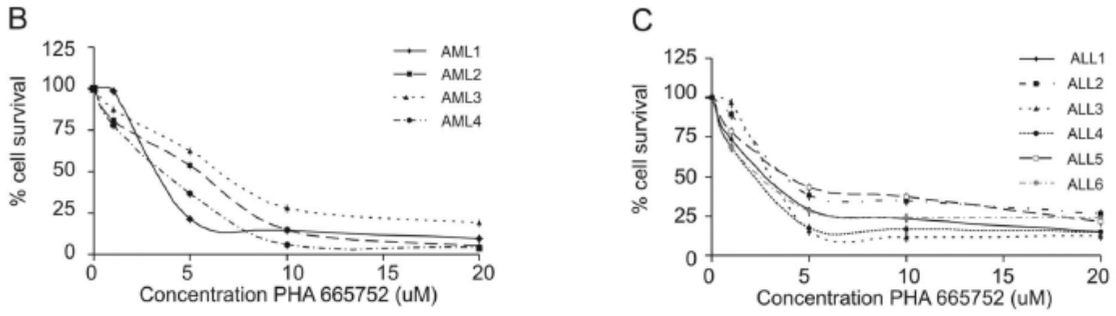
Confirming PamChip® data: Selected examples from Literature

Data generated using PamChip assays need to be confirmed or validated. Published examples of different approaches and methods are provided here, to illustrate possibilities for your research.

Using Kinase inhibitors to test inhibition of cell cultures *in vitro*

Ter Elst A *et al.*, (2011) Anal Biochem. Leuk Lymphoma. 52(1):122-30

1. PamChip® tyrosine phosphorylation profiles clinical leukaemia samples showed increased phosphorylation of 44 peptides common to all 3 types (AML, CML, CLL).
2. PamChip data corresponded to literature for leukaemia targets, such as EGFR, PDGFR, NTRK1 and NTRK2, upstream of RAS-RAF-MEK-ERK pathway. Additionally RON (MSTR1) was discovered as a target and was activated.
3. Cell survival assays with a MET/RON inhibitor for all three leukaemia types (*CLL not shown*) showed a dose-dependent inhibition.

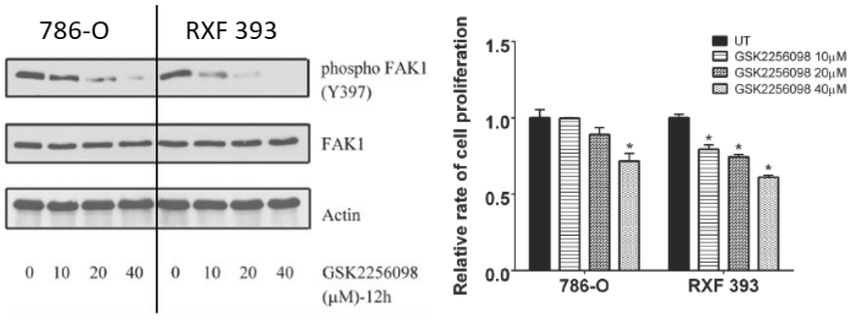


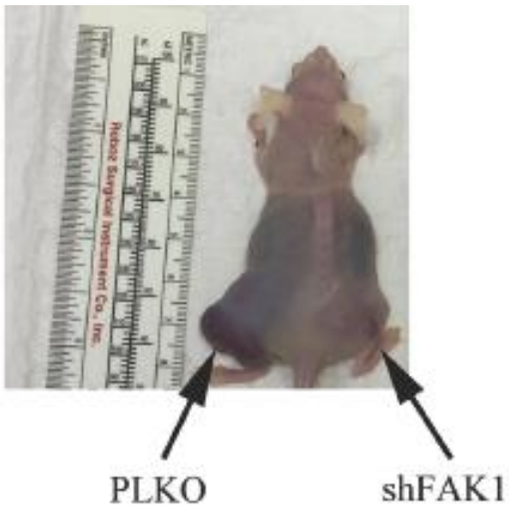
MET/RON could be a potential therapeutic target for Leukaemia.

Using Kinase inhibition to test inhibition of kinases *in vivo* (Mouse model)

Ghosh AP (2017) *et al., (2017)* Oncotarget, 8, (No. 17), pp: 29220-29232

1. Comparing kinomic profiles of primary and metastatic RCC tumours in clinical samples revealed top kinase networks activated in mRCC, FAK1 ranking highest
2. Pharmacologic inhibition of FAK1 with GSK2256098 suppresses *in vitro* tumour phenotypes
3. FAK1 knockdown in RCC cells suppresses *in vivo* tumour growth in subcutaneous nude mouse models





FAK could be a potential therapeutic target for metastatic ccRCC.

