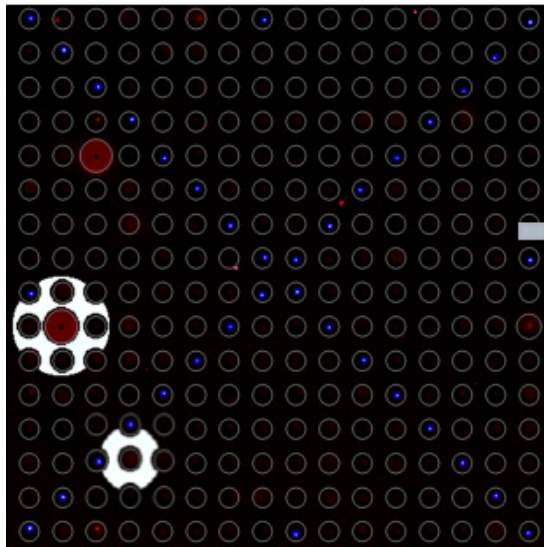


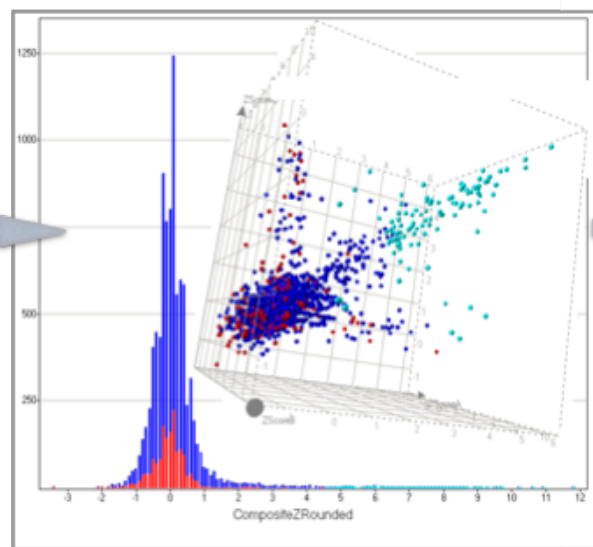
L5 – A Probe Discovery Vignette

March 2, 2017

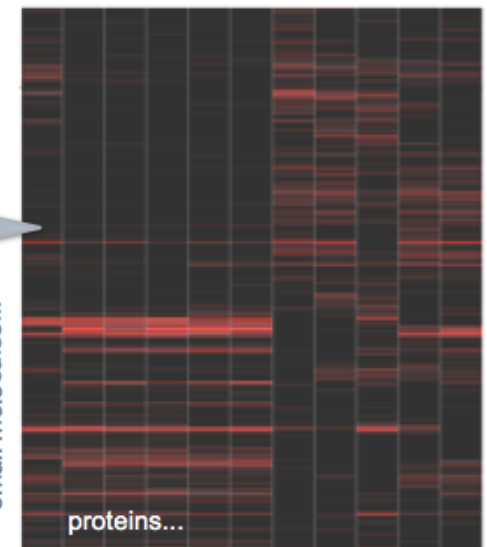
SMM hits to chemical probes



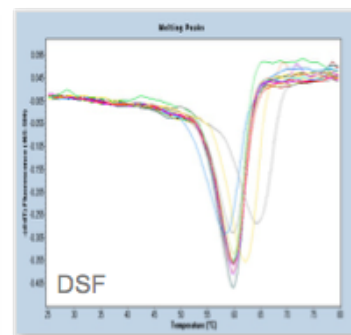
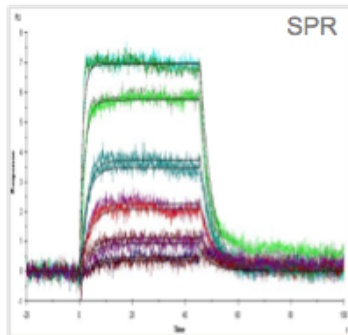
fluorescent features reveal putative interactions



