cys398 may disrupt nuclear transport, as well as ubiquitination.

PREKKLI is a putative nuclear transport sequence.

Lys401 (Lys411) and 402 (Lys412), part of the PREKKLI seq are putative sites for ubiquitination.

Cys388 (Cys398 in Uniprot sequence) is very near this space.

Frank Lovering



Note: In this Xtal, Cys340 and 342 are numbered 330 and 332 respectively, (n-10)

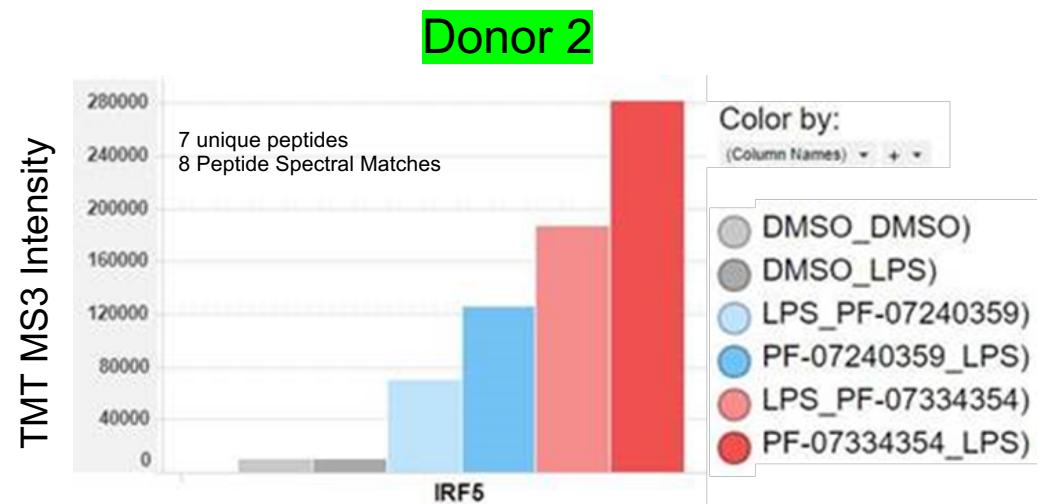
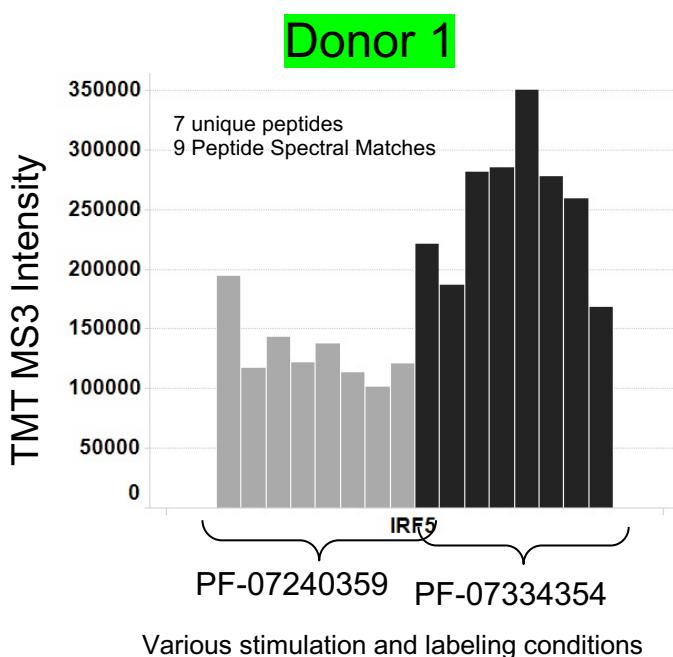
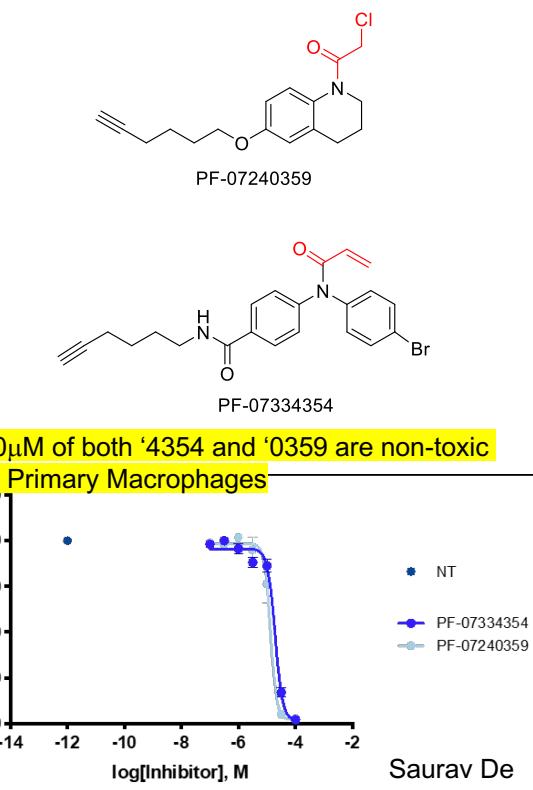
Inflammation & Immunology

Pfizer Confidential

Confidential

45

PF-07240359 and PF-07334354 label IRF5 *in live cells @ 10μM*



Key Unknowns

What Cysteine is being labeled?

More than 1 cysteine labeled?

Stoichiometry of labeling?

Will biotinylated versions of '0359 and '4354 label IRF5?