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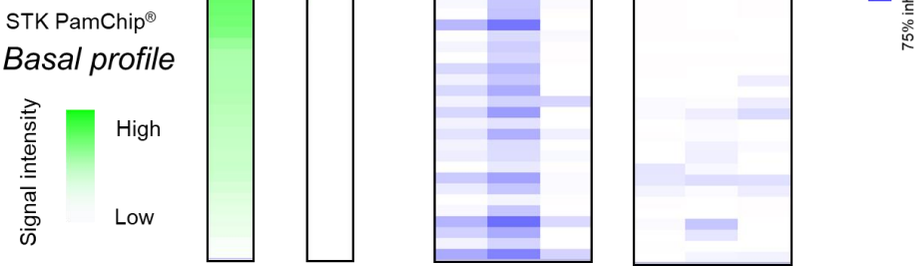
Using Kinase inhibitors to test inhibition of kinases *ex vivo* (Spike-in)

Hilhorst R *et al.*, (2011) AACR Poster

AKT activation when PTEN is knocked down in prostate cancer, can be confirmed by showing higher inhibition in PTEN knock down lysates compared to WT, when AKT pathway inhibitors are directly spiked-in to lysates before the PamChip assay.

PTEN ⊣ PI3K
↓
AKT

AKT activity pharma-cologically shown



Using Kinase inhibitors to test inhibition of recombinant kinases

Hoozemans *et al.*, (2014) J Clin Cell Immunol 5:4

1. IRAK-4 Kinase Activity is increased in Alzheimer's Disease. Human temporal cortex specimens (n=13) Alzheimer's Disease patient (n=7) versus control (n=6).
2. Confirmation by kinase inhibitors and recombinant kinases. Peptides on PamChip showing significant increase in phosphorylation by protein lysates derived from AD temporal cortex compared to control temporal cortex also showed increased activation by recombinant IRAK-4 kinase. The same peptides showed significant inhibition by an IRAK-4 inhibitor.

Peptides showing increased phosphorylation by human recombinant IRAK-4

PamChip Peptide ID	p-value
ACM1_421_433	0.046
ACM4_456_468	0.015
ACM5_498_510	0.032
ADDB_696_708	0.022
CDK7_163_175	0.032
CSK21_355_367	0.018
FOXO3_25_37	0.015
H32_3_18	0.045
INSR_1368_1380	0.032
MP2K1_287_299	0.022
P53_308_323	0.038

Peptides showing decreased (>50%) phosphorylation in the presence of 2500 nM IRAK-1/4 inhibitor

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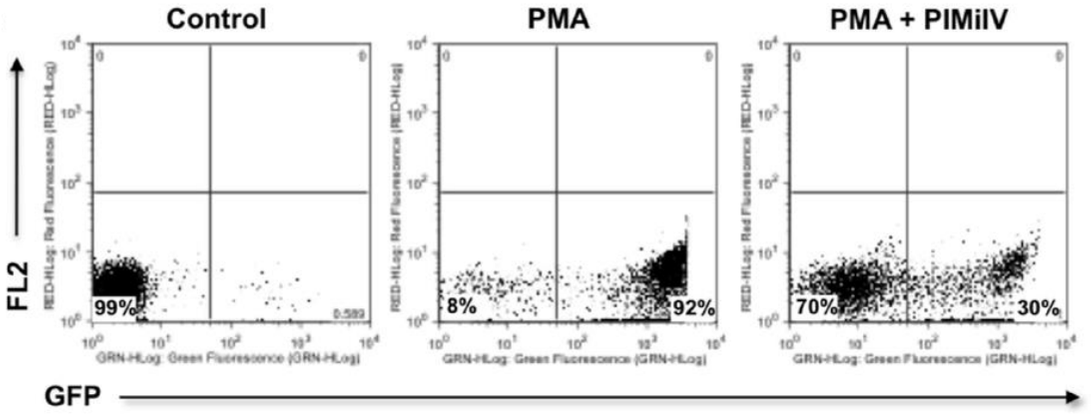
Using Reporter Cells

Duverger A *et al.*, (2014) J Virol. 88(1):364-76

Kinase activity profile of latently HIV-1-infected T cells is altered relative to that of uninfected T cells.
1. Ranking of the altered kinases predicted PIM-1 kinase as a key switch involved in HIV-1 latency control.
2. PIM-1 inhibitor IV prevents activation induced HIV-1 reactivation. Latently **HIV-1-infected CA5 reporter T cells** were stimulated with the PMA +/- PIMi IV. Reactivation was measured as the percentage of GFP-positive cells by using flow cytometry.

