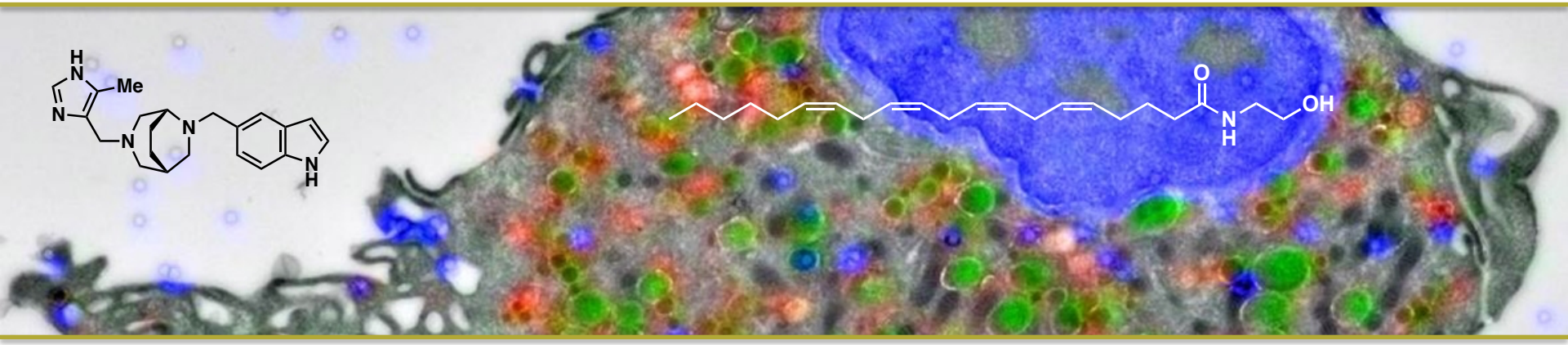
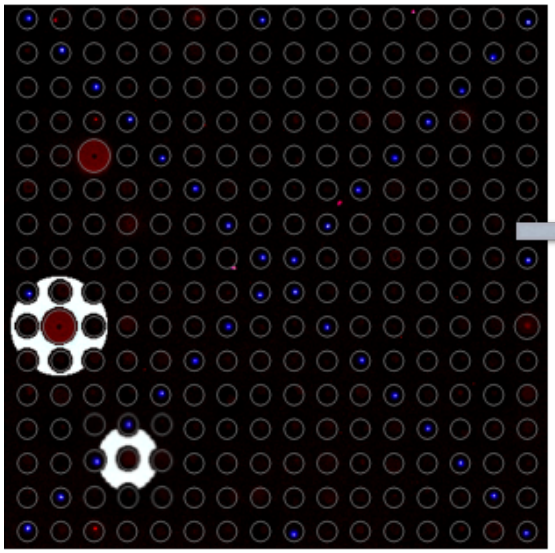


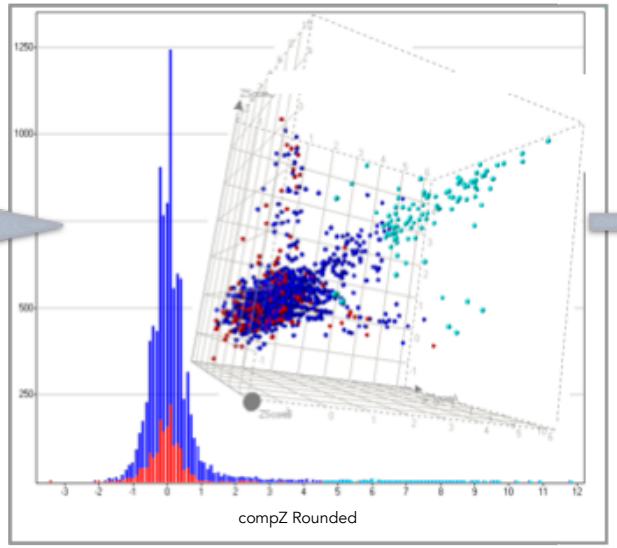
L5 – A Probe Discovery Vignette

February 25, 2020

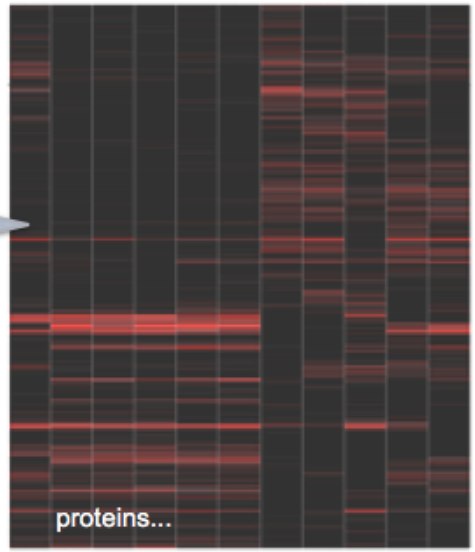
From hits to probes –> validation



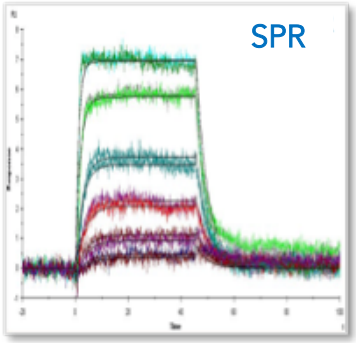
fluorescent features reveal putative
TDP43-ligand interactions

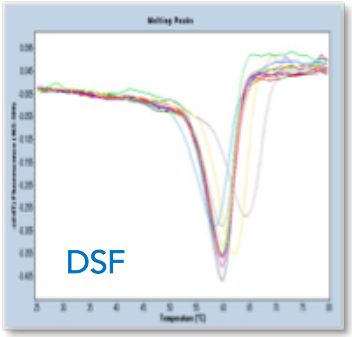


compute composite Z-scores, 'hit' calls



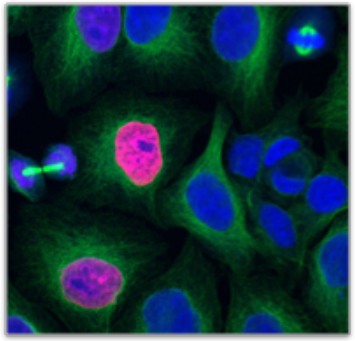
specificity analysis across proteins

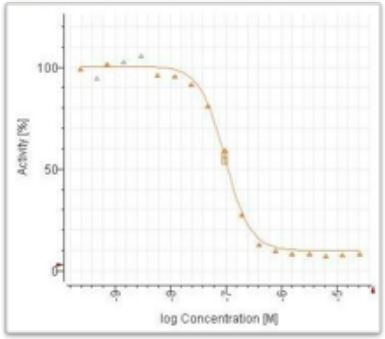




secondary, quantitative binding assays

+

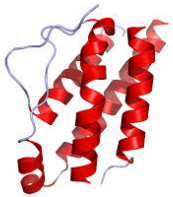




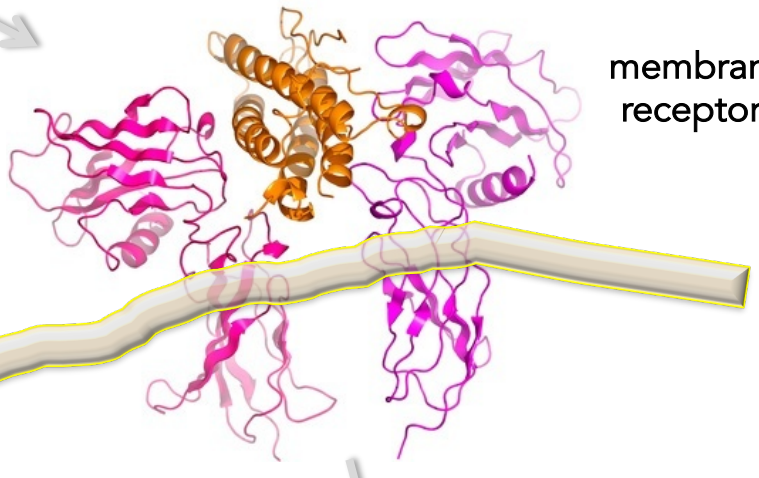
functional assays (e.g. cellular, biochemical)

the '20.320 version' of biology

extracellular factors



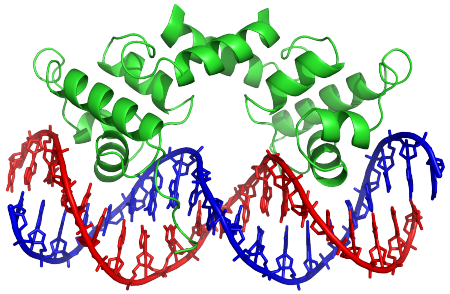
membrane receptors



intracellular signaling proteins

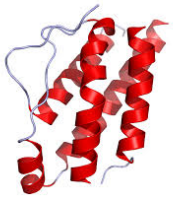
cellular response

transcriptional regulators



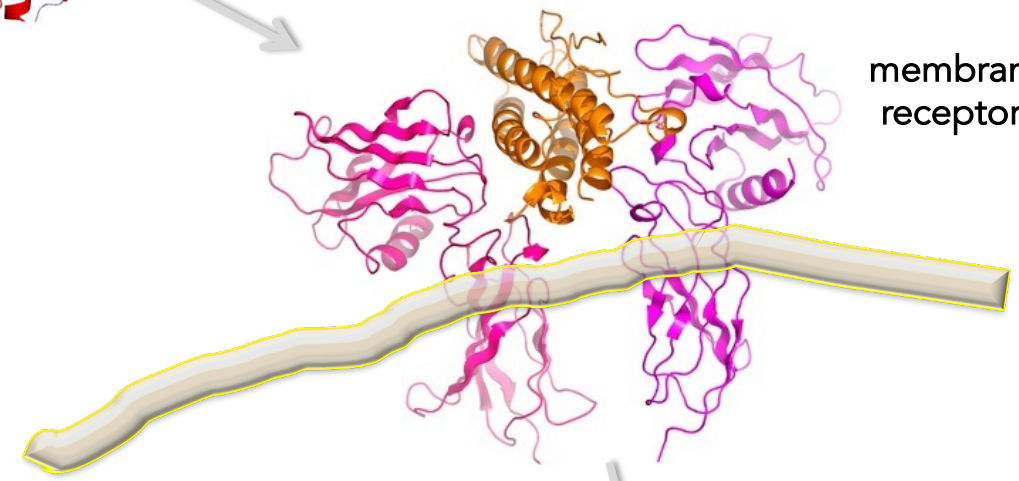
experimental methods
mathematical models
manipulate targets/systems

extracellular factors

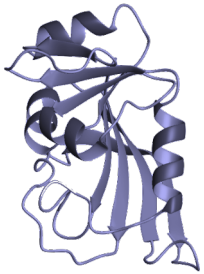


ion channels
receptor kinases
(oncology, neurology, mood disorders, inflammation)

membrane receptors

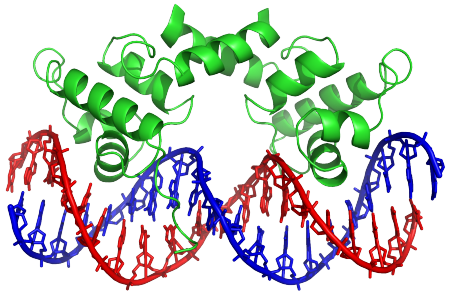


cellular response



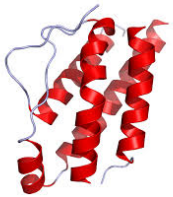
intracellular signaling proteins

kinases
metabolic enzymes
(many diseases)