compute composite Z-scores, "hit" calls

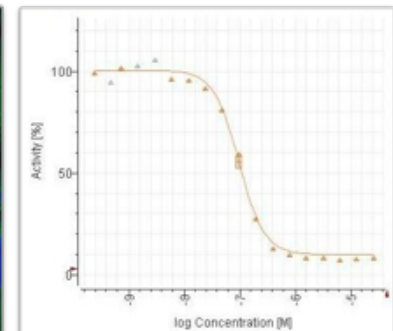
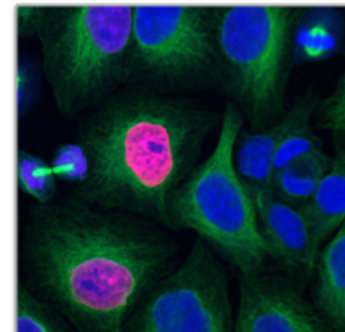


specificity analysis



secondary binding assays

+



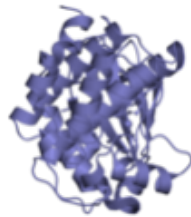
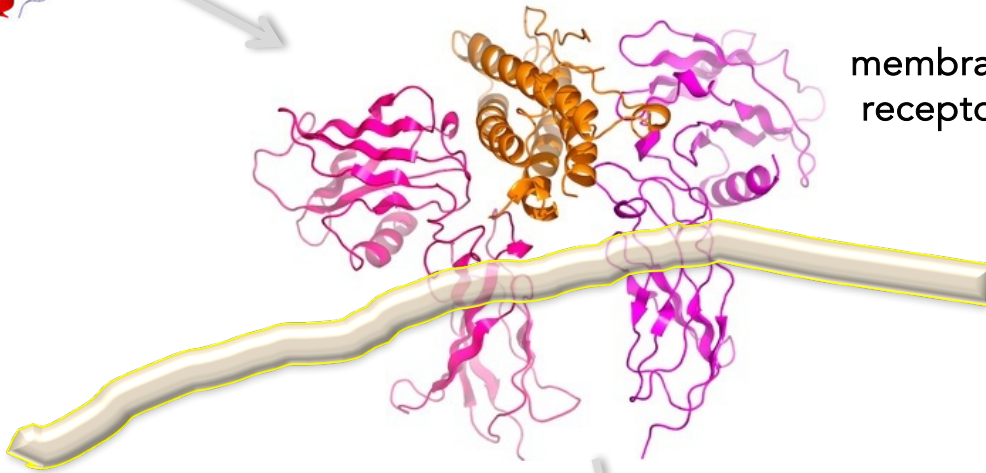
functional assays