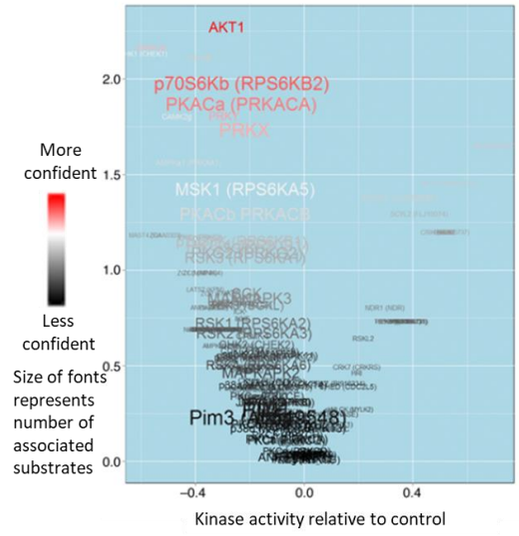


PIM-1 activity is required for HIV-1 reactivation in T cells

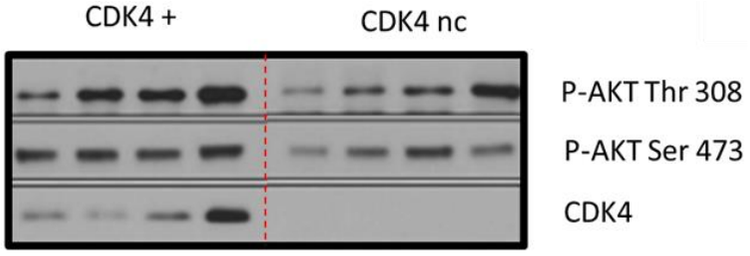
Using Western blotting

Lagarrigue S *et al.*, (2016) J Clin Invest. 126(1):335-348.

1. To analyze how Cdk4 is involved in the regulation of insulin pathways in adipose tissue, Cdk4nc mice and control mice (n = 4) were injected (portal vein) with insulin for 3 minutes. In the PamChip assay, lower activity of AKT pathway was seen in Cdk4nc samples compared to control samples, suggesting that CDK4 activity played a role in the regulation of AKT



2. Western blot analyses confirmed that AKT activity, as measured by phosphorylation in Ser473 and Thr308, was decreased in Cdk4nc mice in response to insulin



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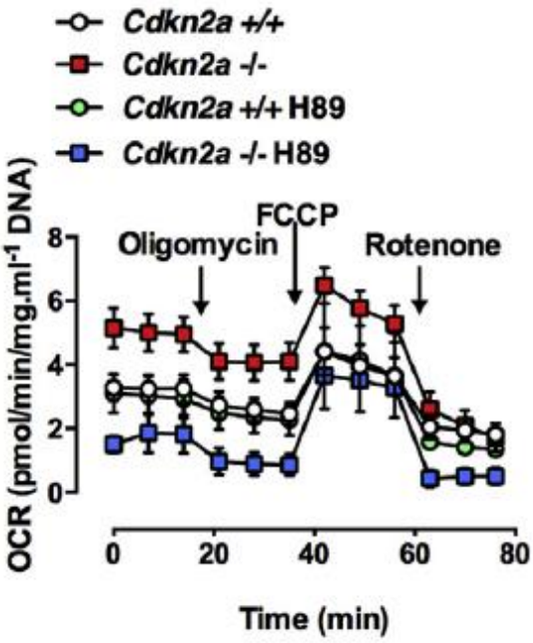
Using Cell metabolism assays (e.g. Agilent Seahorse)

N. Rabhi *et al.*, (2018) Mol Metabolism 126(1):65-76

1. Differences in Cdkn2a-regulated signalling cascades in adipose tissue browning were studied by comparing kinase activities between control and Cdkn2a-/- mice using the PamChip assay. 47 peptides showed significant differences in phosphorylation levels between Cdkn2a +/+ and -/- mice. Using the HPRD database PKA was picked as the top canonical pathway.

2. Oxygen consumption rate (OCR) was measured using the Seahorse XFe24 platform. After 8 days of differentiation (D8), OCR was measured in Cdkn2a-/- and+/+ differentiated adipocytes -/+ a PKA inhibitor. PKA inhibition of Cdkn2a-/- cells lowered OCR, confirming PamChip data of PKA involvement. In adipose tissue browning.

Top canonical pathways	p-value
Protein kinase A signaling	$3.00e^{-16}$
AMPK signaling	$4.44e^{-13}$
Synaptic long term potentiation	$1.43e^{-12}$
G-Protein Coupled Recpetor Signaling	$4.42e^{-11}$
Prostate cancer signaling	$1.38e^{-10}$

