



The Alliance for Cellular Signaling will be holding its 4th Annual Meeting in Dallas, Texas May 23rd through 26th, 2004. Work this year centered on the RAW 264.7 macrophage cell line. Alliance scientists will report progress and challenges and make plans for the future. We look forward to sharing our results with the cell signaling community in our upcoming summer Newsletter. PowerPoint presentations will be available on the Signaling Gateway after the meeting. We invite you to review briefly several of this year's highlights and to explore promising data and resource links that have recently become available.

The Year in Review Ligand Screen in RAW 264.7

The ligand screen assesses the extent of signaling interactions among numerous ligands acting on the same cell. This well-defined database of selected biochemical responses to a variety of ligands serves as the entry point for the perturbation studies and modeling efforts of the AfCS. In the spring of 2003, it became clear that the initial cells chosen for study (primary B cells, adult cardiac myocytes, WEHI-231 lymphocytes) were not amenable to certain approaches for manipulation of gene expression, most notably, techniques using RNA interference. Seven new cell lines were selected for

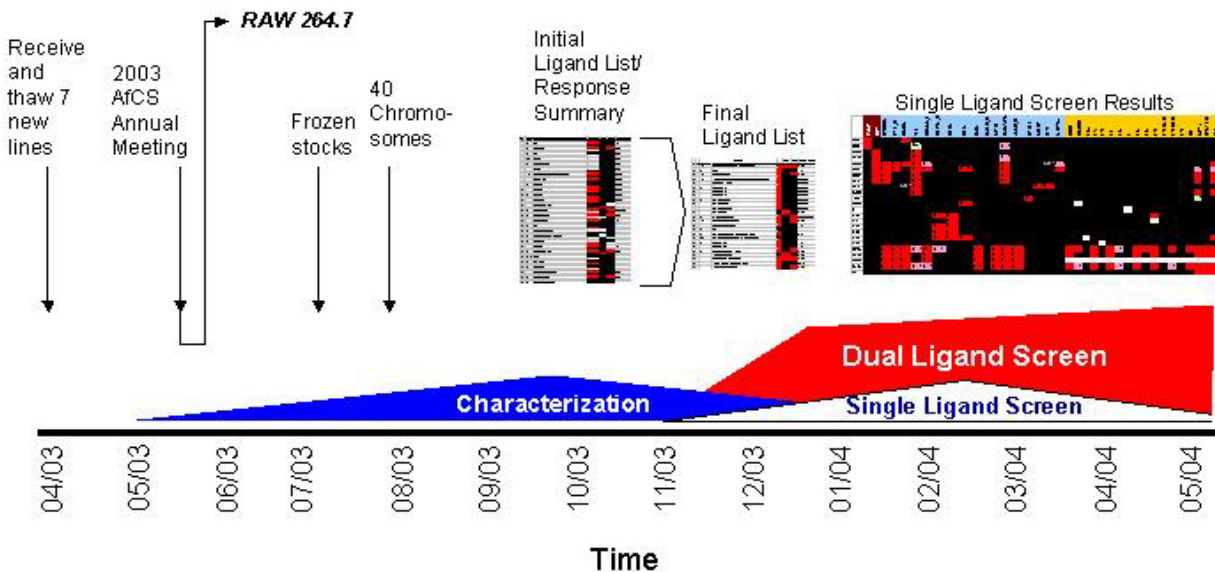


Figure 1. Time line of the progress achieved by the AfCS on the RAW 264.7 ligand screen.

examination. Initial characterization of these cells in the four weeks leading up to the 3rd annual AfCS meeting led to the choice of RAW 264.7 cells.