IRF5 Covalency: The Known-Unknown Matrix

	Knowns	Unknowns
Known	IRF5 can be liganded by covalent tool compounds in live cells	Are any IRF5 cysteines relevant to function?
Unknown	What Cysteine is targeted? Multiple cysteines being targeted?	Covalent IRF5 binders will be functional

Each quadrant can be experimentally tested in a hypothesis-driven manner

Path Ahead for Direct Binder Cellular Assay

Cysteine Mutation study

Tier 1: Cys340; Cys342; Cys398
Tier 2: Cys92 & Cys272

Mutants in THP1 (C→A & C→Bulky)

Response to stimuli
CETSA

Changes to stability &/or
stimuli suggest functional
relevance

Cysteines chosen based on conservation through
evolution and structural location near
binding/modification sites

Will enable counter screens for both efforts
on right

Development of Gain of
signal plate-based
covalent ELISA

Primary Macrophages

Use PF-07240359 and PF-
07334354 as positive controls

Translate MS assay to ELISA
format

Is IRF5 enriched?

Proof of principle & tool compounds available
Can start assay development now
Assay good for subset of covalent library

Do biotinylated versions of '0359
and '4354 label IRF5?
"IRF5 Scout Probe"

If yes, What Cysteine(s)

<2 Cysteines?

Develop Competitive (Loss of
signal Covalent ELISA)

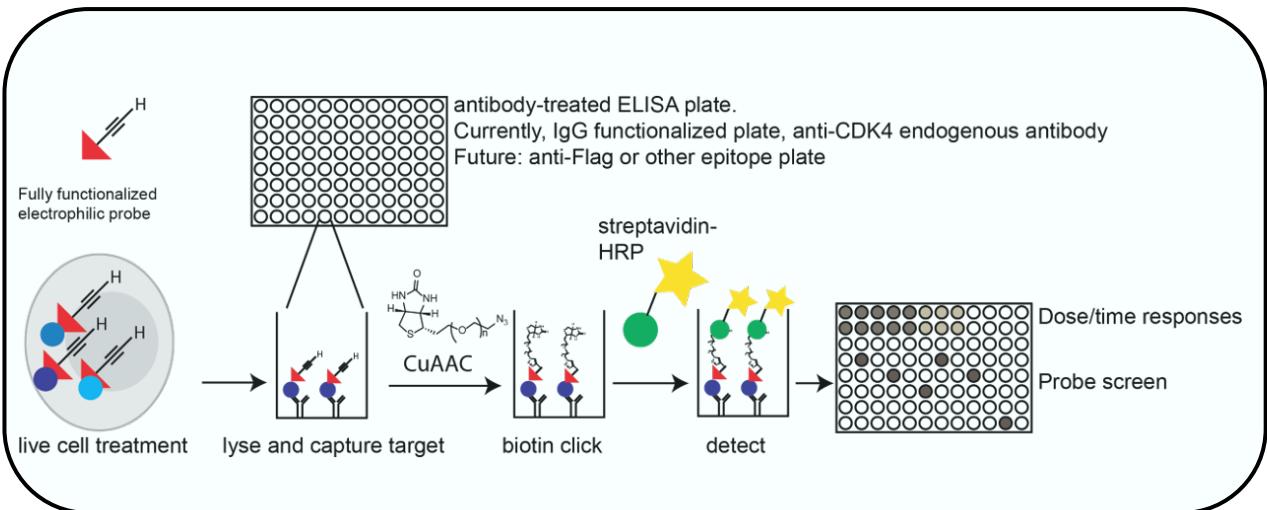
Screen 10K compounds

Assay good for entire covalent library

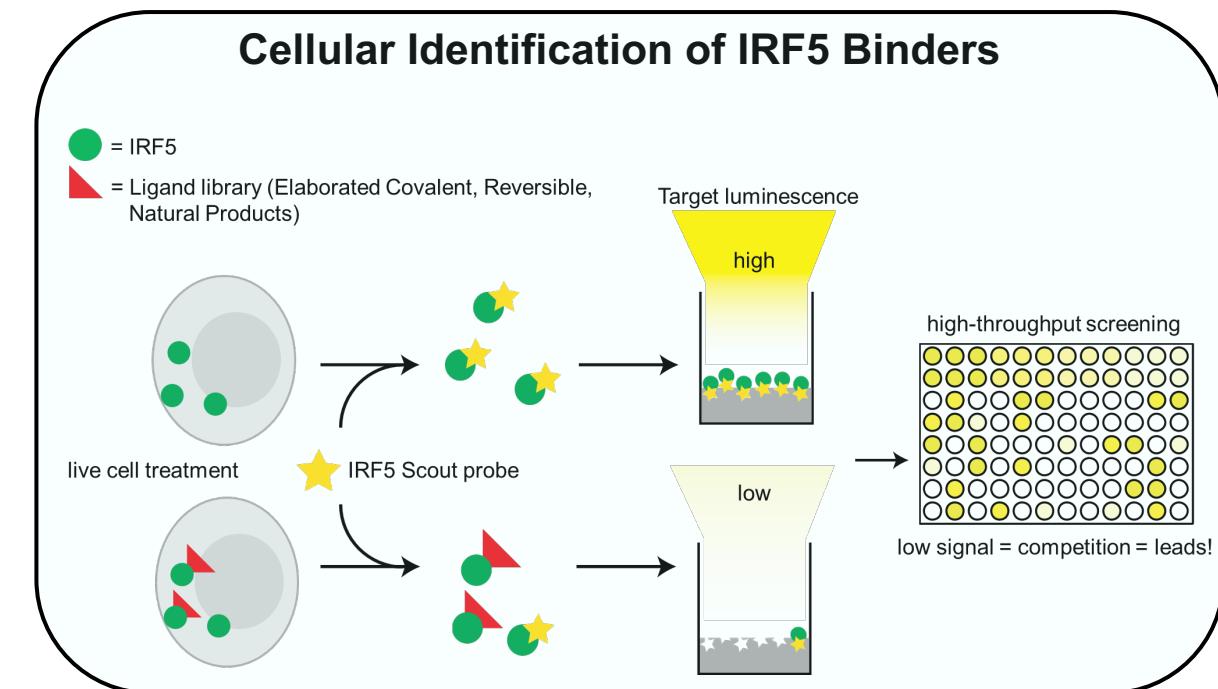
Tool compounds need to be defined
Pre-work (3-4mo) needed before assay dvlpm

Plate-based Covalent Binding Assay

Gain of Signal Covalent ELISA



Competitive/Loss of Signal covalent ELISA



Cellular binders most relevant

Relevant Stimuli

Tool/Positive Controls available

Challenge: Will Covalent binding disrupt IRF5-Ab binding?
Challenge: S/N on ELISA?