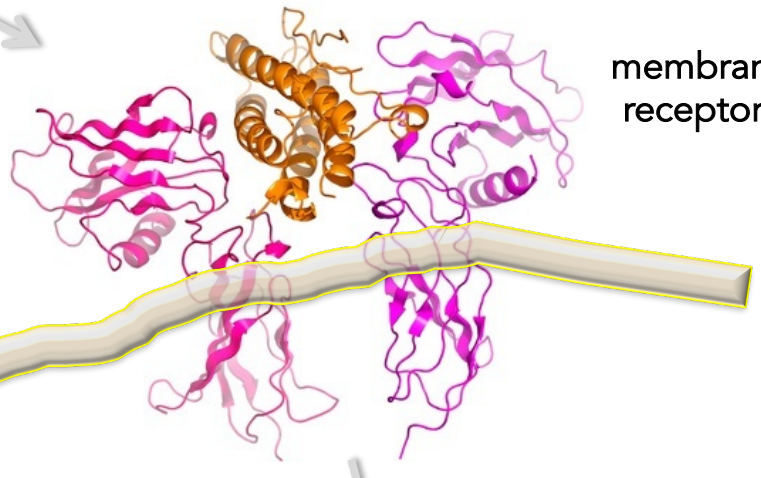


transcriptional regulators

extracellular factors



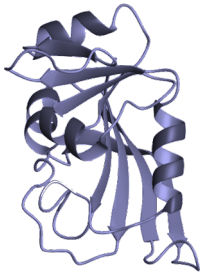
clotting factors
(DVT, hemophilia)
one of the best drugged classes!



membrane receptors

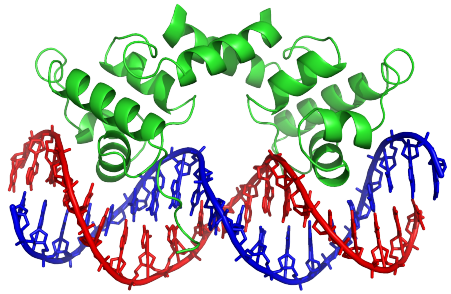
ion channels
receptor kinases
(oncology, neurology, mood disorders, inflammation)

cellular response



intracellular signaling proteins

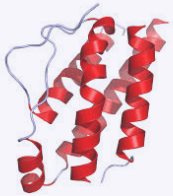
kinases
metabolic enzymes
(many diseases)



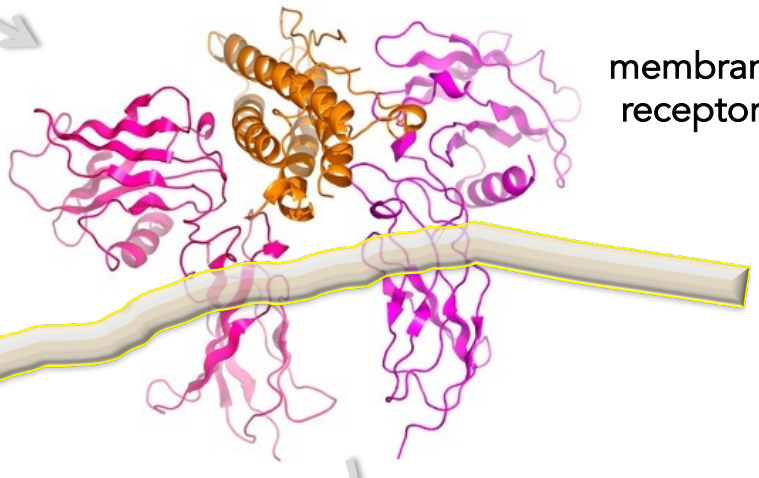
transcriptional regulators

nuclear hormone receptors
(many diseases)

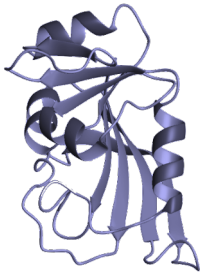
extracellular factors



cytokines – IL-4
chemokines – MCP-1
growth factors - SHH



membrane receptors

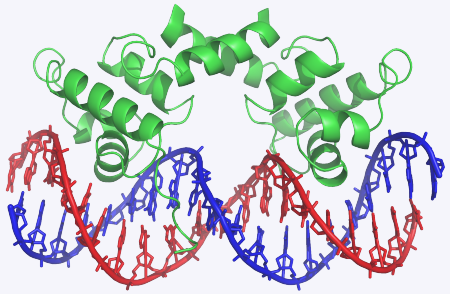


intracellular signaling proteins

cellular response

epigenetic enzymes - HDACs
latent cytoplasmic TFs – NF-kappaB
nuclear hormone receptors – FOXA1
classic transcription factors – MYC, MAX

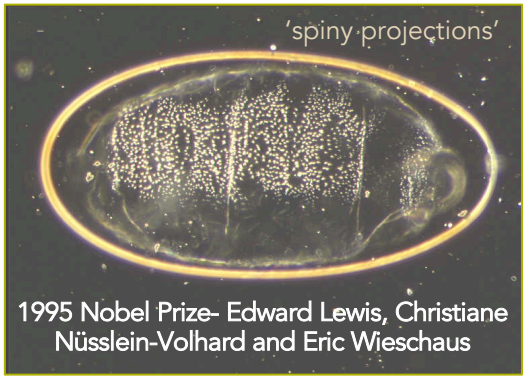
transcriptional regulators



Sonic hedgehog protein

important role in development including limb and brain development

1978- Embryogenesis
Mutational Genetic Screen
mutant hedgehog drosophila larva



1995 Nobel Prize- Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus

3 Hh genes

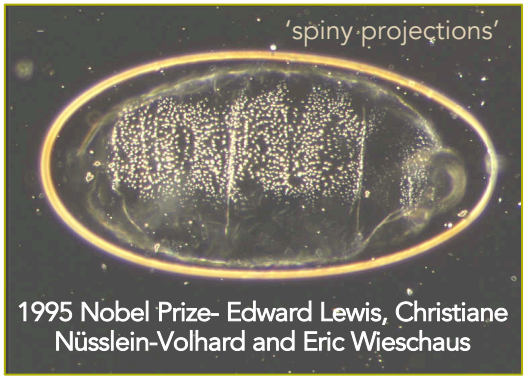
Desert and Indian
(Dhh and Ihh)

Sonic
(Shh)

Sonic hedgehog protein

important role in development including limb and brain development

1978- Embryogenesis
Mutational Genetic Screen
mutant hedgehog drosophila larva



mutations in Shh are linked with
Holoproscencephaly (HPE)

'cyclopia'

M. Muenke, *Seminars in Developmental Biology* Vol. 5, 293-301, 1994

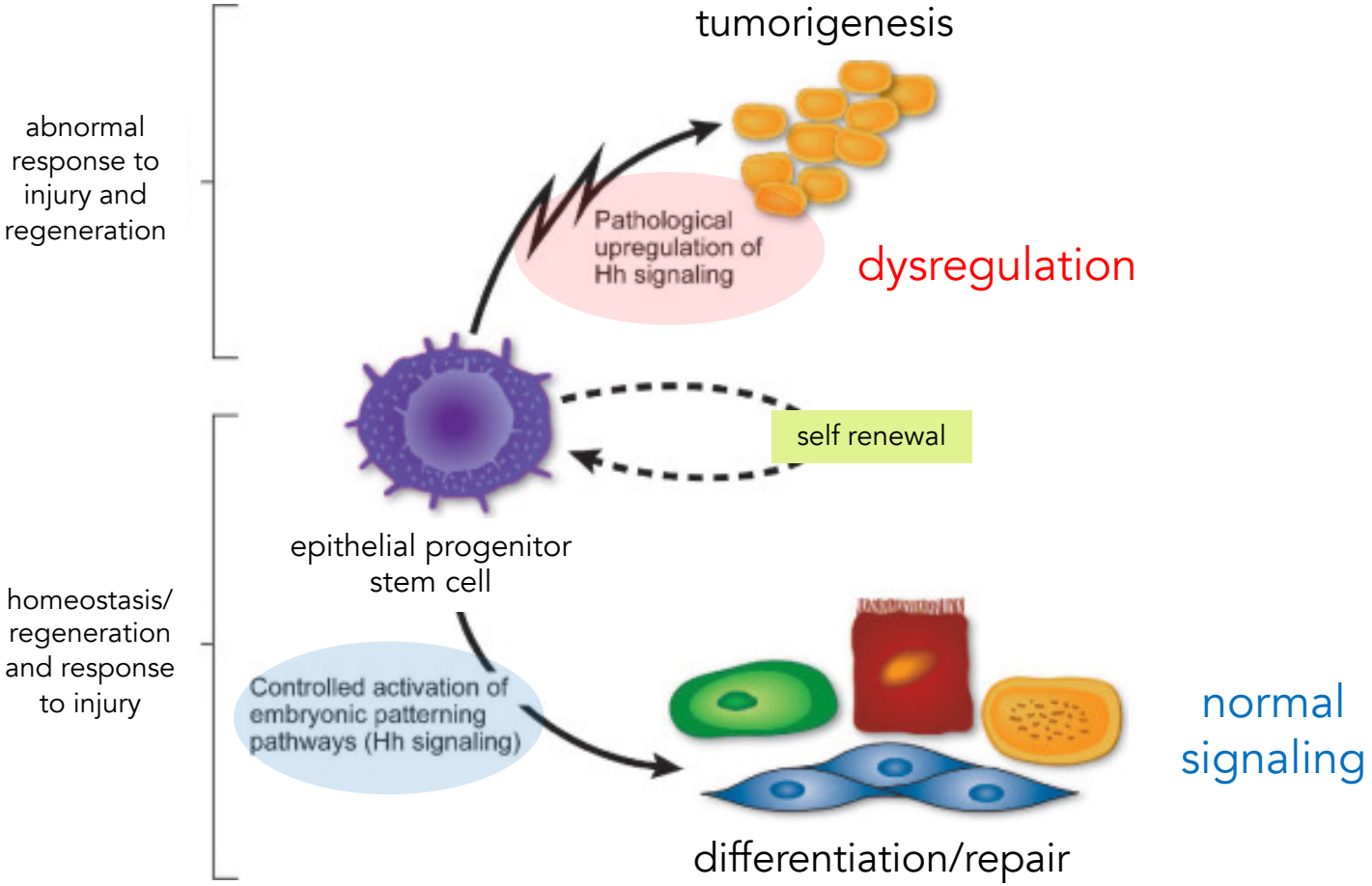
3 Hh genes

Desert and Indian
(Dhh and Ihh)

Sonic
(Shh)

Hedgehog signaling goes beyond embryogenesis

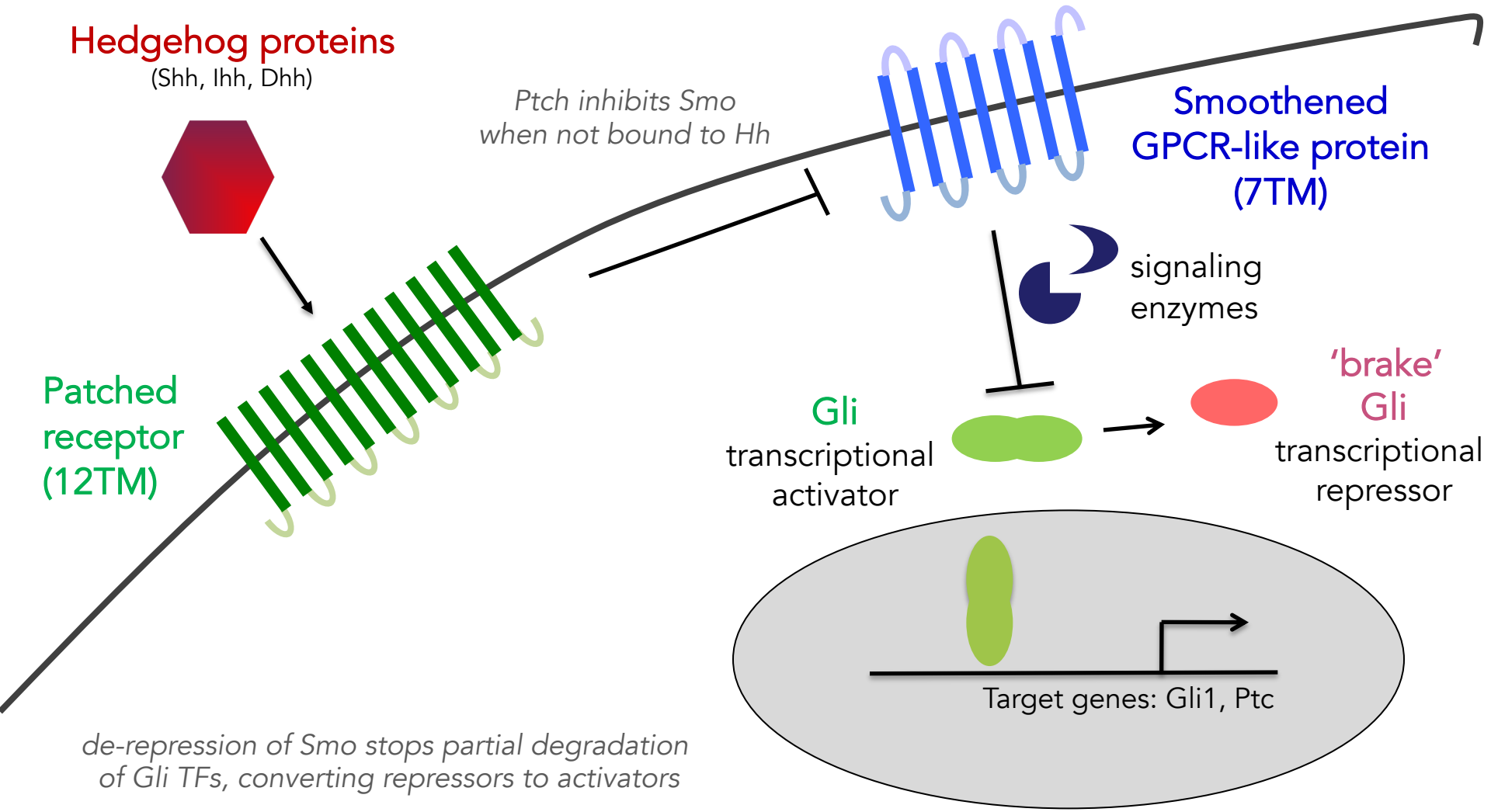
development, differentiation, and disease



signaling pathways responsible for embryogenesis play a critical role in the maintenance of stem cells in adult life and cellular responses to injury