extracellular
factors

the '20.320 version'
of biology



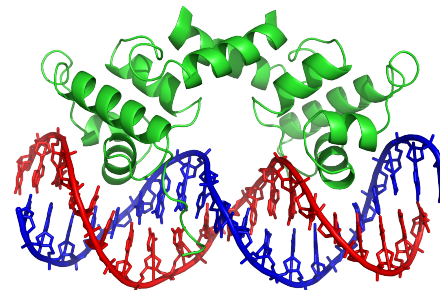
membrane
receptors



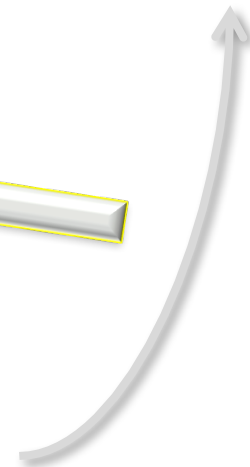
intracellular
signaling
proteins



transcriptional
regulators



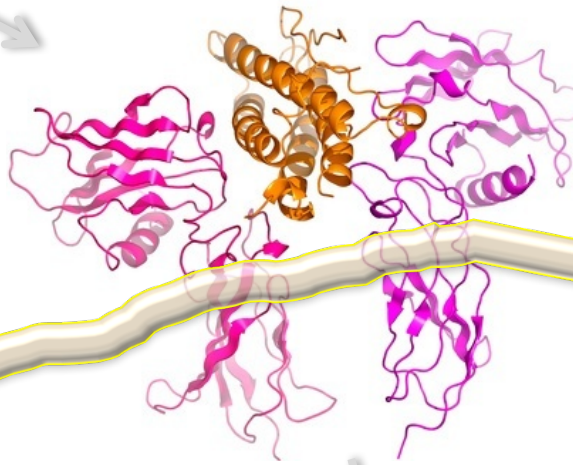
cellular
response



extracellular
factors

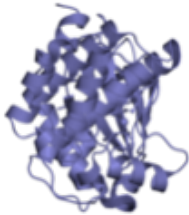


membrane
receptors



GPCRs
ion channels

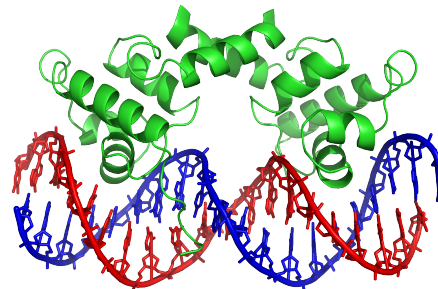
intracellular
signaling
proteins



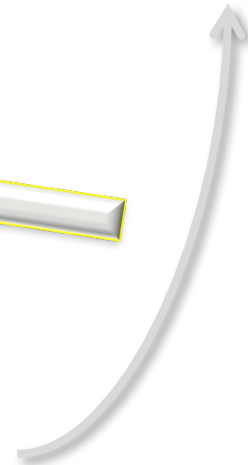
kinases
metabolic enzymes



transcriptional
regulators



cellular