Cellular binders most relevant

Relevant Stimuli

IRF5 Scout Ligand

Scout ligand: A promiscuous covalent ligand that binds IRF5 with high enough occupancy at the Cys of interest, but is relatively promiscuous to other proteins

Cysteine Mutation Experiment

Hypothesis: Mutation of functionally relevant cysteine would alter IRF5 response to stimuli

Cysteine	Human	Chimpanzee (99% Identity)	Mouse (85% Identity)	Zebrafish (50% Identity)	Chicken (51% Identity)	Tortoise (58% Identity)
Cys28				Gly	Gly	Asn
Cys44			Tyr	Arg	Val	
Cys92						
Cys121						Tyr
Cys272						
Cys340						
Cys342						
Cys350				Gly		
Cys357				Pro	Arg	Gly
Cys398						

 Higher priority

 Second round of experiments

Cysteine	Conserved?	Hypothesis	Priority
Cys 340	Yes	Near pLxIS binding. Disulfide modulation?	High
Cys 342	Yes	Near pLxIS binding. Disulfide modulation?	High
Cys 398	Yes	Near site of Ub/Modulate Nuclear translocation?	High
Cys 272	Yes	Unknown function; but “reactive” & detectable by MS	Medium
Cys 92	Yes	Unknown function	Low

CETSA and Response to stimuli

Outcome can guide to prioritizing targeting of specific cysteine(s)

IRF5 Cysteine Detectability Experiment

Cysteine Detectability



V5 tagged IRF5 (THP1)



Pulldown



Various Digestion Conditions



Trypsin/LysC
LysC
GluC + LysC
Limited proteolysis?

What Cysteines can be detected?

IRF5 Cysteine	Peptide	Mass	Proteomics DB	Gygi TMT Database	SRM/MRM Compatibility	Hydrophobicity
Cys28	LKPWLVAQVNS C QYPGLQWVNGEK	2756.4110	Yes (7)	No		41.46
Cys44	LFC IPWR	991.5182	Yes (15)	Yes (11)		33.11
Cys92	C ALNK	605.3075	No	No		5.21
Cys121	IYEVC S NGPAPTD SQPPEDY SFGAGEEEEEEELQR	4087.7145	No	No		31.93
Cys272	ALTISNPH GCR	1225.610	Yes (21)	Yes (5)		13.94
Cys340	LC QCK	708.3167	No	No		3.36
Cys342	LC QCK	708.3167	No	No		3.36
Cys350	VFWSGP C ASAHDSCPNIQR	2286.0178	Yes (3)	No		30.53
Cys357	VFWSGPCASAHD C CPNPIQR	2286.0178	Yes (3)	No		30.53
Cys398	GQTNTPPPFEIFF C FGEEWPD R	2671.1921	No	No		47.92
			(Proteotypicity)	(PSMs)		

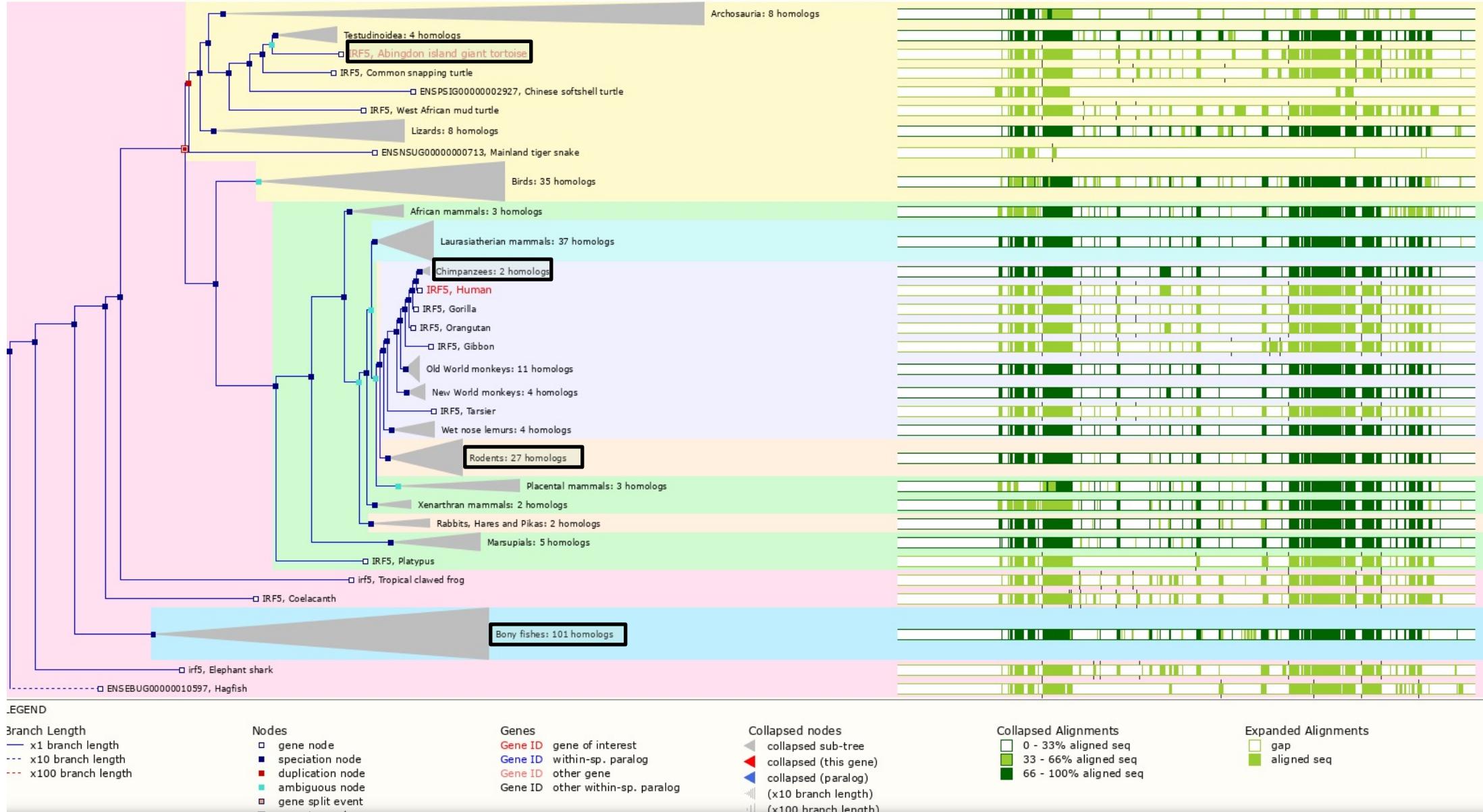
Do any of the “non-traditional” cysteines come into play?