Hedgehog proteins 'de-repress' Smoothened

Hh-Ptch binding interaction activates Gli-driven transcription

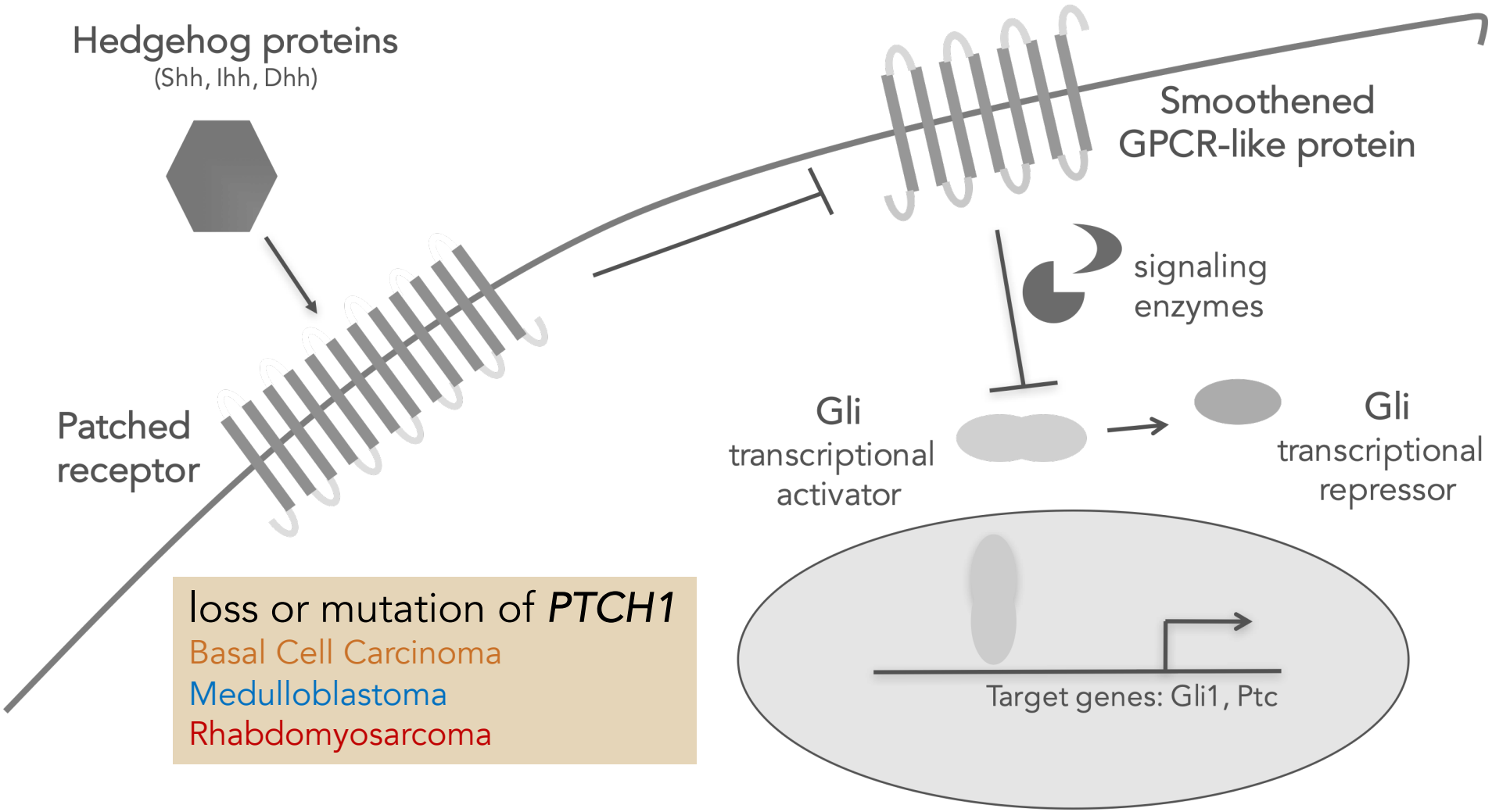


overexpression of *SHH*

Pancreatic Cancer (70%) Prostate Cancer
Gastric Cancer Lung Cancer
Medulloblastoma Ovarian Cancer

activating mutations in *SMO*

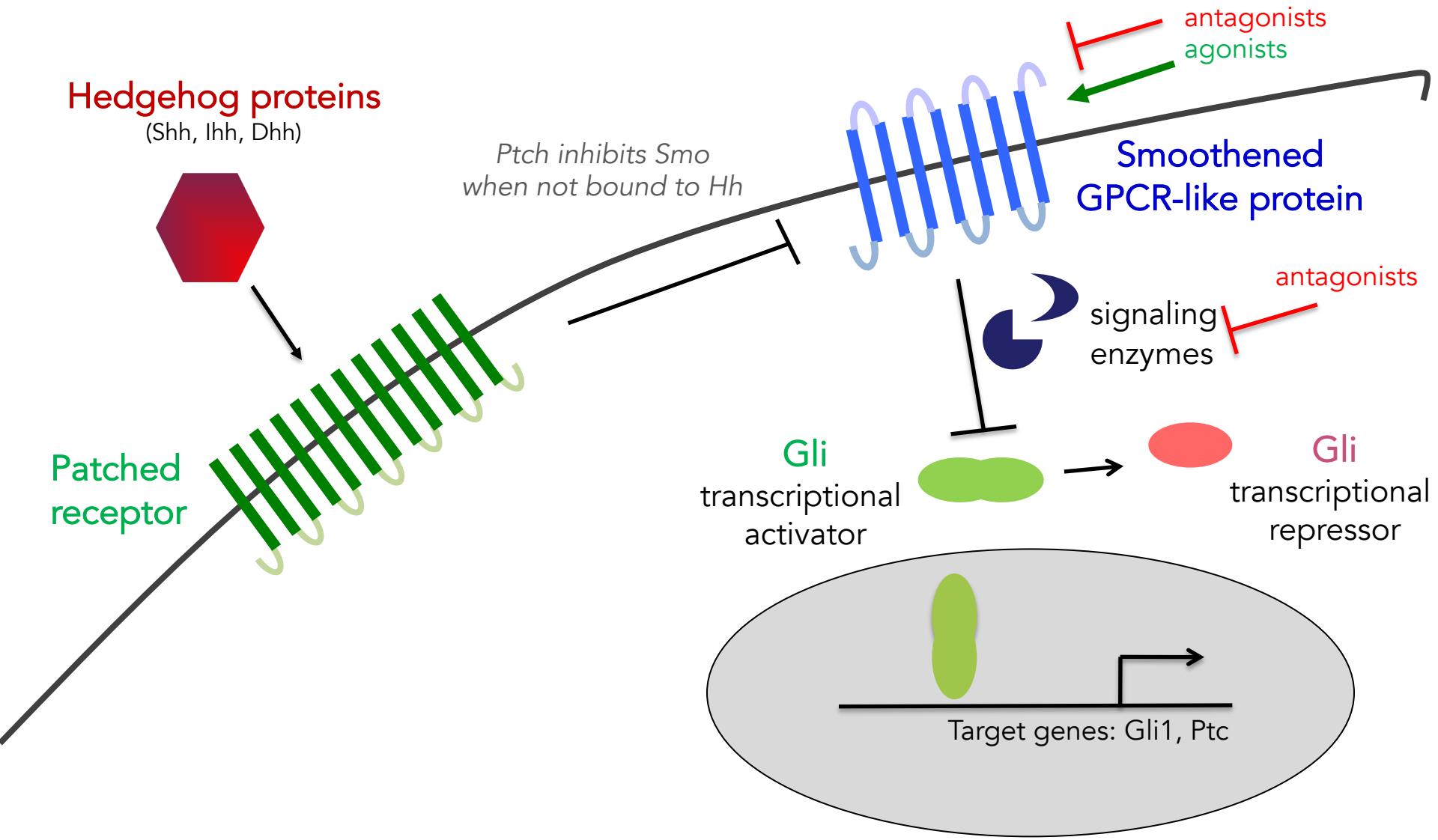
Basal Cell Carcinoma
Ovarian Cancer



loss or mutation of *PTCH1*

Basal Cell Carcinoma
Medulloblastoma
Rhabdomyosarcoma

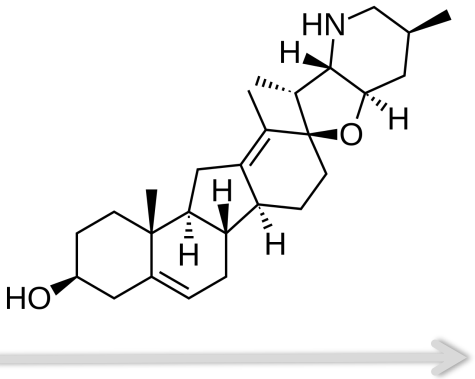
Drugs targeting Hedgehog pathway



Cyclopamine

Smo antagonist and Hh pathway inhibitor

Veratrum californicum
wild corn lily



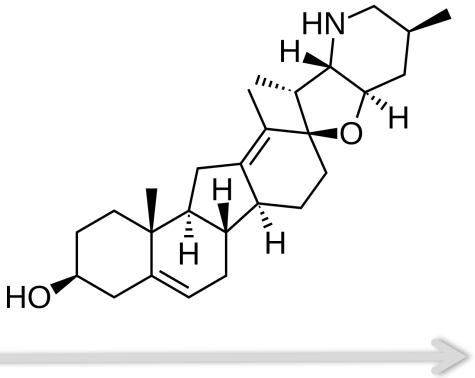
11-yr investigation
By US Dept of Agriculture

cyclopic lamb born of
a sheep that ate corn lily
(Idaho farm, 1957)

Cyclopamine

Smo antagonist and Hh pathway inhibitor

Veratrum californicum
wild corn lily



11-yr investigation
By US Dept of Agriculture

cyclopic lamb born of
a sheep that ate corn lily
(Idaho farm, 1957)

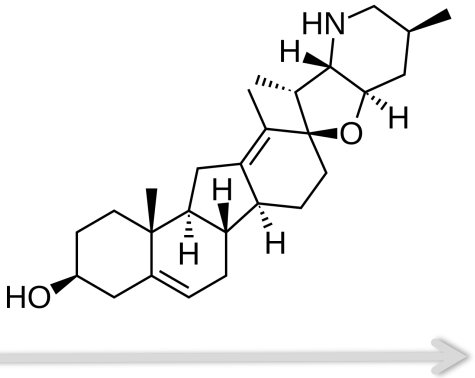
Beachy Lab (Stanford): Cyclopamine inhibits Hh signaling by influencing the balance of active and inactive Smoothened protein

How did they arrive at this conclusion?