Once the official decision to study to RAW 264.7 cells was made, we used our experience accrued with the other cell lines and our now established systems for data acquisition, transfer, storage, and display to facilitate implementation of the ligand screen in these new cells. A time line of our efforts is shown in Figure 1. From June to November of 2003, AfCS lab efforts established procedures for consistent culture of RAW 264.7 cells as measured by viability, chromosome stability, and retention of characteristic calcium responses (to C5a, PAF, LPA, and UDP) and stimulation of cAMP (by ISO and PGE). While establishing culture and assay conditions, 56 ligands were evaluated for their ability to elicit changes in one of three assays (calcium, cAMP, or selected phosphoproteins). Twenty five of the ligands elicited one or more responses in these assays and were chosen for the ligand screen. The ligand screen was started in December of 2003. It has been performed in a dual ligand format that examines responses to both single ligands as well as their combinations. Because of the frequent repetition of single ligands as the controls for dual ligand experiments, sufficient data has accumulated rapidly to define single ligand responses, which are presented in the single ligand screen at <http://www.signaling-gateway.org/data/cgi-bin/table.cgi?cellabbr=RW>.

The dual ligand screens for calcium and cAMP are essentially completed, although data are still being curated and some ligand combinations require additional data to assess statistical significance. We anticipate that the dual ligand screen for phosphoproteins will be completed by December. Our progress for sample production is illustrated in Figure 2. The goal is to complete at least three repeats of each