Are IRF5 Cysteine Mutations Common in the Population?

- IRF5 generally appears to be fairly conserved in the population (gnomAD & centoMD databases)
- Only cysteine mutations seen on IRF5 are from gnomAD:
 - Cys28Arg
 - Cys28Tyr "Loss of function and missense variants in general are relatively fewer in IRF5 than average genes; we do not know about the conservation of cysteine residues specifically" – Xinli Hu
 - Cys358Tyr
 - Each mutation is found in one heterozygous carrier with no phenotypic association reported
- No IRF5 cysteine mutations found in centoMD database
- Does high degree of conservation suggest functional importance of cysteines in IRF5?
 - The hypothesis being that “functionally detrimental mutations are less frequently found in the population”

Xinli Hu and Isac Lee

IRF5 between species



Five IRF5 Cysteines are conserved across species

Cysteine	Human	Chimpanzee (99% Identity)	Mouse (85% Identity)	Zebrafish (50% Identity)	Chicken (51% Identity)	Tortoise (58% Identity)
Cys28				Gly	Gly	Asn
Cys44			Tyr	Arg	Val	
Cys92						
Cys121						Tyr
Cys272						
Cys340						
Cys342						
Cys350				Gly		
Cys357				Pro	Arg	Gly
Cys398						

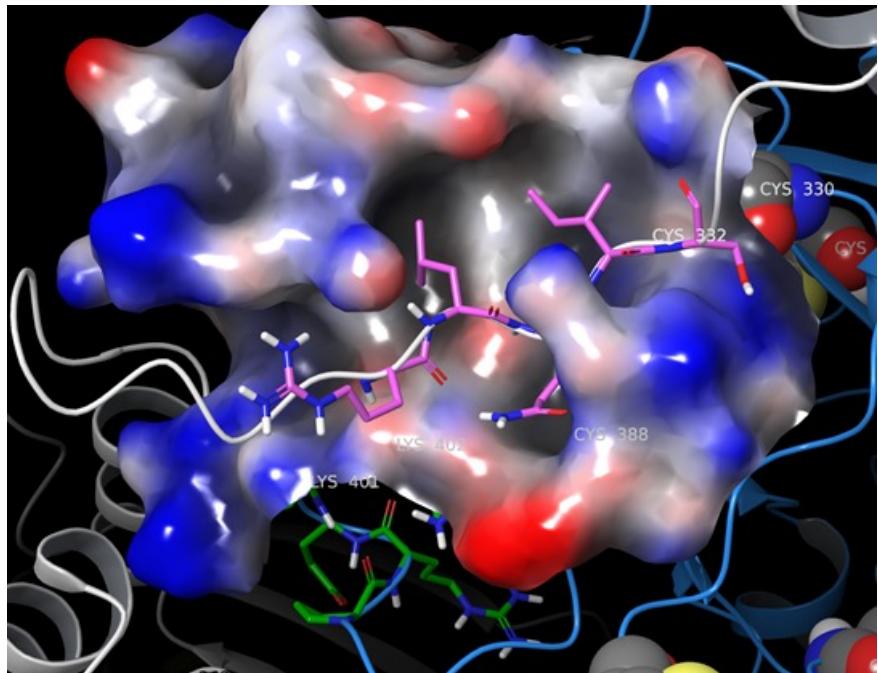
A much wider comparison needed to draw solid inferences, but it does appear that certain Cys residues are conserved