Cyclopamine

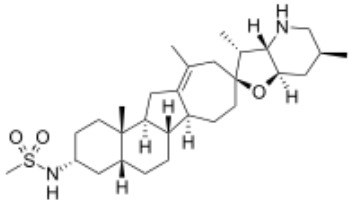
lead for development of anti-cancer agents

Veratrum californicum
wild corn lily

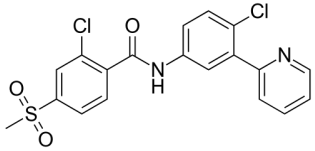


cyclopic lamb born of
a sheep that ate corn lily
(Idaho farm, 1957)

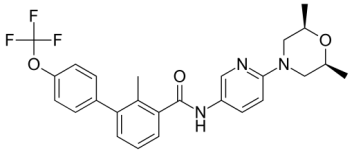
Adult cancers - basal cell carcinoma, medulloblastoma, prostate, breast, pancreas



saridegib

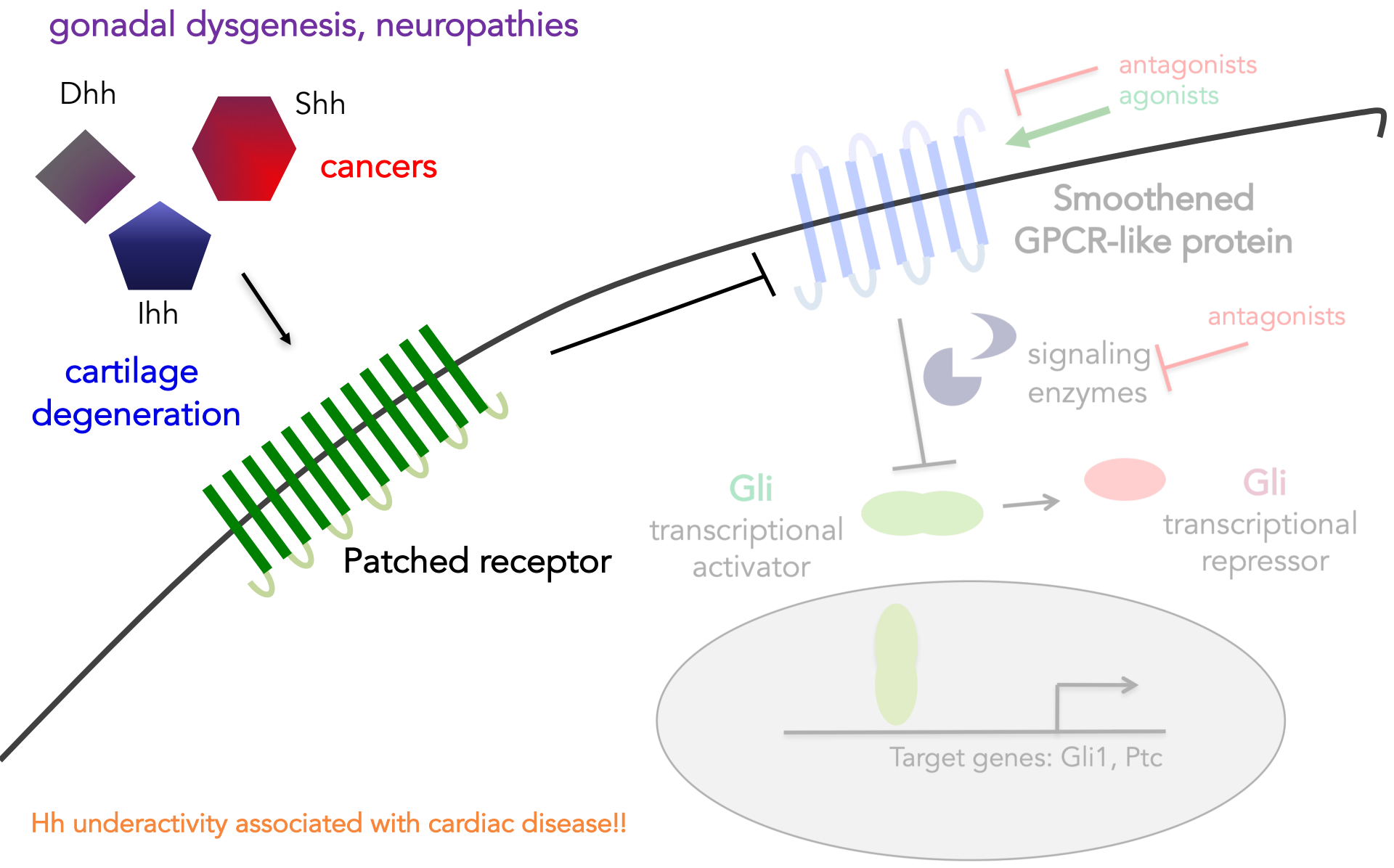


vismodegib



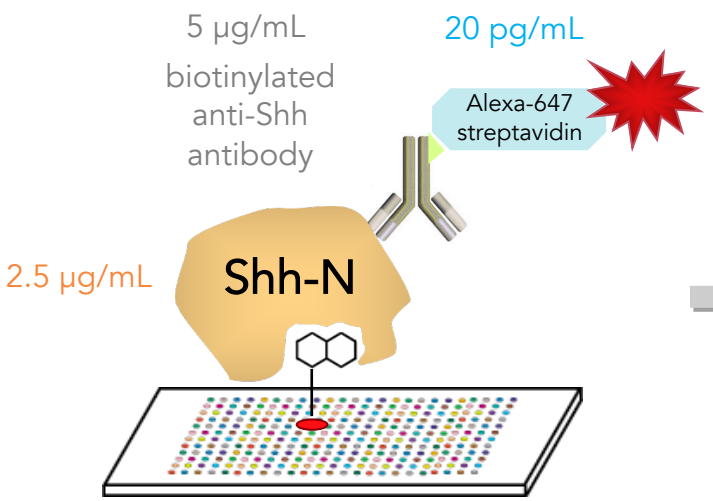
sonidegib

Selective targeting of Hh signaling upstream of Smo



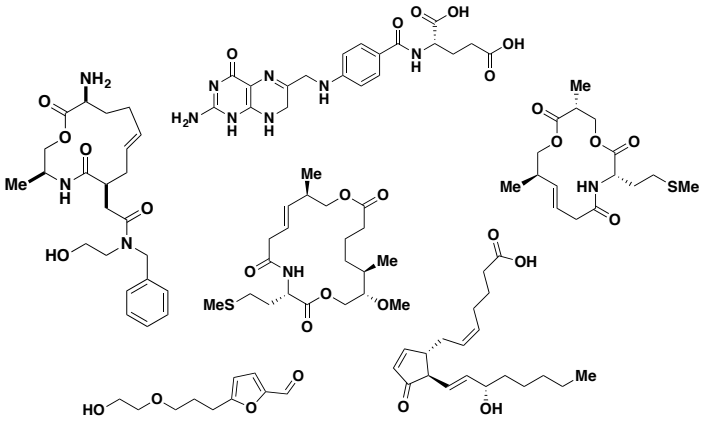
Hh underactivity associated with cardiac disease!!

SMM assay: 20 kDa Shh N-terminal fragment



~10,000 printed compounds
(small molecule microarray)

19 SMM hits



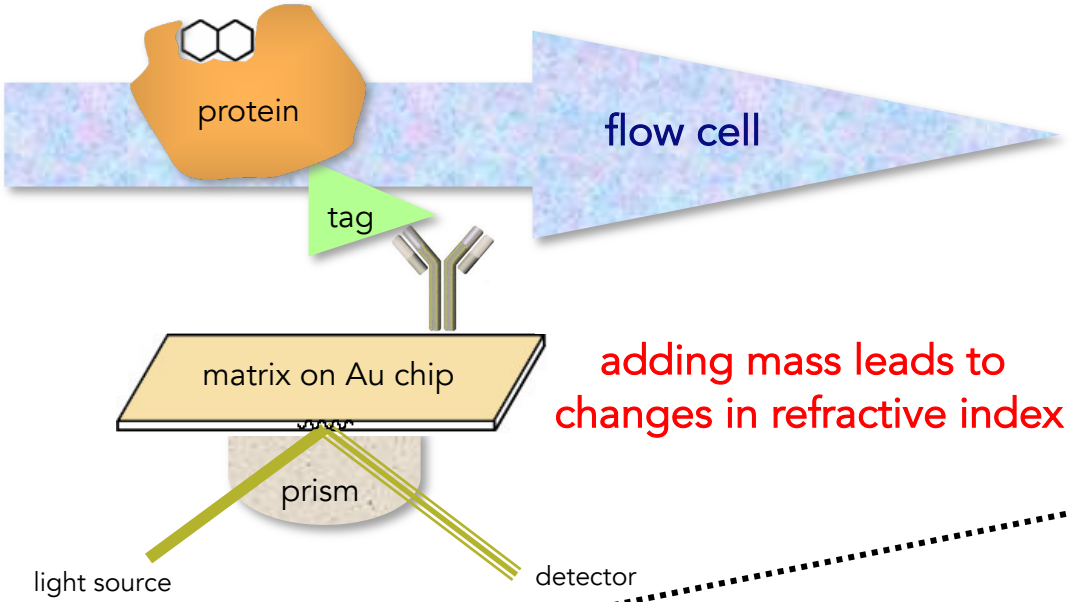
Angela, Broad Fellow

Lee Peng, MGH

Ben Stanton, Harvard

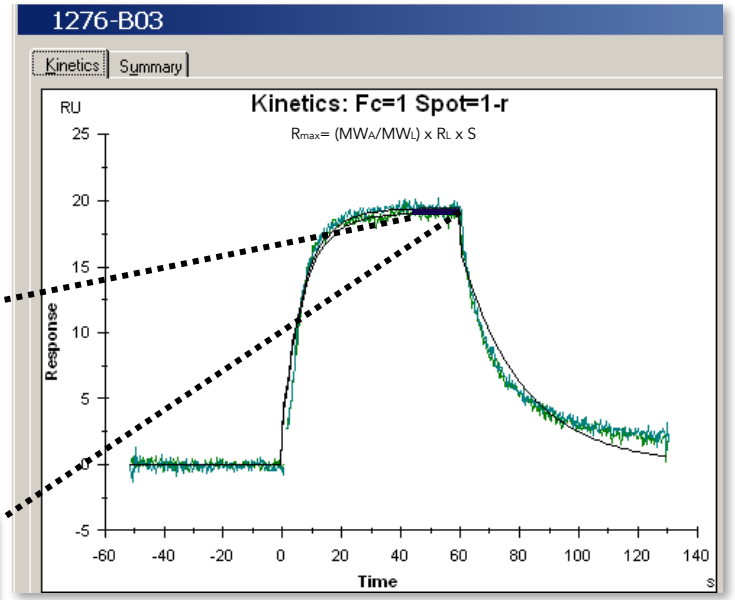
Validating assay positives in secondary binding assays

'mass sensing' by Surface Plasmon Resonance (SPR)