	TGF	MCF	P2C	IFB	C5A	I1B	IFG	UDP / UTP	I06	I10	ISO	PAF	LPS / LBP	GMF	2MA	LPA	I04	P3C	PGE	IFA	SIP	848		
TGF																								
MCF	31106A 31114A 31113A 31118A 40407A																							
P2C	31106A 31106A 31114A 31113A 31118A	31104A 31106A 31114A 31113A 31118A																						
IFB	31104A 31106A 31114A 31113A 31118A	31104A 31106A 31114A 31113A 31118A	31104A 31106A 31114A 31113A 31118A																					
C5A	AA 40422B	31222A 31222A 40408B	40422B 40422B 40428A	40322A 40326A 40326B																				
I1B	31222A 31222A 31222A	40113A 40408B	31211A 40122A	40319A 40326A 40427B	40113A																			
IFG	31230A 40122B	40123A 40128A	40120A 40128A	40212A 40326B 40427B	40212A 40326B 40427B	40227C 40227C 40227C																		
UDP / UTP	40206A 40401A AD		40213A 40317A	40217A AM	31216A 40427A	40407A																		
I06	40210A 40226A AE	40123A	31211A	31230B 40122A	40304B 40326A 40427B	31211A 40109B	40123A 40127A 40507B	AU 40422A																
I10	40206A 40401A AD	40407B	40116A 40330A	31230B 40122A	40322A 40115B	AR 40415A AT 40420B	40407A 40420B																	
ISO	40205B	31218A 31222A 40113A	40211A 40401B AI	40423A 40113A	31218A 40423A	40113A 40423A	40407A	31222A 40109A 40407A 40422A	40219A 40316A 40407A 40323A	40109A 40407A														
PAF	40122B 40428B 40407B	40414A 40428A	40120A 4020A 40428A	40123A	40304A 40326A 40427A	40220A 40128A 40427A	40122B 40128A BF 40507B	40303A 40122A 40427A	40122A 40316A 40407B	40217B AZ														
LPS / LBP	40205B 40506A	40120A 40506A	40213A AI 40414B 40428A	40203A 40325A BH	40304B 40326A 40427A	40120A 40420B	40213A AI 40427A 40317A	40304B 40325A BH	AR BB 40420B	40205B	40318A BA 40427A													
GMF	31125A 31202A 31209A 40506A	40115A 40506A	AA 40210A AB 40428A	AA 31230B 40203A AA	AB 40428A	31230B 31209A 40109B AA	40120A 40127A	40416A AX	31230B 40109B 40316A	40217B AZ	40120A 40217B AZ	40503A 40318A 40227B 40506A	AA BA 40318A											
2MA	AA 40422B	40311A AF	AA AP 40422B	40319A AH	AA BC 40422B	40319A AV	40310A AV	AU 40422A	AU BC 40422A	40311A AF	BG 40422A	40318A BA	40318A BA 40318A											
LPA	40206A 40401A AD	40123A 40408B	40211A 40401B AL 40428A	40203A	40304A 40401A 40408B	40415A AD 40507B	40123A AD 40507B	40303A BP	40205B 40401A AD AL 40416A 40430A 40423A	40304A 40401B 40507B	40203A 40203A	40310A BD												
I04	31230A 40205B	40115A	40115A 40402A	40115A 40420A	40322A 40115B	31211A 40302A BH	31230A 40127A	40303A AW	31211A 40127A	40322A 40115B	40420A 40423A	40205B AW	40205B 40402A	40115A 40127A	40310A BM	40310A BD 40402A								
P3C	31125A 31202A 31209A	AJ	40128A	40217A AM 40428A	40322A 40115B	31125A 31202A 31209A	40128A 40310A AV	40217A AM	40219A AY 40115B BB	40322A 40115B BB	40128A	40429A BB	40310A AV 40430A	31125A 31202A 31209A	40310A BD 40303A	40310A BD 40402A								
PGE	40210A 40326A AE	31218A 31222A 40408B	40116A 40330A	AD 40428A	31218A 31222A 40408B	40220A 40115A 40408B	40310A AX	40116A 40326A 40330A	40219A AY 40326A 40330A	40116A 40326A 40330A	31218A BB 31222A	40220A AS 40428B	40429A BB 40414B	40115A AX 40416A	40319A AV 40430A	40303A SP 40408B	40115A AD 40415B 40408B	40115A AD 40415B 40408B	40310A BB	40116A BB	40303A BB	40310A BB		
IFA	31230A 40122B	40414A AK	40116A 40330A	40319A AH 40414B	40304B 40326A 40402A BH	31230A 40122B	40418A AX	40304B 40326A 40330A	40116A BG 40430A	40414A AG 40414A 40430A	40428B AI 40414A AK 40428B	40327B BK 40415A AT	40418A BK 40415A BM	40319A AH 40415A BM	B1 31230A BH	40309A BP 40415B 40333A	40418B BM	40219A AY 40415B 40333A	40309A BP 40415B 40333A	40116A BB	40305A BL 40414B	40305A BL 40414B		
SIP	AG 40428B AK 40507A	40213A AI 40317A	40212A 40326B AC 40427B	40212A 40326B AC 40427B	40415A AT 40427B	40212A AT 40427B	40213A AI 40306A AY 40427B	40219A AV 40115B BB	40415A AT 40115B BB	40219A AV 40115B BB	40414A AG 40414A 40430A	40327B BK 40415A AT	40418B BK 40415A BM	40418B BK 40415A BM	40211A AW 40401B 40428A	40303A AW 40428A	40305A BL 40414B	40220A AS	40305A BL 40414B	40305A BL 40414B	AG 40428B 40428B 40507A			
848	AG 40428B 40507A	40311A AF 40507A	40211A 40414B AL	BC 40427B	40220A AS 40428B	40227C BJ 40428B	40303A AW	BC 40427B	40311A AF 40428B	40116A AG 40428B	40220A AS 40428B	40227B BK 40415A AT	40227B BK 40415A AT	40311A AF 40428B	40303A AW 40428B	40305A BL 40414B	40220A AS	40305A BL 40414B	40305A BL 40414B	AG 40428B 40428B 40507A				
TGF																								

Figure 2. Combinations of ligands for assay of phosphoproteins. Combinations completed are tracked by the displayed barcodes.

combination of ligands, while minimizing the repetition of control treatments (single ligands alone). In the case of phosphoproteins, experimental restraints define the optimal single experiment as four ligands and their six associated combinations.

The measurement of cytokines as an output for RAW 264.7 cells was explored at the beginning of 2004. Verification that several cytokines were secreted and could easily be measured led to initiation of a full dual ligand screen with this assay. Rapid implementation, ease of assay, and a dense output allow rapid progress, and it is anticipated that this portion of the screen should be completed in June. A matrix of the experimental plan and progress is shown in Figure 3. Efforts to complete curation of data and design of displays are a high priority, and more complete display of these data should be available soon.