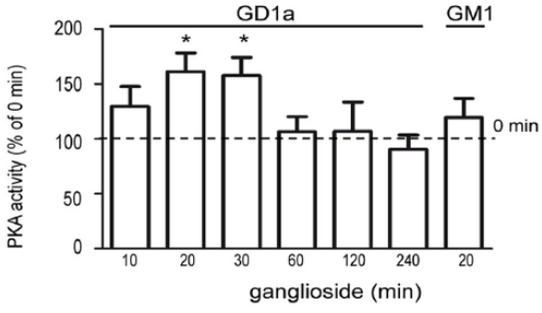


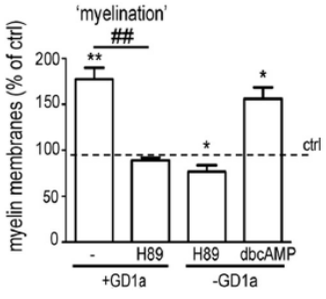
Using Enzyme activity assays

Qin J *et al.*, (2017) The Journal of Neuroscience, 37(41):9925–9938

In multiple sclerosis (MS), fibronectin aggregates (aFN) prevent myelin formation in oligodendrocytes (OLGs). This is overcome by GD1a, a ganglioside.

1. STK PamChip profiling identified that PKA is significantly activated in GD1a-treated oligodendrocytes cultured on aFN
2. PamChip® results were confirmed by measuring PKA activity*, which increased at 20-30 minutes and decreased to basal levels after 1h. *(* nonradioactive PKA activity assay (Enzo Life Sciences)*
3. PKA inhibitor H89 resulted in decreased myelination, and PKA activator dbcAMP increased it, showing that the process is mediated via PKA signaling.





Ganglioside GD1a might act as a potential novel therapeutic tool to selectively modulate the detrimental effects of aFN that prevents remyelination in MS

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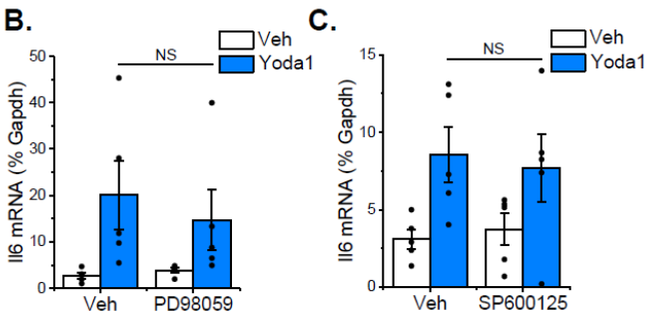
Using RT-PCR for mRNA expression

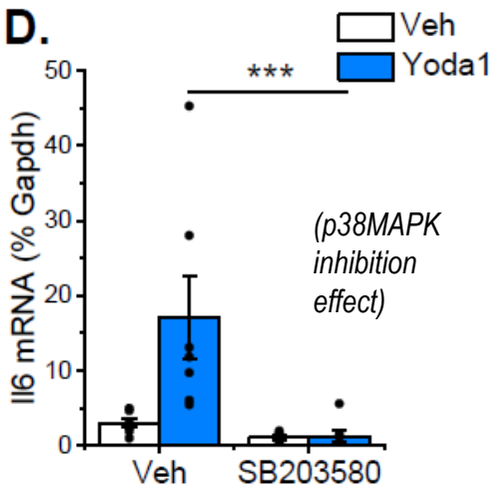
Blythe N *et al.*, (2019) J Biol Chem. jbc.RA119.009167

1. Cardiac fibroblasts express mechanically activated Piezo1 ion channels coupled to secretion of the signalling molecule IL-6, important in regulating cardiac remodelling. STK PamChip assay was used to assess differences in kinase activity following treatment of murine cardiac fibroblasts with Yoda1 (channel agonist) for 10 min.

2. Of the Top 20 kinases, MAPK signalling was significant (extracellular signal-regulated kinases ERK1/2/5, c-Jun N-terminal kinases JNK1/2/3, p38 mitogen-activated protein kinases p38α/β/γ/δ), of which only p28MAPK inhibitor SB203580 (D) significantly reduced the Yoda1-induced increase in Il6 mRNA levels back to basal levels.

(No effect of ERK and JNK inhibition)





(p38MAPK inhibition effect)

P38 MAPK activity is important for Yoda1-induced Il6 gene expression in cardiac fibroblasts.

Additional examples and links*

*(*not illustrated here)*

AKT was found to be activated in Osteosarcoma cells using the PamChip assay and confirmed using *in vitro* cell culture inhibition with AKT inhibitors.
Kuijjer ML *et al.*, (2014) BMC Med Genomics. 21;7:4

In a subset of 14 thyroid tumours, BRAFV600E could be classified from WT with *ex vivo* kinase inhibition profiling using spike-in of Dabrafenib
Hilhorst R *et al.*, (2015) AACR Poster 4322

New EPHB4 mutations were identified in NSCLC that lead to increased cellular proliferation and motility. Altered kinome signature (PamChip assay) showing increased phosphorylation of several targets in EPHB4 mutants (v WT) and re-expressed in Knockdown cells, was validated by immunoblotting.
Ferguson BD et al., (2015) Sci Rep. (Nature) 5:10641.

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