Low Conservation of Cysteines between different IRFs

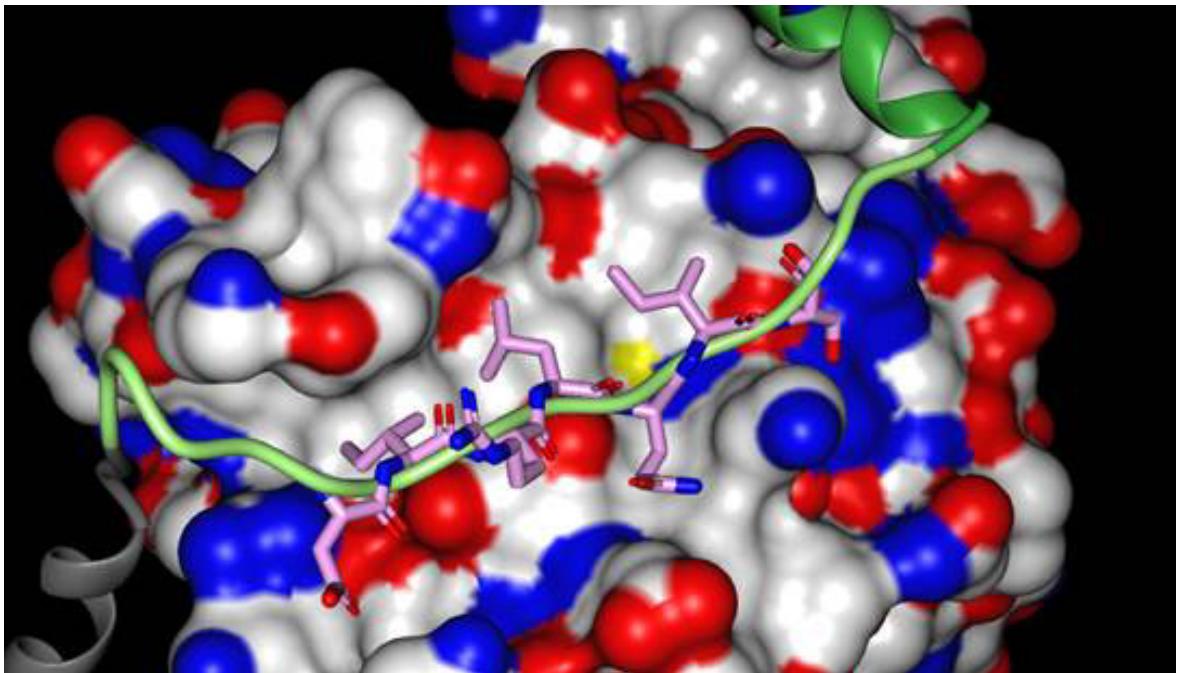
Cysteine On IRF5	IRF3 (26.5%)	IRF2 (14.8%)	IRF1 (15.3%)	IRF7 (24.9%)	IRF4 (27.2%)	IRF8 (27.8%)	IRF6 (45.7%)	IRF9 (23.4%)
Cys28	G	N	N	G	G	S	G	G
Cys44	R	Q	Q	R	R	R	Q	R
Cys92	S							
Cys121	V	L	L	S	V	V		L
<u>Cys272</u>	L	E	I					
Cys340	G	M	-	G				
Cys342		-	-		S	G		I
Cys350	E	-	-	G	L	A		Q
Cys357	P	T		P	R	R	A	G
Cys398			P	N	G			N

— IRF5: Druggability prediction

IRF5 pLxIS binding site (Frank L)



IRF5 pLxIS binding site (Xray, PDB 3DSH)



	Drugability	SiteScore	Size	Dscore	Volume	Exposure	Enclosure	Contact	Phobic	Philic	balance
Schrodingers Expected Values	Frank's Score	Avg 1, more is better, below 0.8 bad		Below 0.9 bad		Avg 0.49, lower is better	Avg 0.79, higher is better	Avg 1.0	Avg 1.0	Avg 1.0	Avg 1.6
MMP-9	5	1.02	145	0.96	248.68	0.49	0.73	1.06	0.67	1.29	0.51
Syk	5	1.05	120	1.08	381.42	0.55	0.75	0.89	1	0.94	1.07
BACE	4	1.06	113	1.08	579.67	0.59	0.76	0.91	0.39	0.97	0.4
IRAK4	5	1.04	166	1.02	516.56	0.48	0.77	1.01	0.78	1.16	0.67
PTP-1b	2	0.94	66	0.53	90.9	0.37	0.81	1.2	0.15	2.22	0.07
BRD4	4	1.07	95	1.12	170.8	0.5	0.73	1.02	1.32	0.71	1.85
3DSH - monomer - apo		0.92	74	0.94	343	0.57	0.67	0.93	0.84	0.84	1
3DSH - monomer - fake Ligand		0.88	62	0.89	278	0.54	0.69	0.98	0.89	0.82	1.1