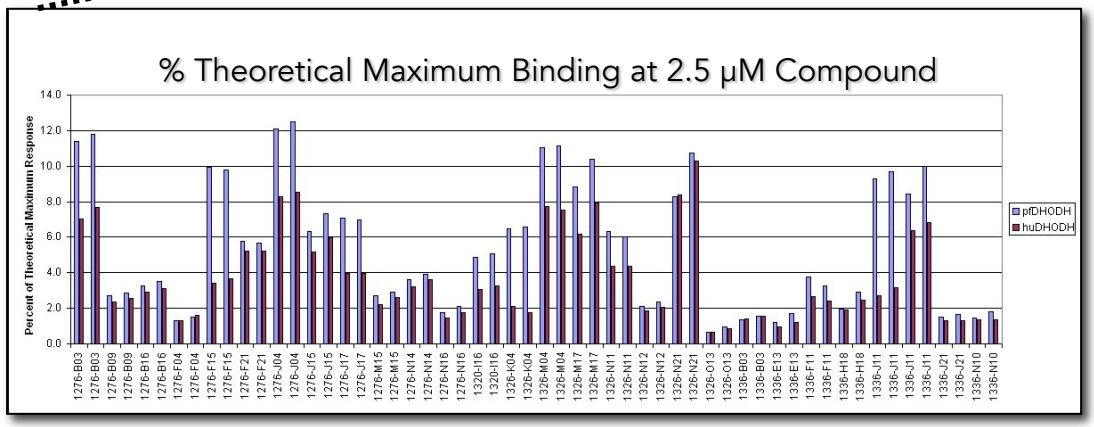


adding mass leads to changes in refractive index

kinetic assay – real time measurement

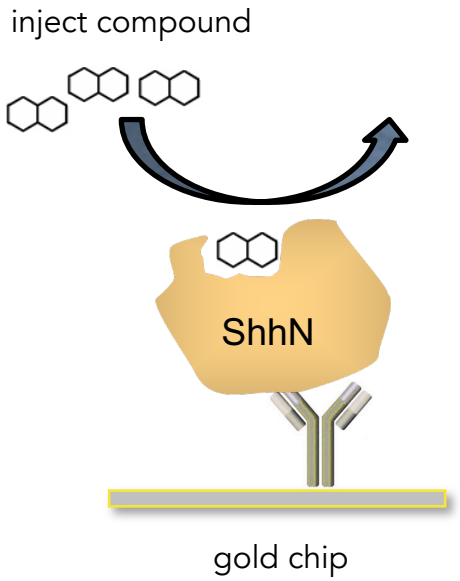


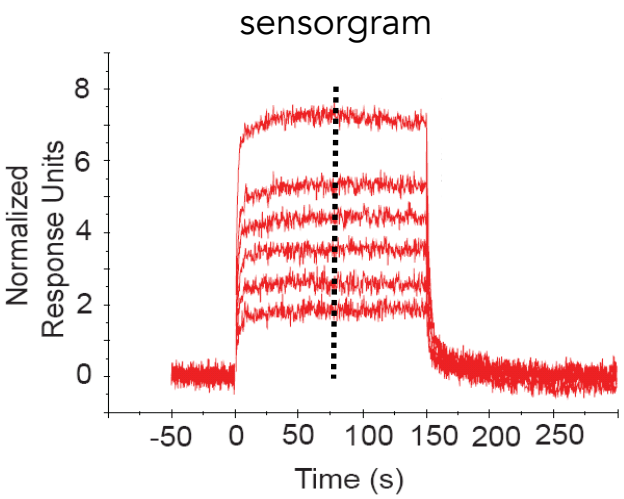
76% of SMM interactions retest by SPR

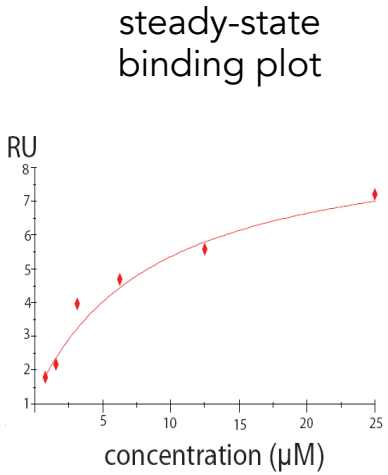


For Video on SPR, go to:
https://www.youtube.com/watch?v=o8d46ueAwXI

SPR experiments for Shh SMM hits







K_D = 9 μM

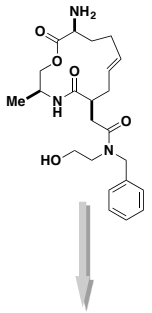
- reverses orientation from primary assay
- measures binding between immobilized protein and compounds injected in solution
- kinetic measurements
- ranking assays (k_{on} vs. $k_{off,}$ % Ru_{max})
- compound affinity characterization

Measuring *GLI*-dependent transcriptional activity

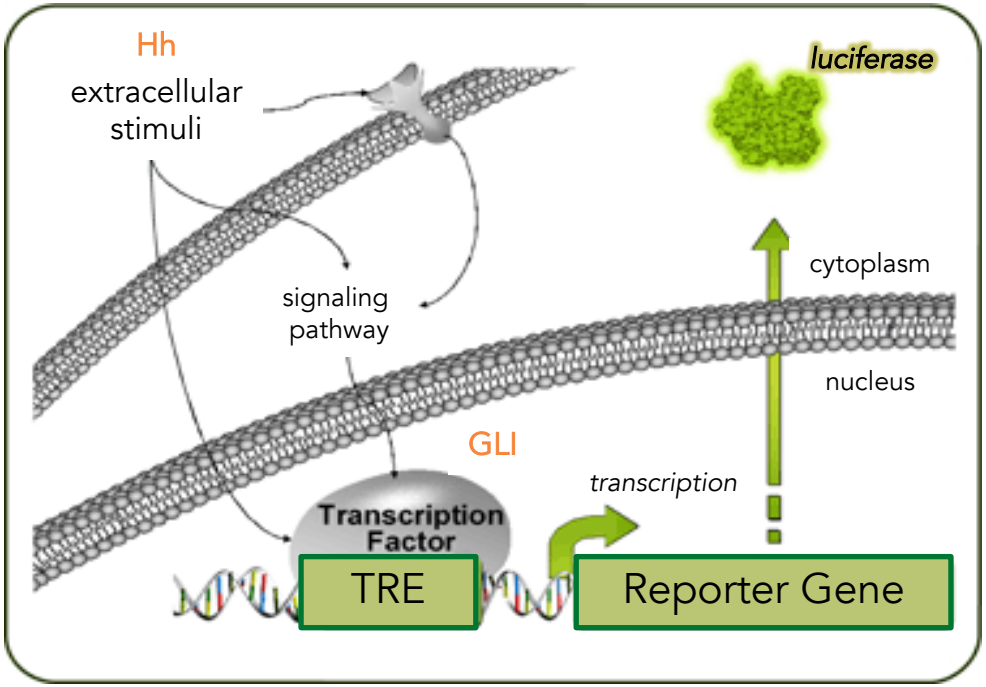
quantitative assay for hedgehog signaling

1

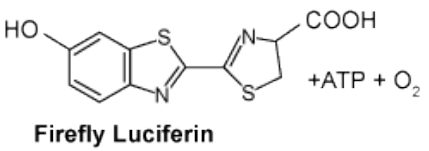
K_D = 9 μM



NIH/3T3 cell line transfected with GLI-responsive reporter assay vector

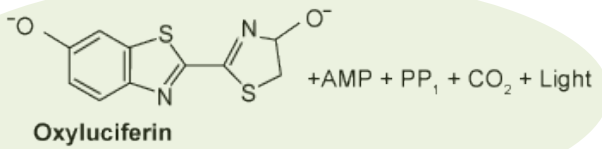


luciferase reaction

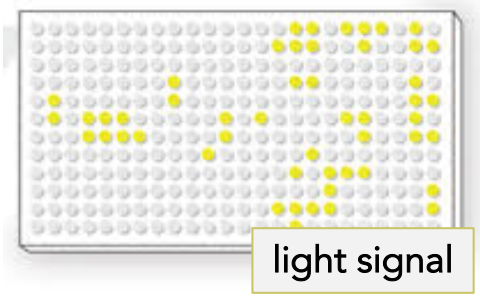


Firefly Luciferin

Firefly Luciferase **Mg^{2+}**



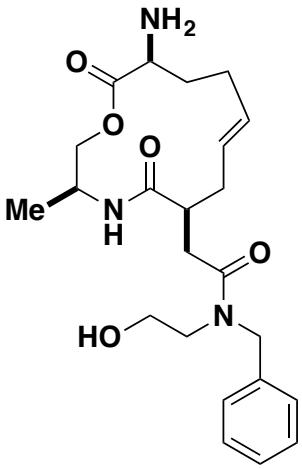
Oxyluciferin



light signal

Measuring *GLI*-dependent transcriptional activity

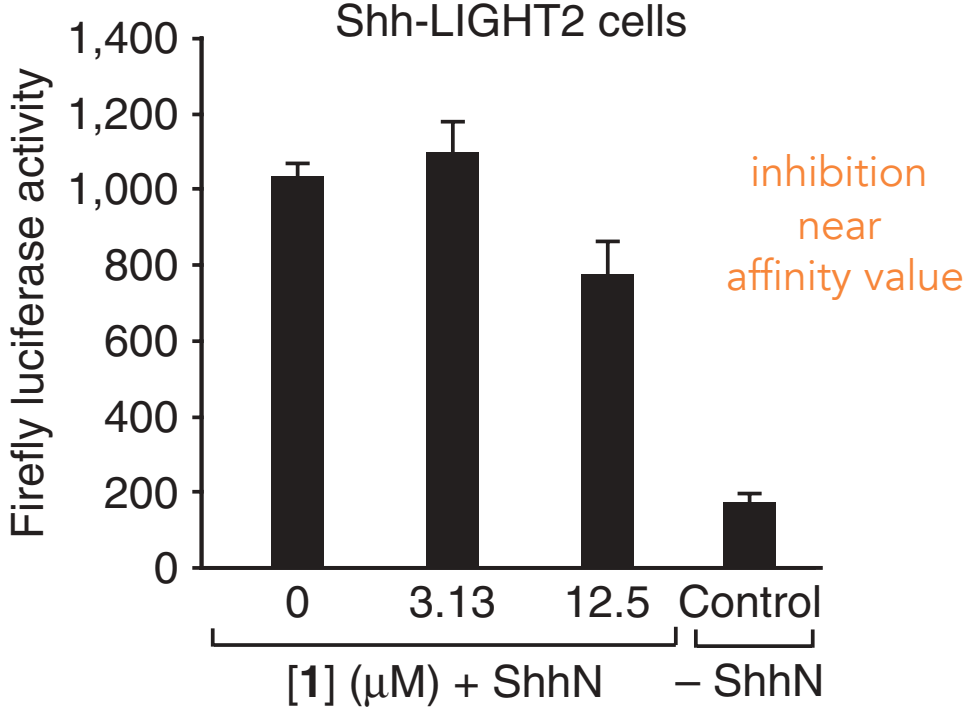
SMM hit modulates transcriptional output in preliminary experiment



1

K_D = 9 μM

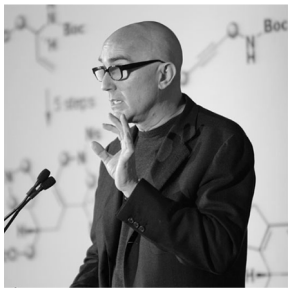
reporter assay



inhibition near affinity value

each value represents 5 technical replicates
error bars denote standard deviation