response



extracellular
factors

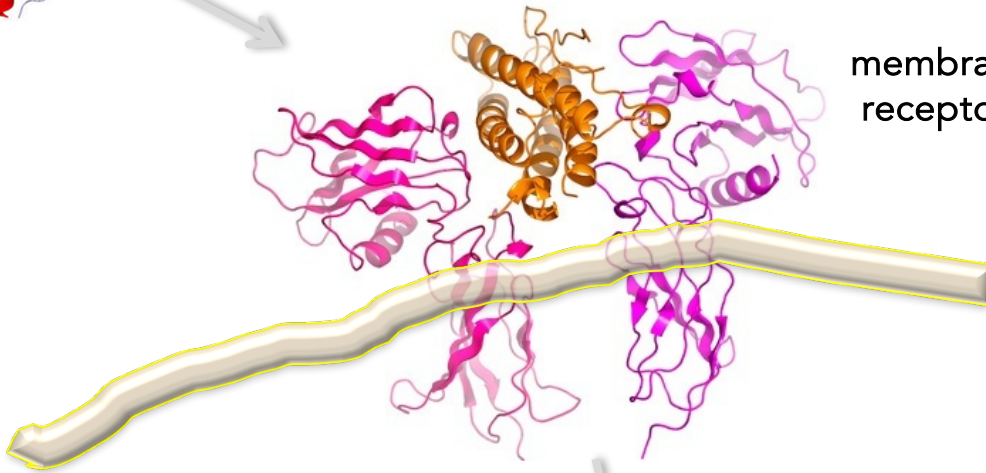


clotting factors

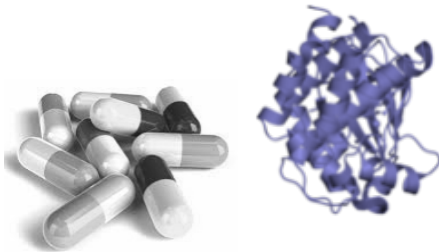
membrane
receptors



GPCRs
ion channels



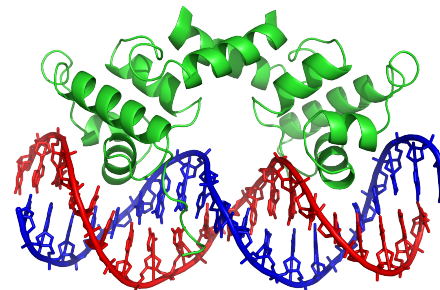
intracellular
signaling
proteins



kinases
metabolic enzymes



transcriptional
regulators

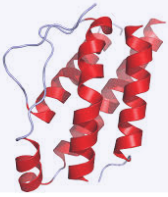


cellular
response



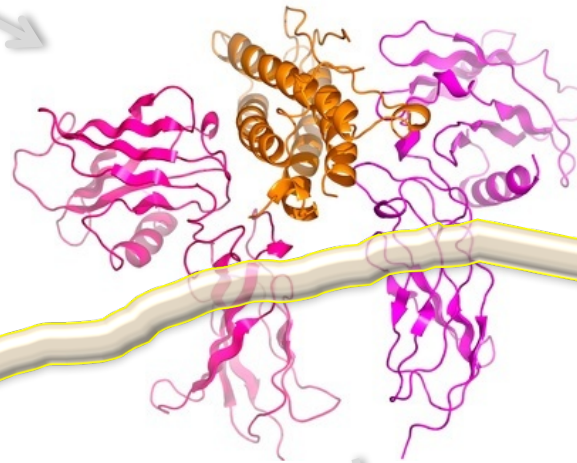
nuclear hormone
receptors

extracellular
factors

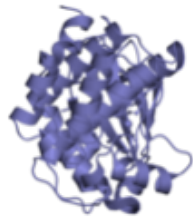


cytokines – IL-4
chemokines – MCP-1
growth factors – SHH

membrane