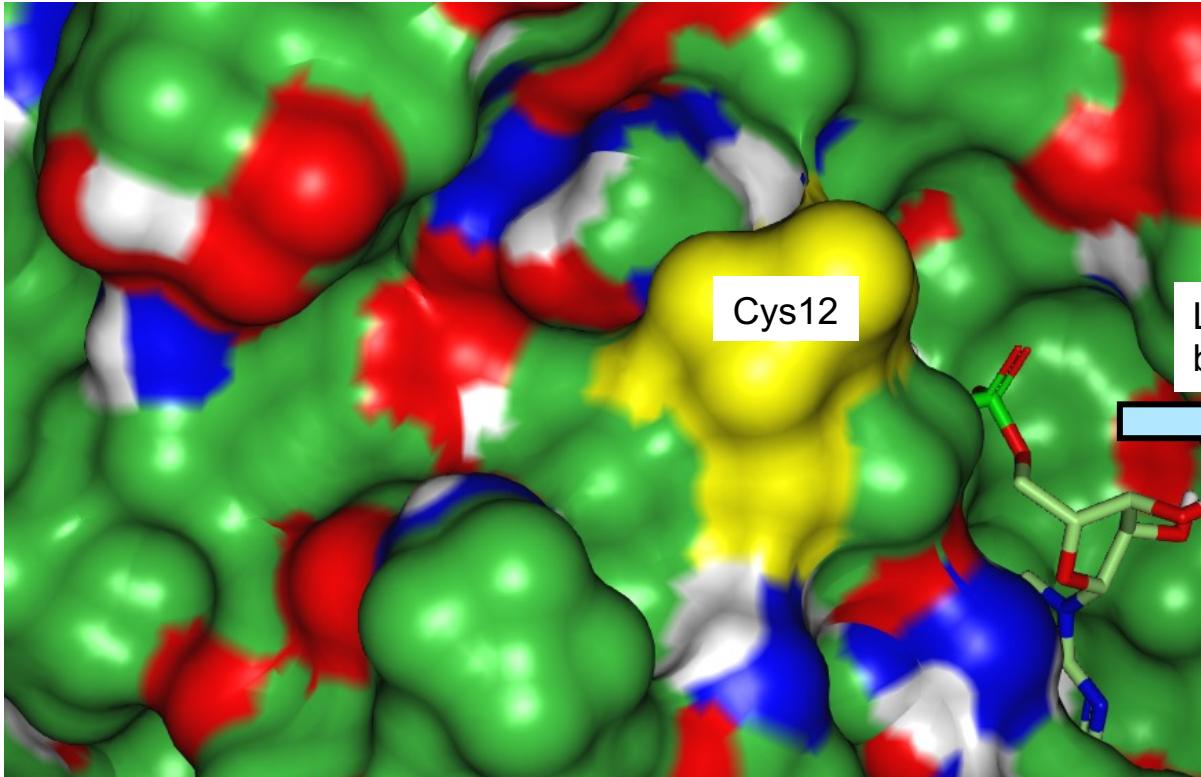
Method	Predicted druggability	Score	Threshold
Pfido_deltaG	NO	-9.37	-9.5
Sitemap_DScore	NO	0.64	0.9
Vertex	NO	1 of 5	5 of 5

Potential druggability opportunity for covalent approach.

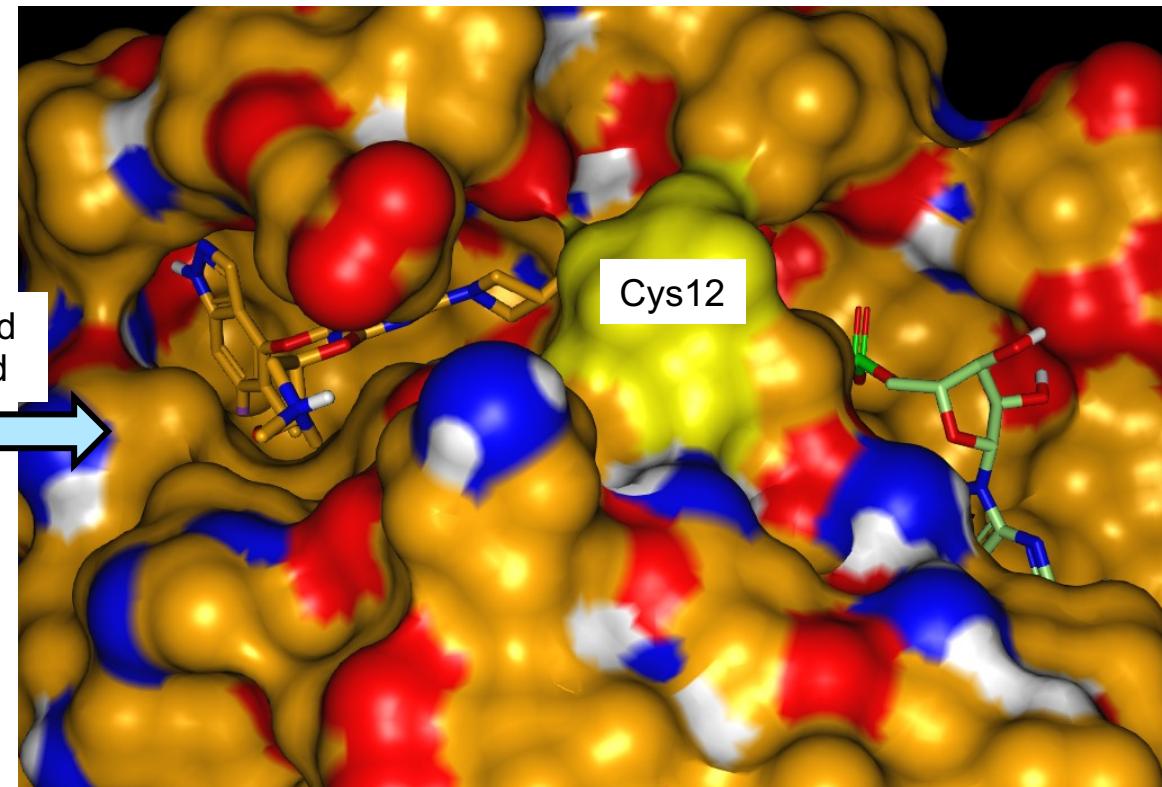


Reference comparison: Druggability for K-RAS G12C

K-Ras apo, with GDP bound



K-Ras complex ligand, with GDP bound

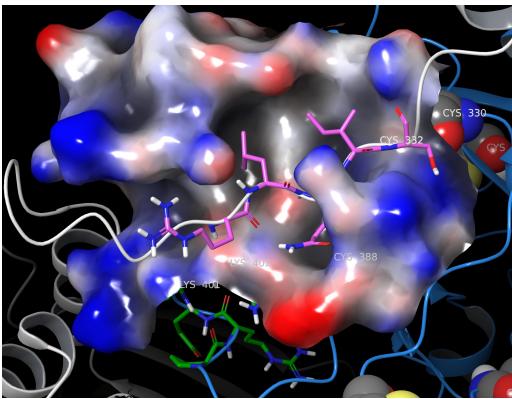


Method	Predicted druggability	Score	Threshold
Pfido_deltaG	NO	-8.68	-9.5
Sitemap_DScore	NO	0.66	0.9
Vertex	NO	2 of 5	5 of 5