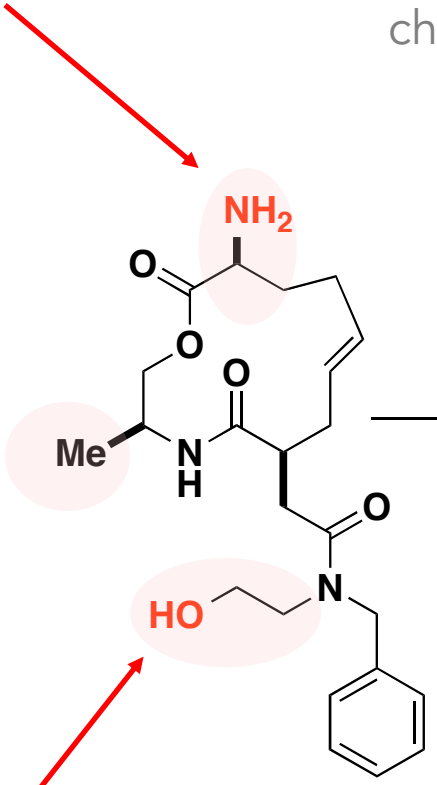
Stuart Schreiber, Harvard



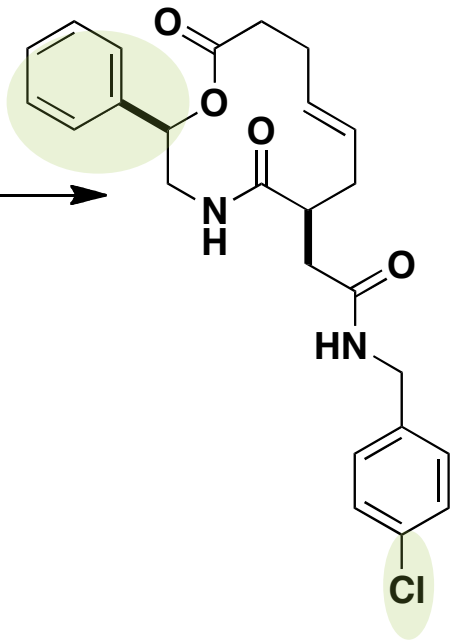
Hit to probe

chemical editing

site of attachment to SMM



18 variants



site of attachment to SMM

K_D = 9 μM

K_D = 3 μM

improved binding affinity

Remove – ethanolamine, methyl, amine and carbon
Add – phenyl, chloro groups

Doctor Ivo "Eggman" Robotnik

Robotnikinin

Shh binder and antagonist

nature chemical biology

A small molecule that binds Hedgehog and blocks its signaling in human cells

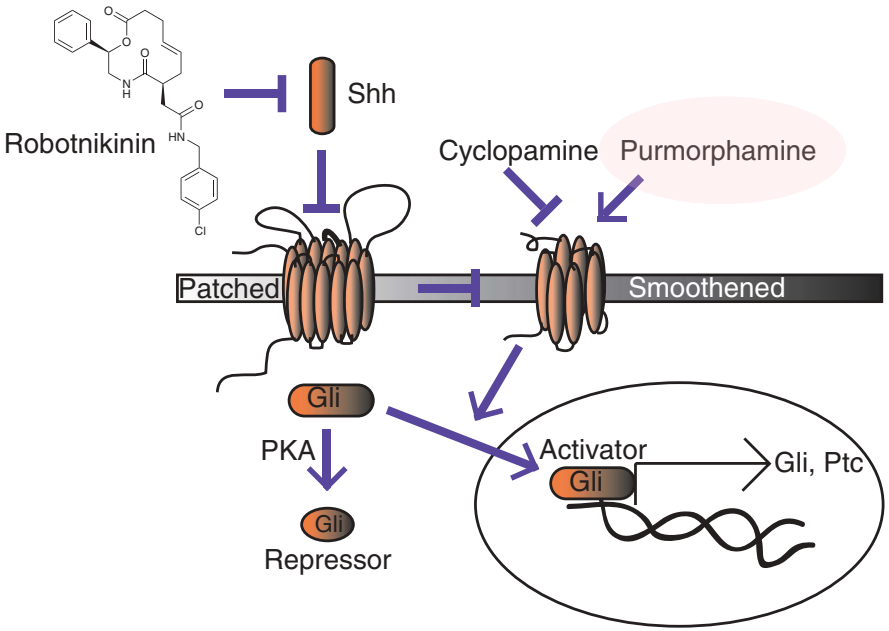
Benjamin Z Stanton[1,2,7], Lee F Peng[1–3,7], Nicole Maloof[1], Kazuo Nakai[2], Xiang Wang[1], Jay L Duffner[1], Kennedy M Taveras[1], Joel M Hyman[4], Sam W Lee[5], Angela N Koehler[1], James K Chen[4], Julia L Fox[6], Anna Mandinova[5] & Stuart L Schreiber[1,2]

Small-molecule inhibition of extracellular proteins that activate membrane receptors has proven to be extremely challenging. Diversity-oriented synthesis and small-molecule microarrays enabled the discovery of robotnikinin, a small molecule that binds the extracellular Sonic hedgehog (Shh) protein and blocks Shh signaling in cell lines, human primary keratinocytes and a synthetic model of human skin. Shh pathway activity is rescued by small-molecule agonists of Smoothened, which functions immediately downstream of the Shh receptor Patched.

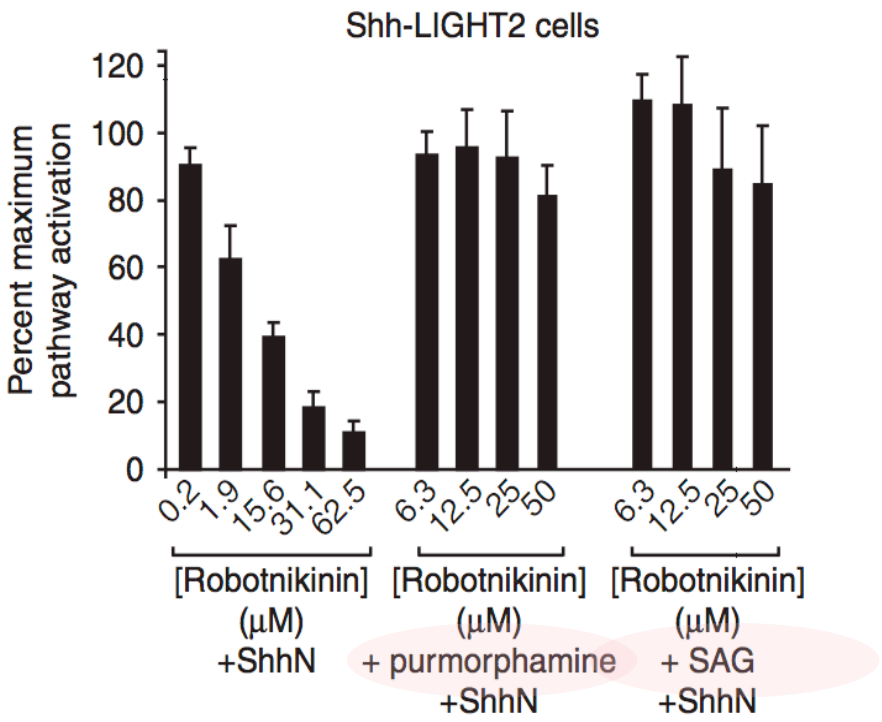
SEGA®

Sonic the Hedgehog

Gli inhibition by Robotnikinin is rescued by a Smoothened agonist

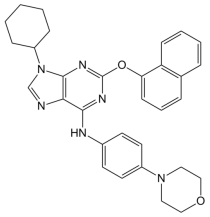


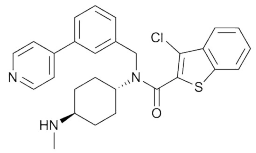
reporter assay



rescue experiments are common in systems biology

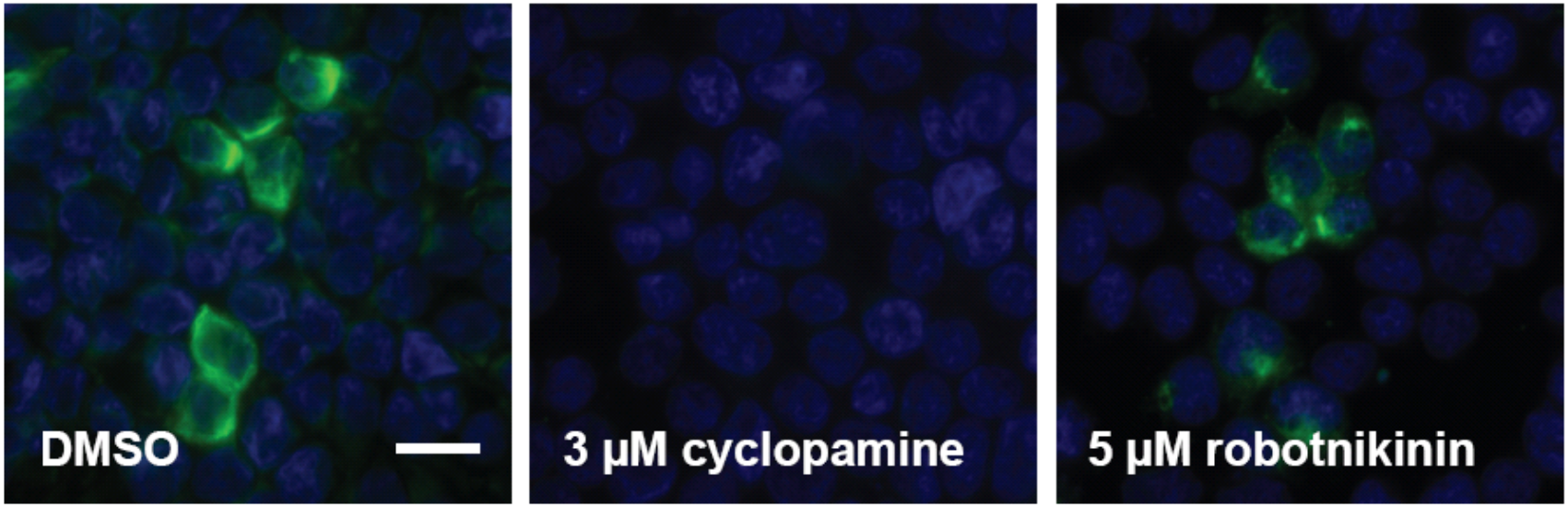
validate mechanistic hypotheses





Ligand competition assays to assess specificity

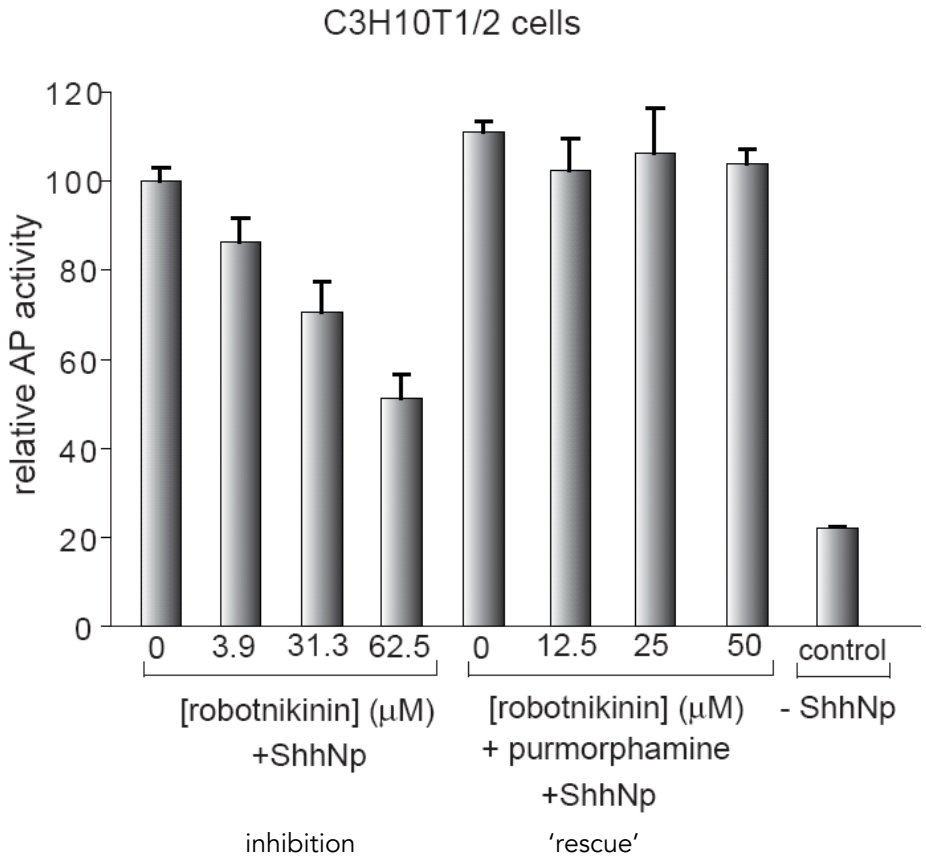
BODIPY-cyclopamine binds to Smoothened at cell surface



Smoothened-overexpressing human embryonic kidney cells

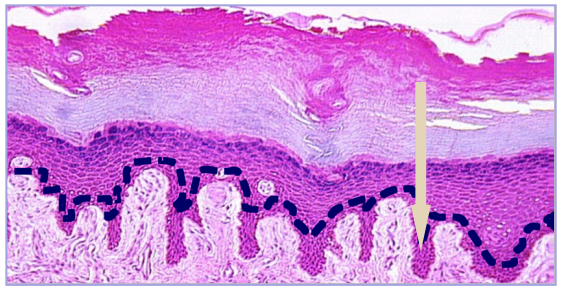
Conclusion: Robotnikinin does not compete with a labeled Smo ligand