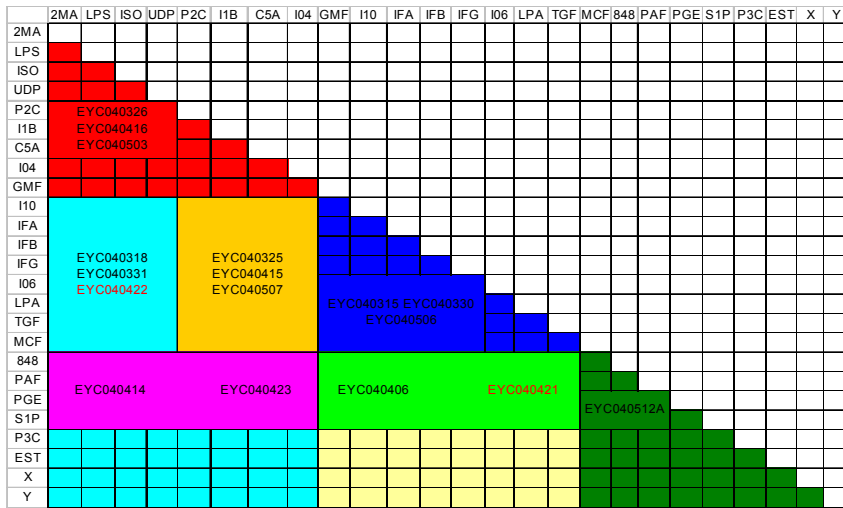


Figure 3. Matrix for the dual ligand screen of cytokine production in RAW 264.7 cells. Each color represents an experiment wherein those combinations and their respective single ligand treatments are examined. Barcodes indicate that treatments have been completed.

Preliminary evaluation of the results of the single ligand screen illustrates a dynamic range of responses by the RAW cells. For example, cytokine receptors elicit unique phosphorylation of a variety of Stat proteins with fast kinetics but do not engage the MAP kinase signal transduction pathways. The agonists for Toll-like receptors and agonists that cause increases in intracellular calcium stimulate activity within MAP kinase pathways. However, the kinetics of this effect are much slower with the Toll-like receptors. Evaluation of cytokine secretion also reveals unique patterns; ligands that stimulate calcium appear to induce secretion of only MIP1 alpha and TNF alpha, whereas the Toll-like receptor agonists enhance production of at least 10 different cytokines.

Since January 2004, we have produced a great deal of data using RAW 264.7 cells. Besides the single ligand responses, much of the dual ligand screen is completed and is being analyzed. Results will be on display at the 4th annual AfCS meeting in Dallas, TX.

FXM Project is a Knockout!