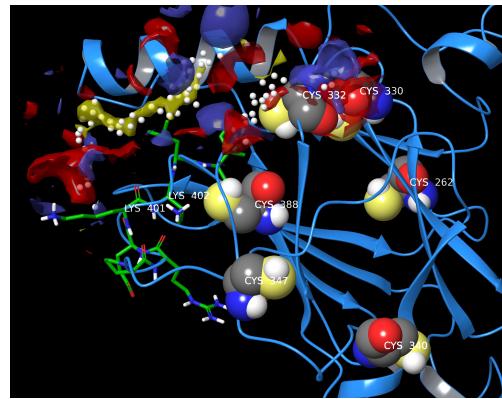
Formation of cryptic pockets creates druggable ligand binding site



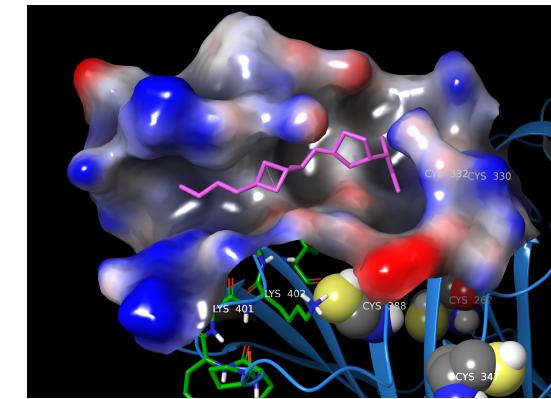
Method	Predicted druggability	Score	Threshold
Pfido_deltaG	YES	-10.08	-9.5
Sitemap_DScore	YES	1.08	0.9
Vertex	NO	4 of 5	5 of 5



3DSH – pLxIS site



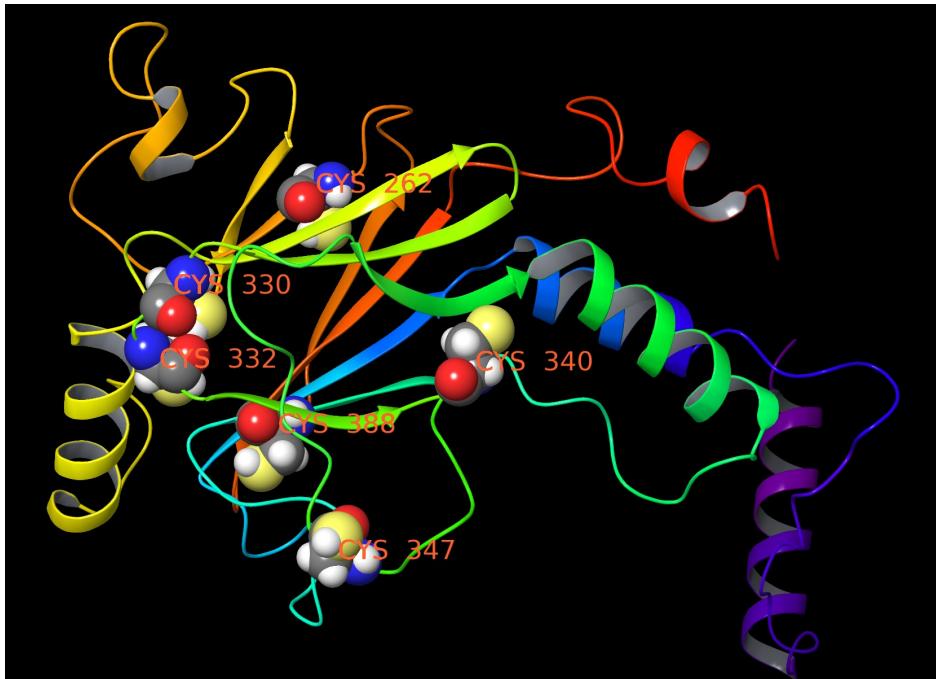
3DSH Monomer Sitemap



3DSH with Fake Ligand

	Drugability	SiteScore	Size	Dscore	Volume	Exposure	Enclosure	Contact	Phobic	Philic	balance
Schrodingers Expected Values	Frank's Score	Avg 1, more is better, below 0.8 bad		Below 0.9 bad		Avg 0.49, lower is better	Avg 0.79, higher is better	Avg 1.0	Avg 1.0	Avg 1.0	Avg 1.6
MMP-9	5	1.02	145	0.96	248.68	0.49	0.73	1.06	0.67	1.29	0.51
Syk	5	1.05	120	1.08	381.42	0.55	0.75	0.89	1	0.94	1.07
BACE	4	1.06	113	1.08	579.67	0.59	0.76	0.91	0.39	0.97	0.4
IRAK4	5	1.04	166	1.02	516.56	0.48	0.77	1.01	0.78	1.16	0.67
PTP-1b	2	0.94	66	0.53	90.9	0.37	0.81	1.2	0.15	2.22	0.07
BRD4	4	1.07	95	1.12	170.8	0.5	0.73	1.02	1.32	0.71	1.85
3DSH - monomer - apo		0.92	74	0.94	343	0.57	0.67	0.93	0.84	0.84	1
3DSH - monomer - fake Ligand		0.88	62	0.89	278	0.54	0.69	0.98	0.89	0.82	1.1

Potential druggability opportunity for covalent approach.



3DSH
Chain A
Monomer 1

262 is in current Reactive DB,
note it is labelled 272.