Inhibition of stem cell differentiation



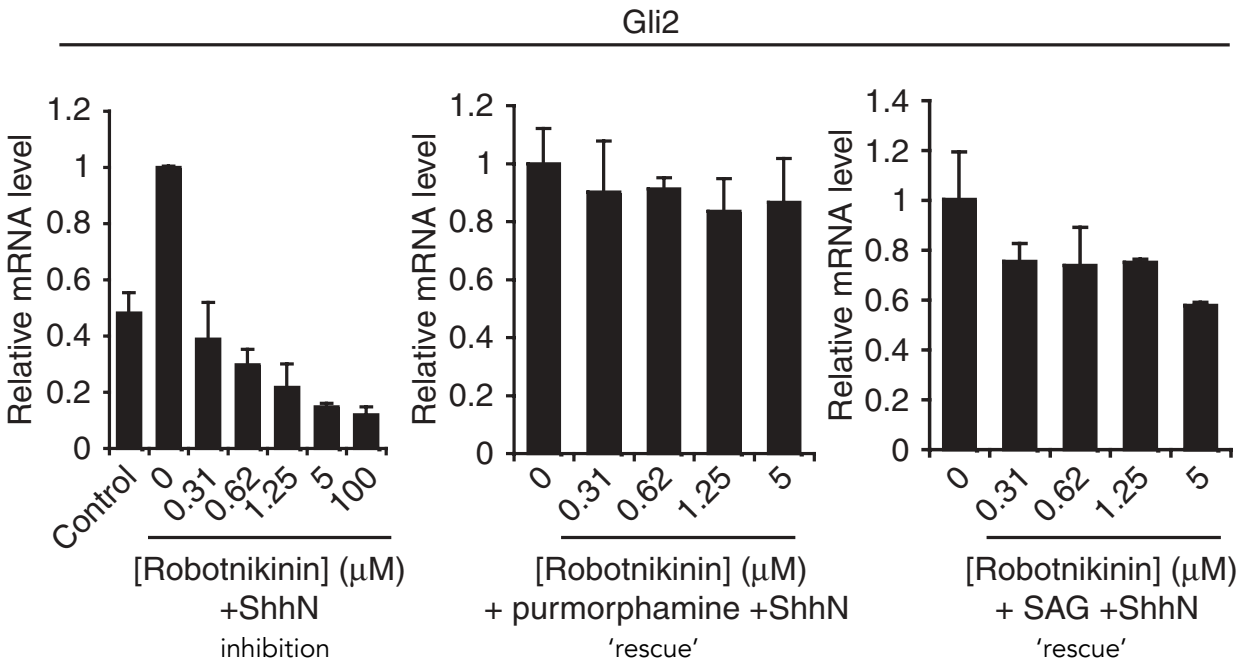
mouse mesenchymal stem cells differentiate into osteoblasts and upregulate alkaline phosphatase (AP) when stimulated with N-palmitoylated ShhN

Skin: Robotnikinin lowers levels of *GLI2* mRNA in primary human keratinocyte cells

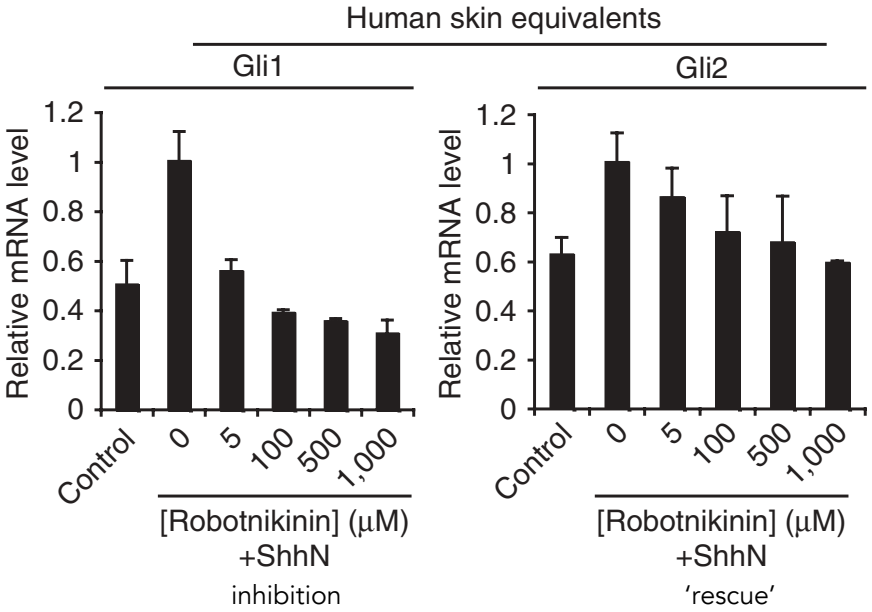


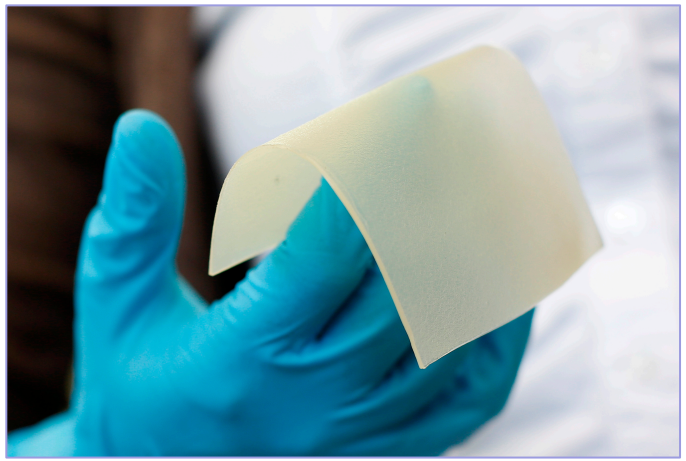
primary human keratinocytes
isolated from the basal cell layer

measure mRNA by quantitative
PCR after 30-hr treatments



Robotnikinin blocks lowers levels of *GLI1 and GLI2* mRNA in synthetic human skin



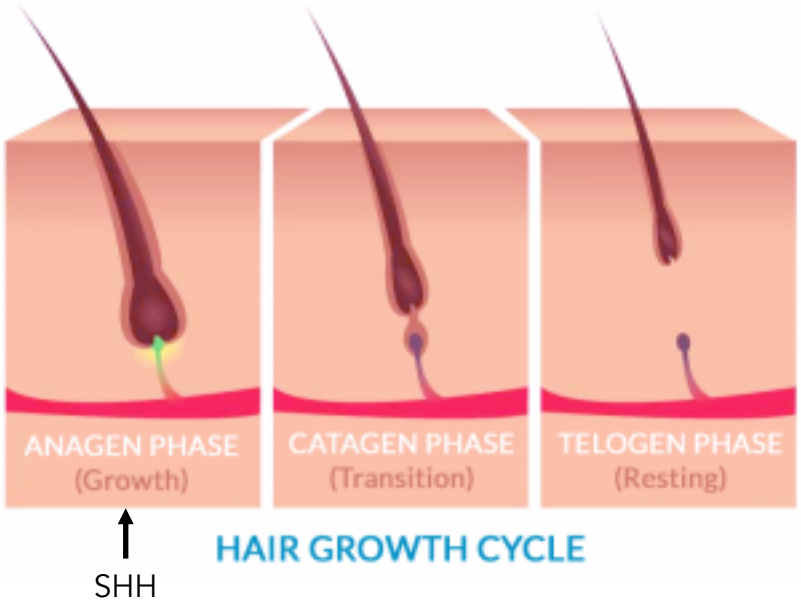


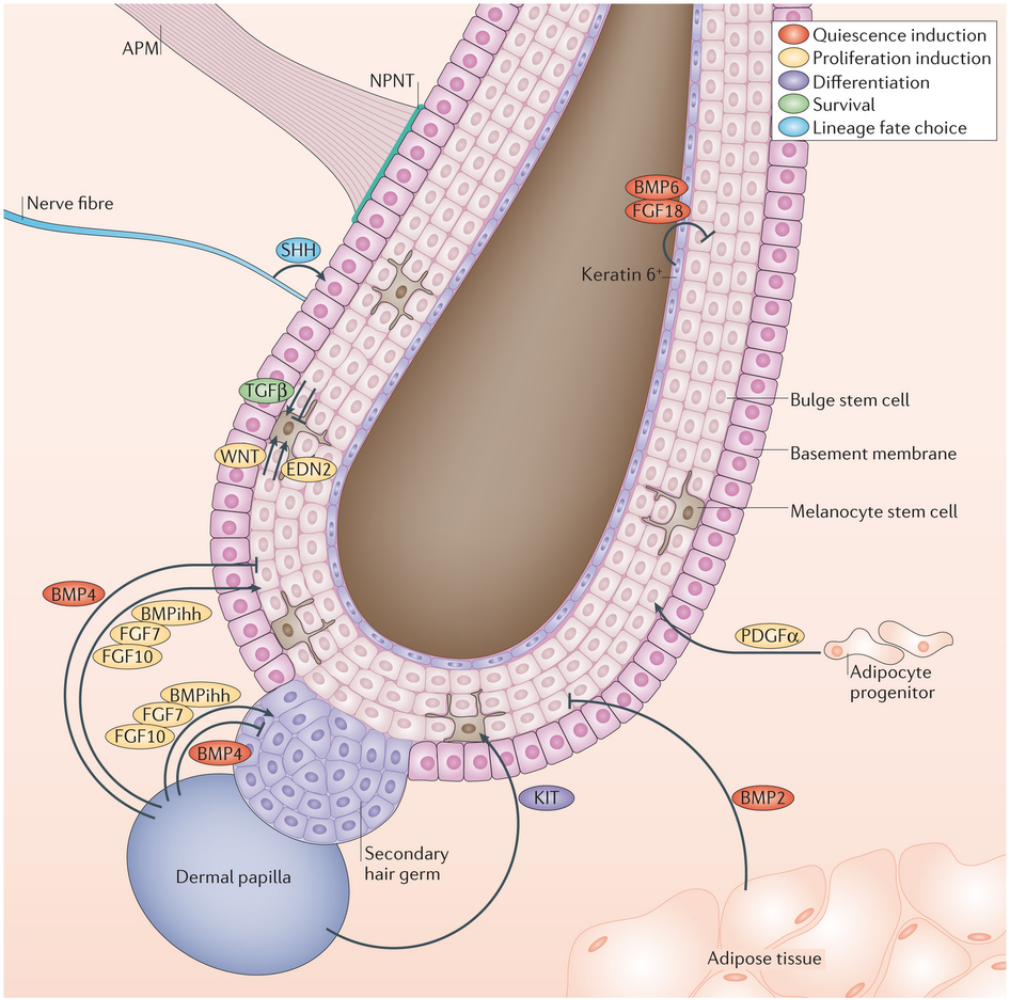
MGH synthetic human skin model:

1. Extract dehydrated collagen matrix from skin grafts
2. Populate matrix with primary keratinocytes
3. Culture to form several dermal layers
4. Incubate with compound, analyze by qPCR and histology

Anna Mandinova, MGH

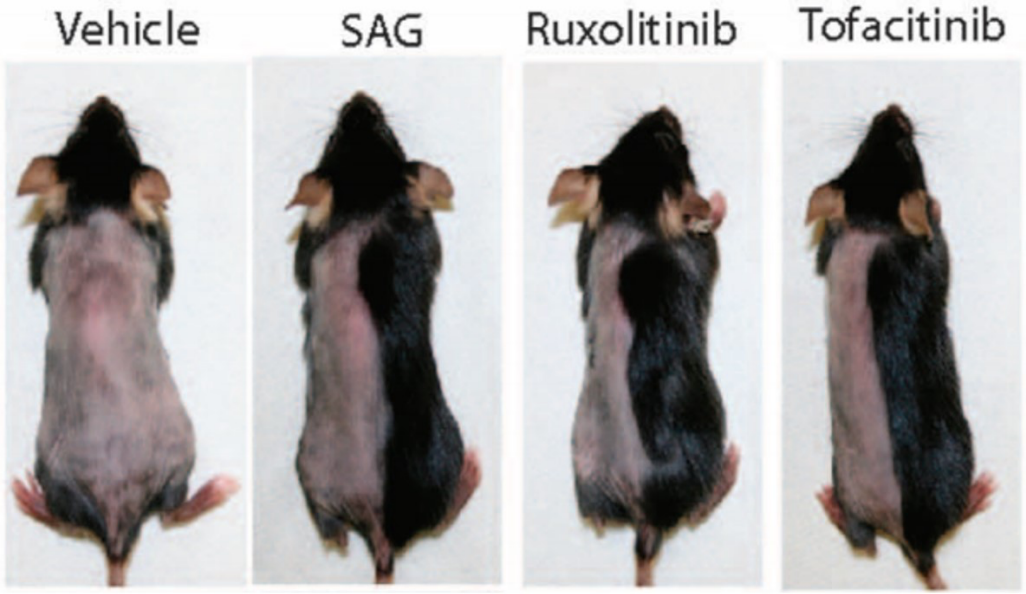
Shh and the hair follicle – a regulator of luscious locks