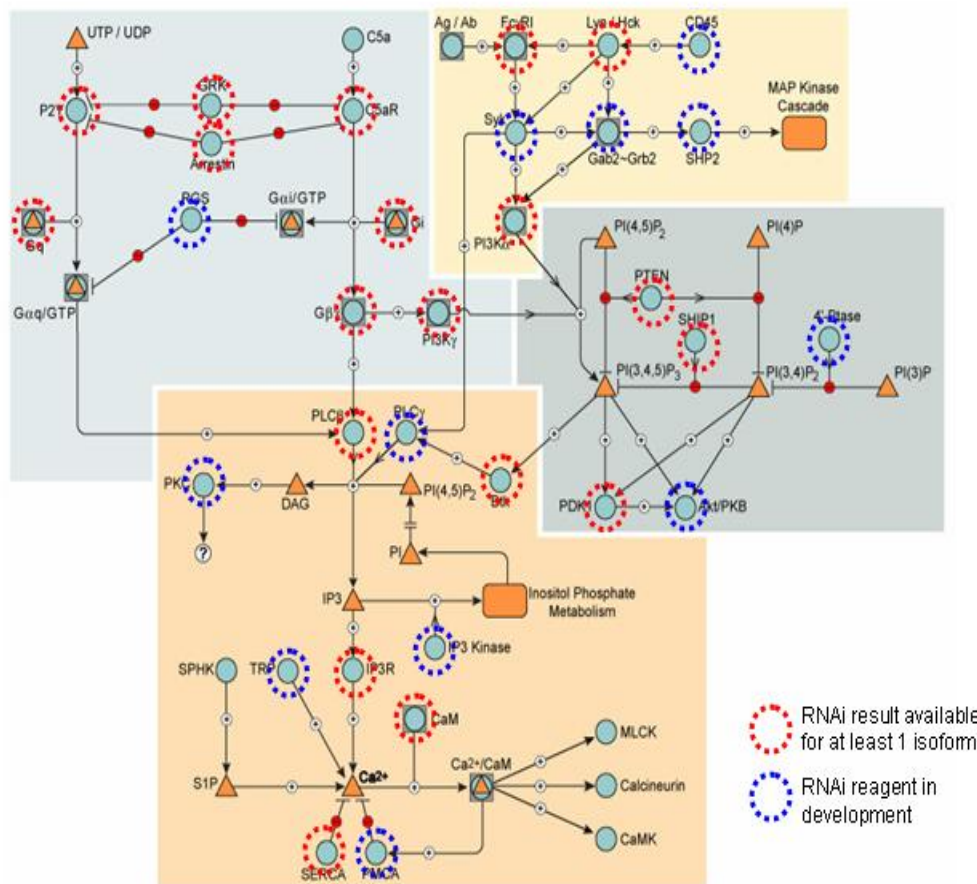
To understand and model a cell's complete signaling network is an audacious goal, ideally suited for the Alliance for Cellular Signaling. Last October the AfCS launched a "Focus on X modules" (FXM) project to pilot the transition from the stage of data collection to that of network modeling. Focused on a small section of a cell's signaling network, this project serves as a test bed for AfCS capabilities and allows us to identify effective strategies for dissecting and modeling cellular signaling. Strategies developed in this project will be employed for understanding more complex signaling networks.

It's all in the timing! The FXM project was launched after the Alliance had established capacities for efficient multi-group cooperation, chosen the mouse macrophage cell line, RAW 264.7, as the primary cell

for study, initiated a screen of responses to many ligands to provide insight into the spectrum of potential pathways in this cell, and found that a major perturbation technology, RNAi, works in RAW 264.7 cells.

The FXM will map and dissect two carefully selected segments ('X') of the overall signaling network, those that control the changes in cytoplasmic Ca^{2+} and a membrane lipid, phosphatidylinositol-(3,4,5)P₃, ubiquitous signaling molecules that mediate many cellular responses to extracellular stimuli. Each of the three ligands chosen for testing activates a different primary signaling pathway: C5a activates a G α -linked chemokine receptor; UDP stimulates G α_q -linked nucleotide P2Y receptors; binding of IgG2a to the Fc γ R1 receptor and subsequent crosslinking leads to tyrosine kinase-coupled activation of this pathway. All three ligands trigger Ca^{2+} responses, and two of the three ligands (C5a and IgG2a) increase PIP₃.

The FXM has already shown considerable progress in mapping, perturbation experiments, and modeling (see Figure below). We began by constructing a literature-based parts list, which includes all known players that may be involved in the signaling cascades from C5a, UDP, and IgG2a to Ca^{2+} and PIP₃, including receptors, adaptors, scaffolds, G-protein components, enzymes for synthesis and degradation, and other signaling molecules. Expression of these proteins in RAW 264.7 cells is confirmed by



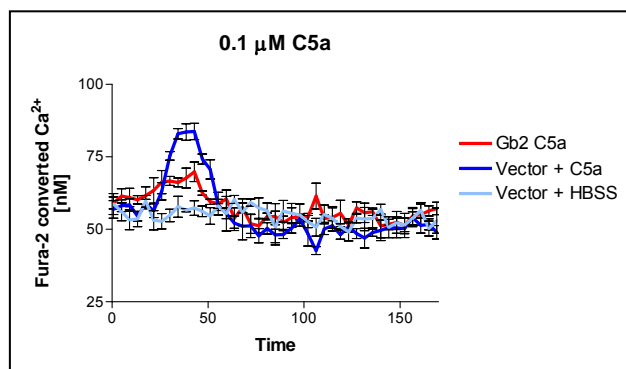
Affymetrix GeneChips and RT-PCR. With the help of many experts in the field, this list has grown to over 200 proteins. It is the basis for coordinating experiments and mapping efforts.