3DSH Dimer

ResNum	Chain	SASA	DistToSurf
262	A	0	4.595
262	A	0	4.62
330	A	0	3.139
330	A	0	3.122
332	A	0	4.848
332	A	0	4.652
340	A	1.682	0
340	A	12.564	0
347	A	12.312	0
347	A	11.859	0
388	A	0	3.357
388	A	0	3.362

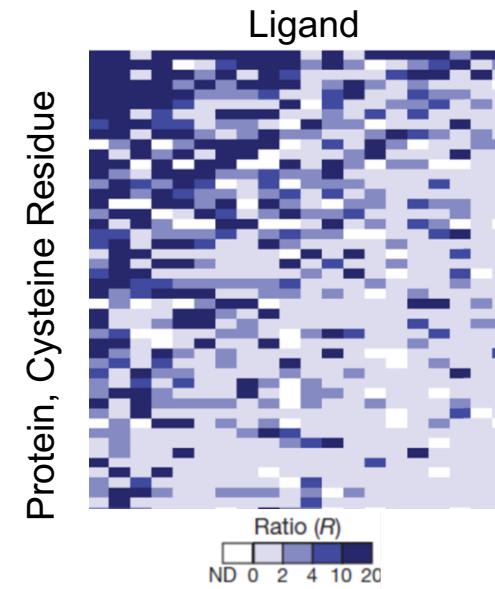
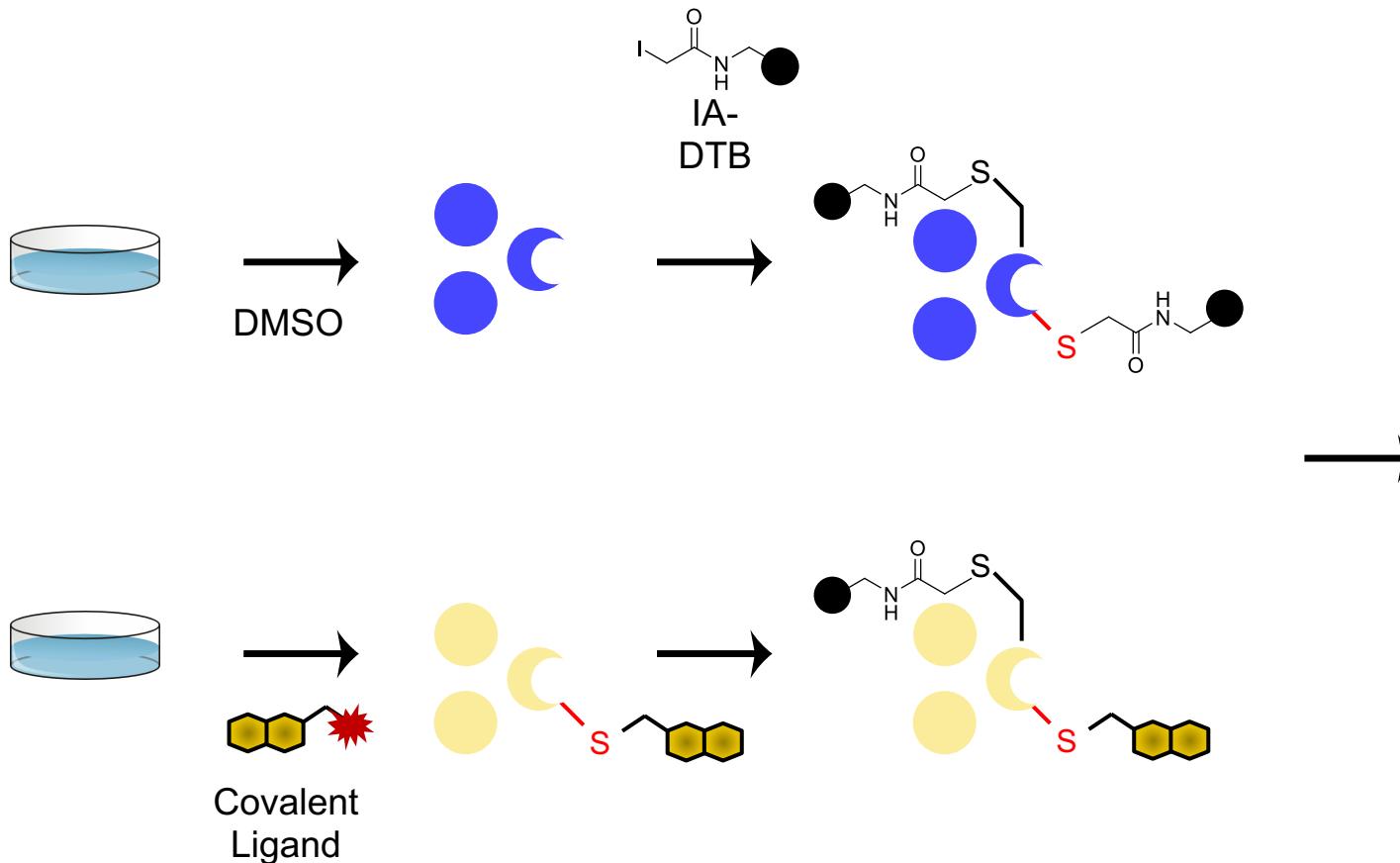
3DSH Monomer

ResNum	Chain	SASA	DistToSurf
262	A	0	4.446
330	A	0	3.244
332	A	0	0.488
340	A	1.913	0
347	A	12.778	0
388	A	0	3.396

SASA = Solvent accessible area
Note DistToSurf = 0 when SASA > 0.

Chemoproteomic Technologies to Define IRF5 Endogenous Cysteine Landscape and Ligandable Sites

Reactivity-based probes enable identification of reactive, functional, and “druggable” pockets and covalent ligands directly in complex proteomes



Weerapana et al. 2010, Nature, 468; 790-795
Wang et al. 2014. Nat Methods, 11; 79-85
Backus et al. 2016, Nature, 534; 570-574
Ward et al. 2017, ACS Chem Biol, 12; 1478-1483