receptors



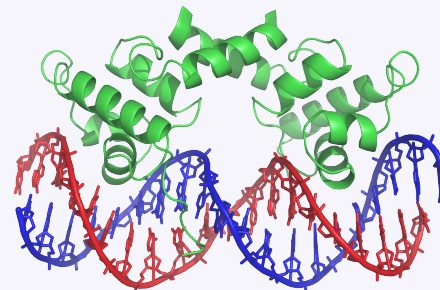
intracellular
signaling
proteins



cellular
response

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

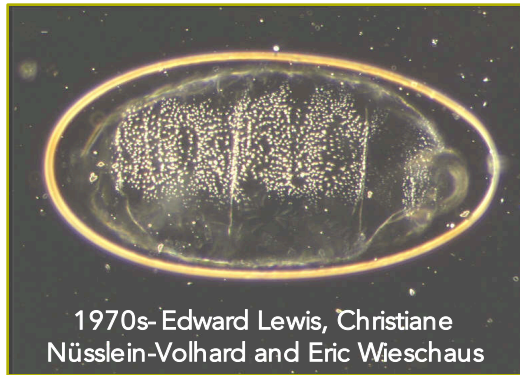
transcriptional
regulators



Sonic hedgehog protein

important role in development including limb and brain development

Reverse Genetic Screen
mutant hedgehog drosophila larva



1970s- Edward Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus



desert and indian



sonic

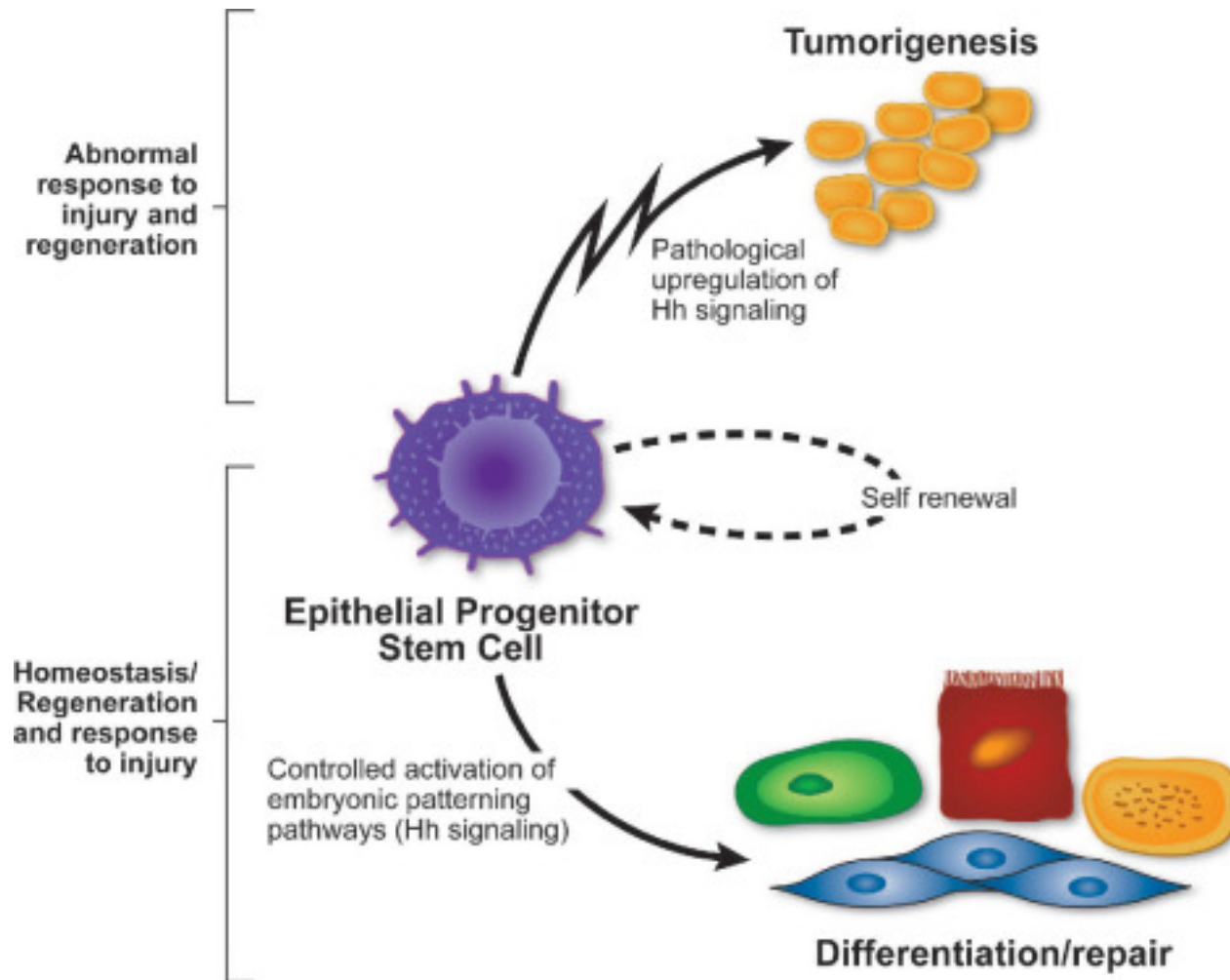
mutations in Shh are linked with
holoprosencephaly