Perturbations of the network, for the most part via RNAi knock-downs of individual proteins, help to map the flow of information from extracellular stimuli to the measured outputs. An effective RNAi sequence is selected and engineered into a lentiviral expression vector by the Molecular Biology Lab at Caltech, in

collaboration with Dharmacon. The lentivirus is then made and transduced into RAW 264.7 cells in the Macrophage Biology Lab at UCSF. Each week these labs generate five lentiviral infected RAW cell lines, including one control and four lines that target a specific gene product. After selection for stable integration and expression, reduction of the targeted protein is assessed by Western blot and/or RT-PCR.

Phenotypes of knock-down cells are initially determined by assessing their responses to the three FXM ligands. Changes in intracellular calcium are assessed by both population assays (Macrophage Biology Lab) and single cell assays (Microscopy Lab). Protein phosphorylations are tested in the Cell Preparation Lab, and translocation of the Akt-PH domain is measured in the Microscopy Lab.

At present 80 genes are in the RNAi pipeline, and 40 lenti-shRNA vector infected lines have been generated. Population Ca^{2+} responses have been examined in cell lines targeting 22 different genes: 16 of these (72%) display altered Ca^{2+} response to at least one FXM ligand. Some of the altered responses are expected, like the decreased Ca^{2+} responses to UDP in Gαq knockdown cells or to C5a in Gβ2 knockdown cells (see Figure below). Some phenotypes are unexpected but interesting, such as the increased Ca^{2+} responses to C5a in PTEN knockdown cells. Others are more puzzling. These unexpected results have generated considerable excitement, but need to be validated as specific consequences of target protein knockdown rather than results of cell line drift or off target effects of RNAi reagents. Efforts to address these issues include replicating knockdown cell lines through independent infections, transient knockdowns by transfection of siRNA or antisense RNA, and expanding the array of measured responses to include multiple intermediate end points.



To model the FXM signaling network, a three-person team, based at UTSW and UCSF, collaborated with Arkin's group at UC Berkeley to develop a comprehensive FXM signaling map. The map breaks down each signaling event into detailed biochemical reactions and connects it to signaling transduction pathways determined from legacy data. This map plays a key role in connecting experiments with the modeling effort in a process that will be dynamic. The first step in modeling will focus on connectivity of the network. The second step will detail behavior of network nodes and quantitatively model the signals.

The FXM, now a major focus of the Alliance, poses many challenges. The FXM is taking the first steps toward developing models that both explain and predict results of experiments. First, we are developing assays for signal flow through intermediate end points, assessed by real-time translocation and FRET assays, as well as high throughput protein phosphorylation and lipid assays. Second, to tackle redundancy of isoforms and epistasis questions, we seek to improve the efficiency of RNAi knockdowns and learn to knock down more than one protein at once. Third, we need to develop effective strategies for data analysis and network modeling. If successful, the resulting models will accomplish the Alliance's ultimate goal: to provide insight into general principles that underlie the structure and dynamic activities of complex signaling networks.