lower levels of Hh expression or signaling is associated with baldness

Exploring stimulation of Shh pathway as a way to promote hair growth

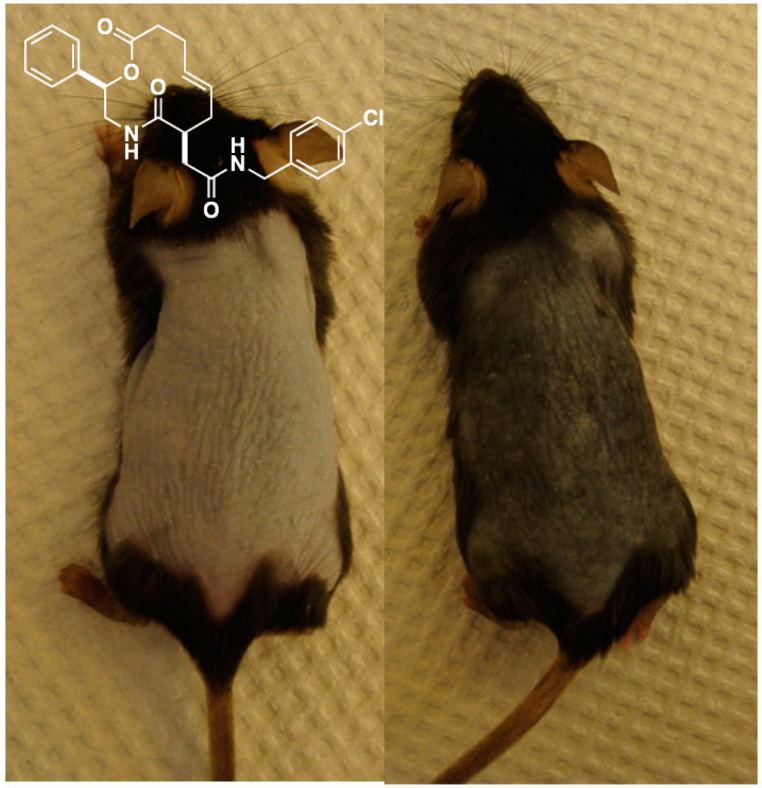


Seven-week-old wild-type mice were shaved and treated daily with either a topical application of vehicle control, sonic hedgehog agonist (SAG), 3% ruxolitinib (JAK1/2 inhibitor), or tofacitinib (JAK3 inhibitor). Skin was harvested at the indicated time points and stained with hematoxylin and eosin (H&E). Images of mice were taken at D21 of treatment. Harel et al. Sci. Adv. 2015

Smoothened agonists used in our rescue experiments

Robotnikinin inhibits hair growth *in vitro*

8 days post depilation



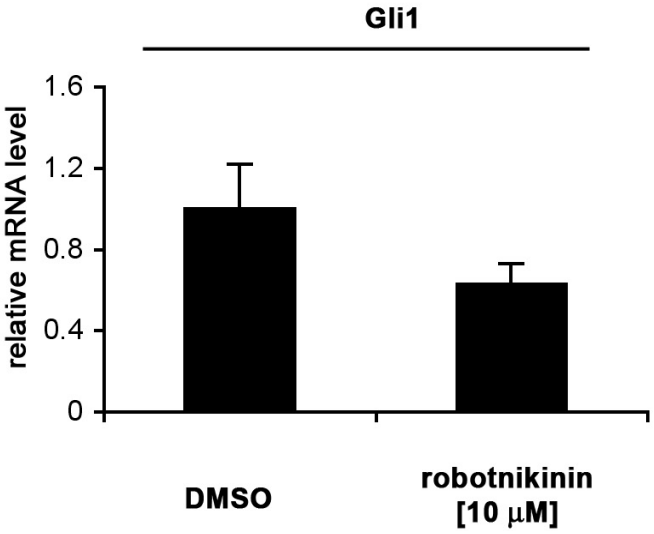
10 uM robotnikinin DMSO

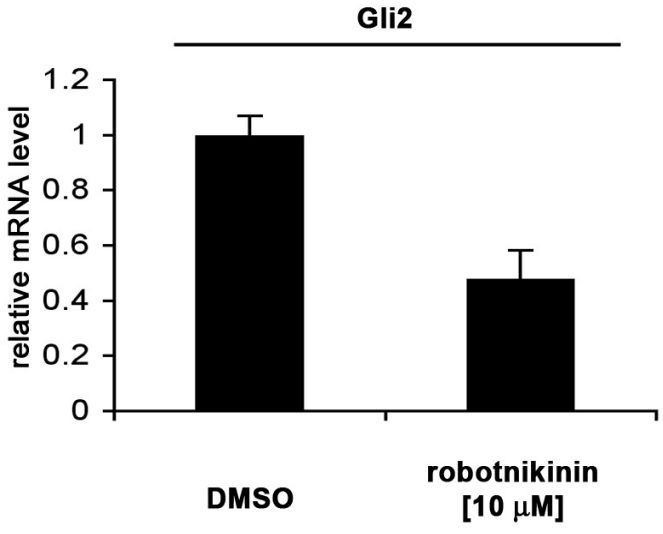
12 days post depilation

10 uM robotnikinin DMSO

Robotnikinin causes hair follicles to fail anagen phase entry

12 days post depilation





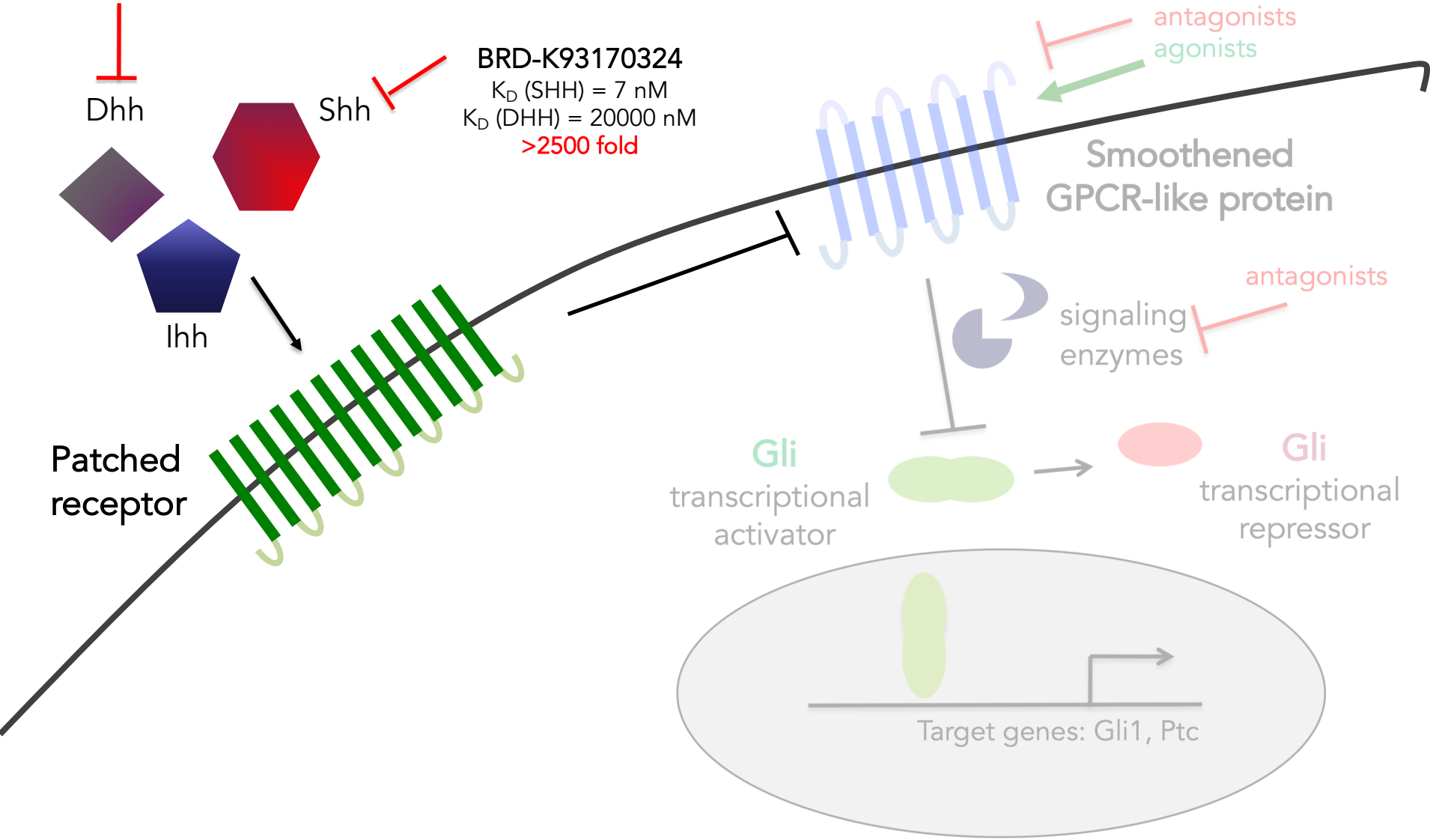
robotnikinin treatment shows no signs of inflammation or failed skin differentiation

Improving Hh homolog selectivity

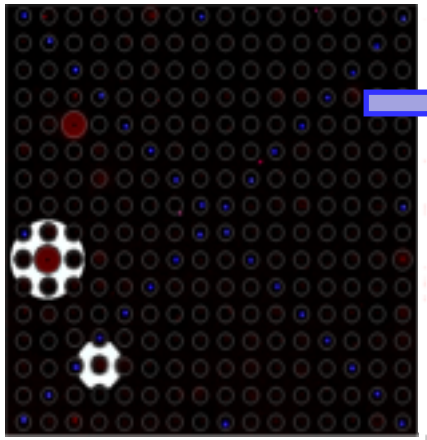
medicinal chemistry

BRD-K81967595
K_D (SHH) = 9500 nM
K_D (DHH) = 13 nM
>500 fold

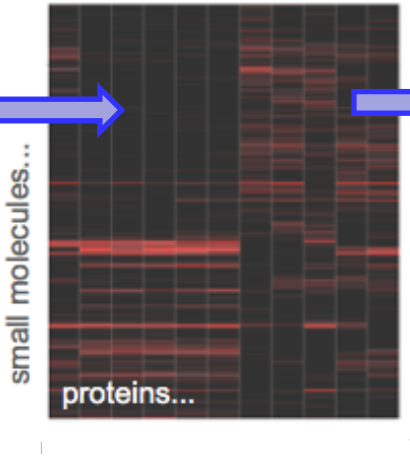
BRD-K93170324
K_D (SHH) = 7 nM
K_D (DHH) = 20000 nM
>2500 fold



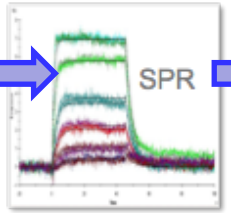
Path for probe discovery, validation, and development

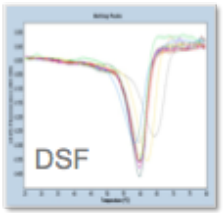


SMM

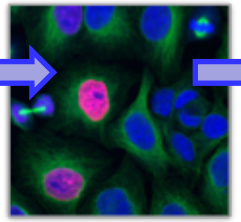


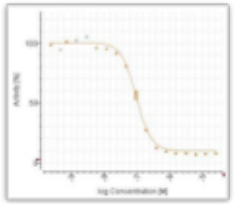
specificity analysis



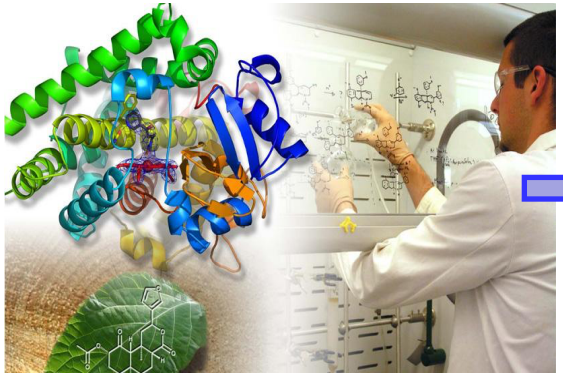


secondary binding





cellular or biochemical assays



optimize molecules using chemistry

additional cell biology animal models

Our path to finding ligands - lectures

2/5/20	Lecture 1	Intro to chemical biology: small molecules, probes, and screens
2/11/20	Lecture 2	Our protein target: TDP-43
2/13/20	Lecture 3	Small molecule microarrays
2/18/20	No Lecture	
2/20/20	Lecture 4	Quantitative evaluation of protein-ligand interactions
2/25/20	Lecture 5	A ligand discovery vignette: sonic hedgehog
2/27/20	Lecture 6	Engineering transcriptional responses with a small molecule
3/3/20	Lecture 7	Wrap up discussion: suggestions for how to report your findings