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

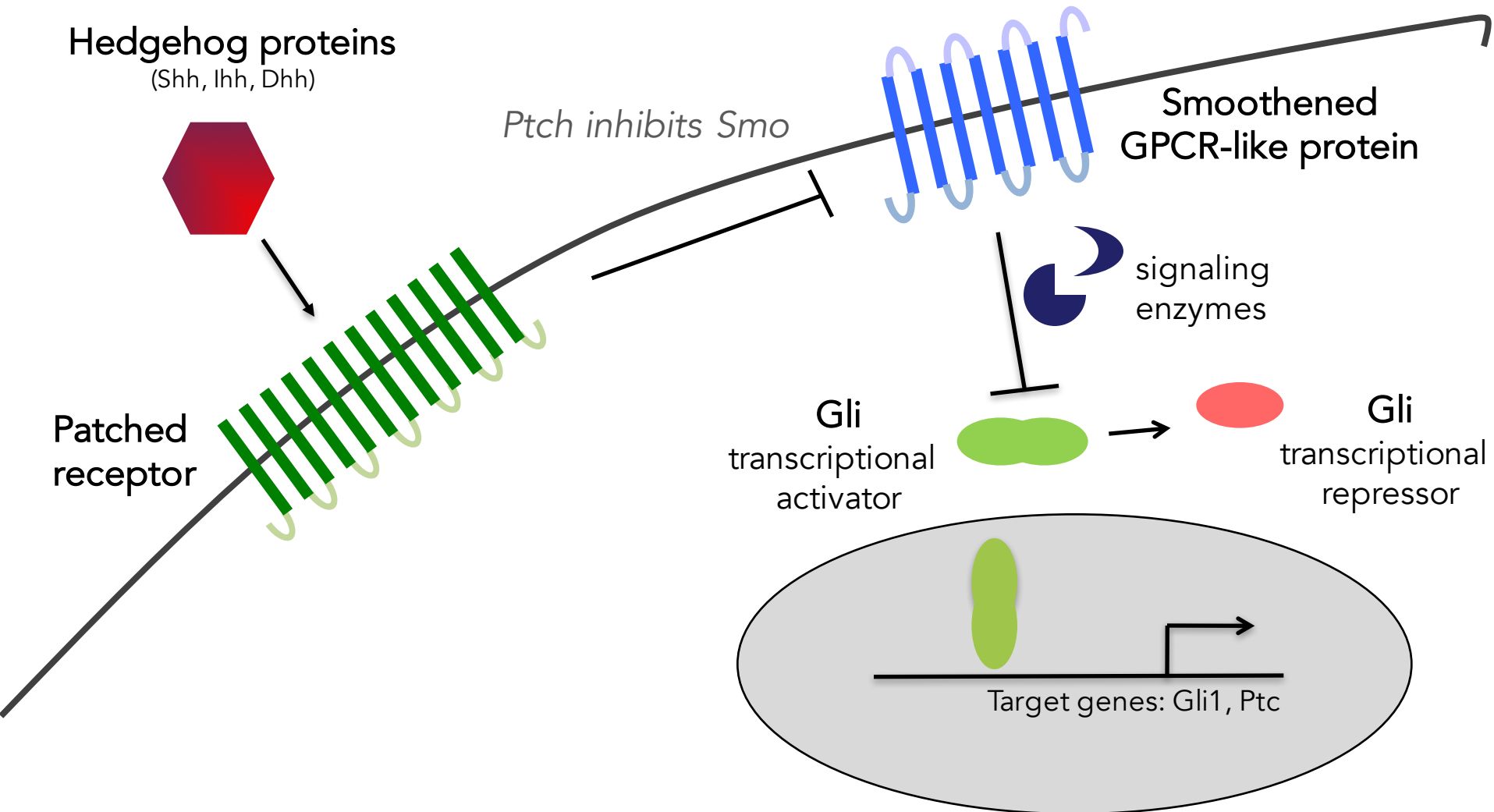
Hedgehog signaling goes beyond embryogenesis