Complete Molecule Pages are Here

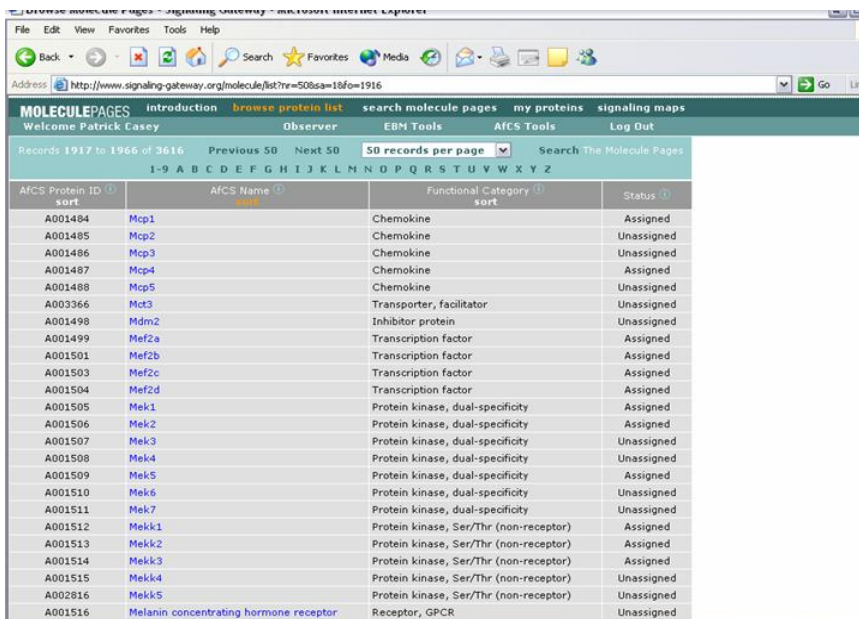
The Molecule Page (MP) initiative recently reached another milestone with the public release of the first full MPs that include complete investigator-curated data on properties and functions of specific molecules. Although >3,500 MPs for signaling proteins have been available on the Signaling Gateway website for some time, these entries are based on automated data-feeds. While the automated sections are updated monthly and provide ready retrieval of essential

molecule-specific information from existing public databases, the availability of full MPs adds a crucial layer to this useful resource, namely a complete analysis of legacy (i.e., literature-derived information) about the molecule. The result is a display of highly-structured quantitative information on the molecule, including subcellular locations, covalent modifications, binding partners, and functions, each coupled to specific references to the literature. The manner in which this information in the full MPs has been captured in an object-relational database has been specifically designed such that it will be equally accessible to computational biologists developing programs to model systems biology and to investigators interested in interrogating molecule-specific functions in signaling pathways. This makes the MP database a unique resource for the scientific community.

Some crucial aspects of the full MPs are that each is authored by investigator(s) that are expert in the particular molecule, and each is subjected to rigorous independent peer review by Nature and the AfCS/Nature Editorial Board. These individuals have been chosen for their broad knowledge of signaling processes.

As a result, MPs are fully citable, ensuring that authors receive the credit they deserve for preparing the equivalent of a scholarly review of the molecule. An additional important feature of the full MPs is that they will be updated annually, ensuring that the database remains an important resource for the scientific community for years to come.

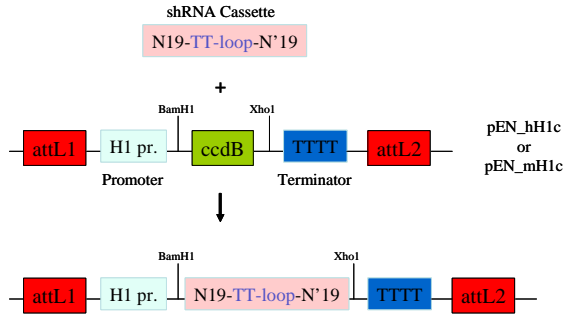
The AfCS extends its most heartfelt thanks to those 800+ investigators who have already committed to author MPs and also to those who have served as peer reviewers. We are actively recruiting new MP authors and hope that our readers will consider such an opportunity if they haven't already agreed to author a MP, or will nominate a colleague for an available molecule. To check out just what the Molecule Pages are, and also to view/search the AfCS Protein List, which indicates whether an author has already committed to any particular molecule, please visit the Molecule Pages section of the Signaling Gateway website at: <http://www.signaling-gateway.org/molecule/>. This site also contains links to an application form to apply for Molecule Page authorship. If you wish to nominate an individual for a specific molecule, please send your nomination to the AfCS Membership and Editorial Office at: afcsed@mc.duke.edu.