development, differentiation, and disease



Hedgehog proteins 'de-repress' Smoothened

Hh-Smo binding interaction activates Gli-driven transcription

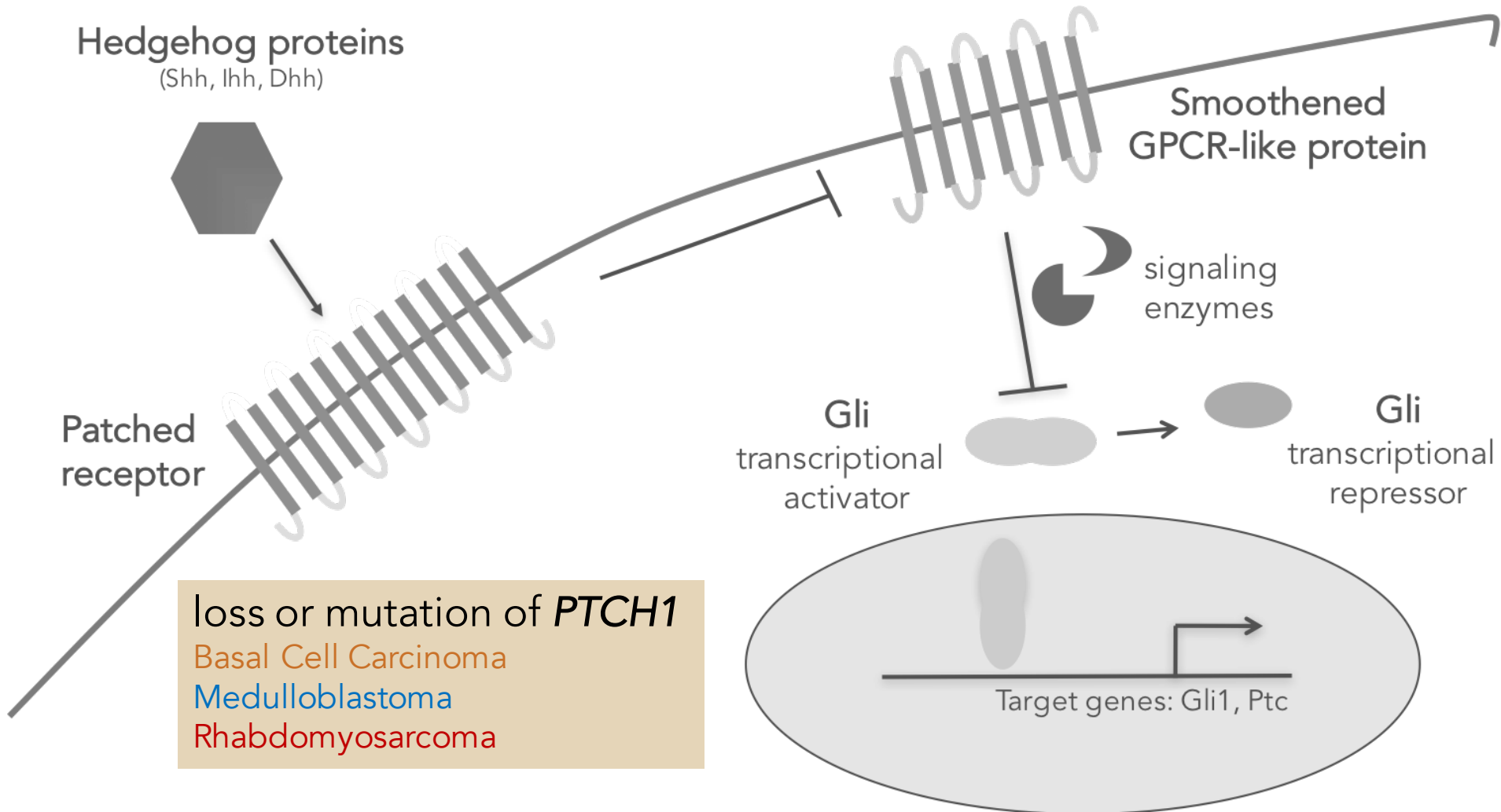


overexpression of *SHH*

Pancreatic Cancer Prostate Cancer
Gastric Cancer Lung Cancer
Medulloblastoma Ovarian Cancer