The screenshot shows a web browser window displaying the 'MOLECULEPAGES' section of the Signaling Gateway website. The page includes a navigation bar with links for 'Introduction', 'browse protein list', 'search molecule pages', 'my proteins', and 'signaling maps'. Below the navigation bar, there is a table listing molecules. The table has four columns: 'AfCS Protein ID', 'AfCS Name', 'Functional Category', and 'Status'. The table contains 20 rows of data, including entries for Mcp1, Mcp2, Mcp3, Mcp4, Mcp5, Mct3, Mdm2, Mef2a, Mef2b, Mef2c, Mef2d, Mek1, Mek2, Mek3, Mek4, Mek5, Mek6, Mek7, Mekk1, Mekk2, Mekk3, Mekk4, Mekk5, and Melanin concentrating hormone receptor.

AfCS Protein ID	AfCS Name	Functional Category	Status
A001484	Mcp1	Chemokine	Assigned
A001485	Mcp2	Chemokine	Unassigned
A001486	Mcp3	Chemokine	Unassigned
A001487	Mcp4	Chemokine	Assigned
A001488	Mcp5	Chemokine	Unassigned
A003366	Mct3	Transporter, facilitator	Unassigned
A001498	Mdm2	Inhibitor protein	Unassigned
A001499	Mef2a	Transcription factor	Assigned
A001501	Mef2b	Transcription factor	Assigned
A001503	Mef2c	Transcription factor	Assigned
A001504	Mef2d	Transcription factor	Assigned
A001505	Mek1	Protein kinase, dual-specificity	Assigned
A001506	Mek2	Protein kinase, dual-specificity	Assigned
A001507	Mek3	Protein kinase, dual-specificity	Unassigned
A001508	Mek4	Protein kinase, dual-specificity	Unassigned
A001509	Mek5	Protein kinase, dual-specificity	Assigned
A001510	Mek6	Protein kinase, dual-specificity	Unassigned
A001511	Mek7	Protein kinase, dual-specificity	Unassigned
A001512	Mekk1	Protein kinase, Ser/Thr (non-receptor)	Assigned
A001513	Mekk2	Protein kinase, Ser/Thr (non-receptor)	Assigned
A001514	Mekk3	Protein kinase, Ser/Thr (non-receptor)	Assigned
A001515	Mekk4	Protein kinase, Ser/Thr (non-receptor)	Unassigned
A002816	Mekk5	Protein kinase, Ser/Thr (non-receptor)	Unassigned
A001516	Melanin concentrating hormone receptor	Receptor, GPCR	Unassigned

RNAi Vectors Now Available from ATCC



The AfCS is utilizing RNA interference (RNAi) to manipulate signaling protein expression in the RAW264.7 macrophage-like cell line. This can be achieved by the expression of gene-specific short hairpin RNAs (shRNAs) as RNA polymerase III (Pol III)-driven transcripts from plasmid or viral vectors. The AfCS Molecular Biology Laboratory has developed plasmids that permit straightforward cloning of short hairpin RNAs (shRNAs) against specific genes and simple subcloning of shRNA expression cassettes into a variety of expression platforms.

A set of these AfCS-developed RNAi vectors are now available from the ATCC. For cloning and testing of shRNAs against specific genes, vectors containing either the human or mouse H1 promoter are available. To permit the simple subcloning of validated H1-shRNA cassettes to either viral or mammalian expression plasmids, we have engineered the vectors to contain Gateway recombination sites. This allows the user to express an shRNA from up to 9 different expression platforms. The expression platforms employ a variety of markers and drug selection options to facilitate enrichment of cells expressing the shRNA.

A detailed document describing the vectors and how they can be used for RNAi studies can be downloaded at the following link http://www.signaling-gateway.org/data/plasmid/RNAi_vector_guide.pdf

SPONSORS

We acknowledge our sponsors with gratitude:

The National Institutes of Health

The National Institute of General Medical Sciences.

The Alliance Project was conceived under the NIGMS Glue Grant Initiative. See: www.nigms.nih.gov/funding/gluegrants.html

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