activating mutations in *SMO*

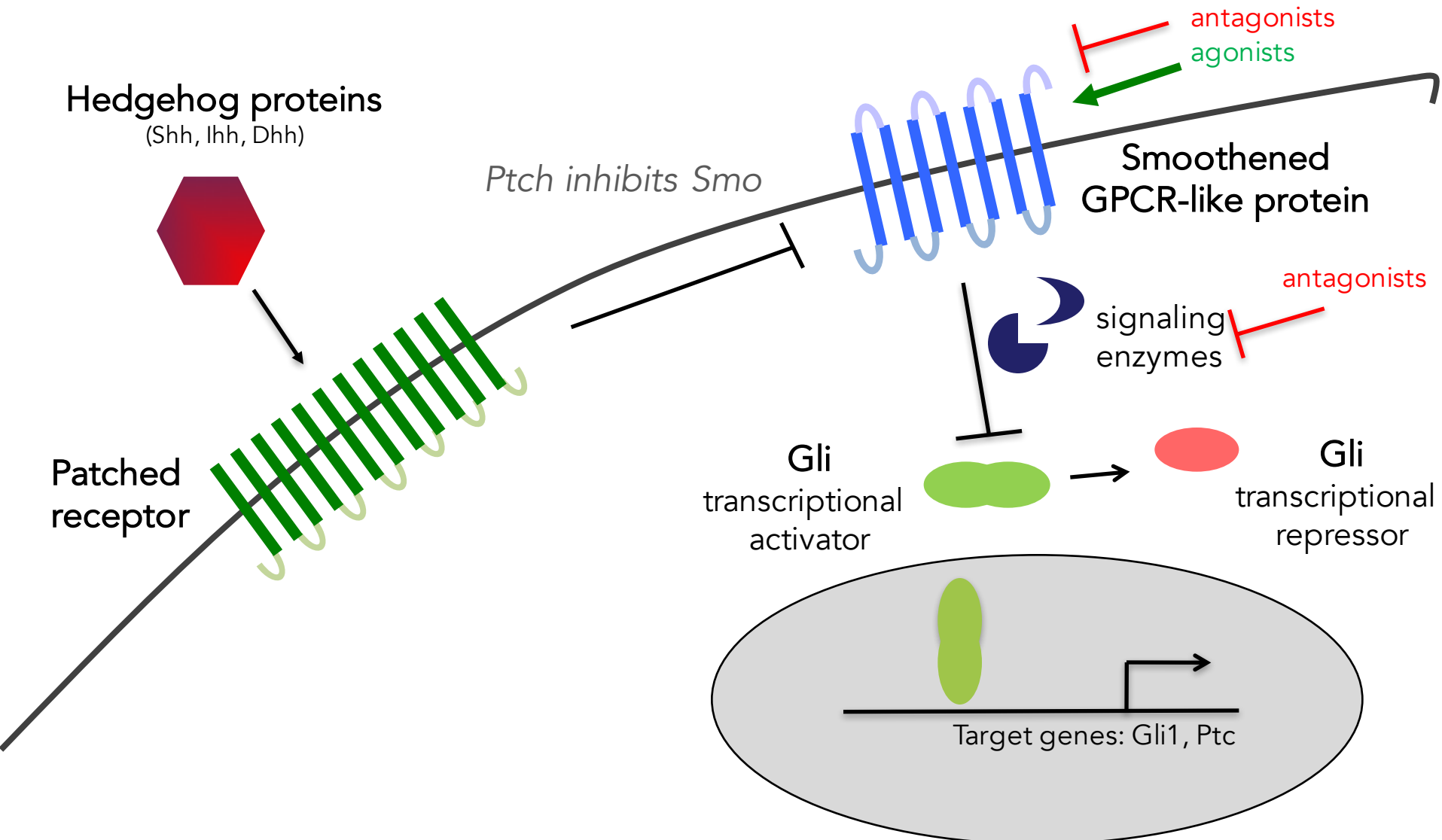
Basal Cell Carcinoma
Ovarian Cancer



loss or mutation of *PTCH1*

Basal Cell Carcinoma
Medulloblastoma
Rhabdomyosarcoma

Drugs targeting Hedgehog pathway

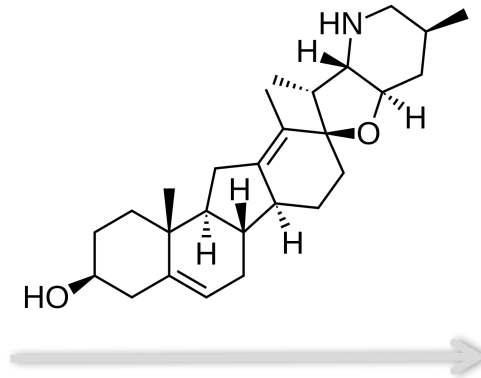


Cyclopamine

Smo antagonist and Hh pathway inhibitor



Veratrum californicum



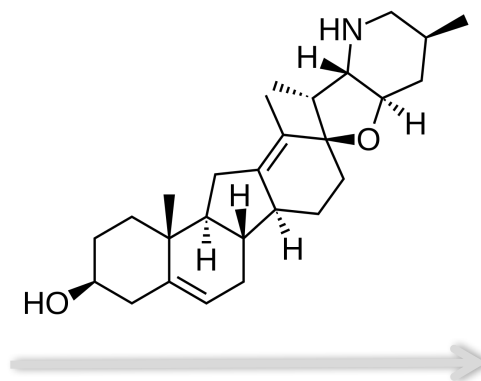
cyclopic lamb born of
a sheep that ate corn lily
(Idaho farm, 1957)

Cyclopamine

lead for development of anti-cancer agents

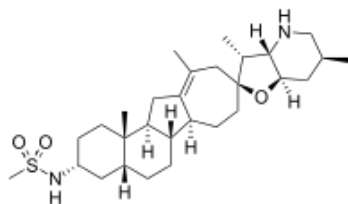


Veratrum californicum

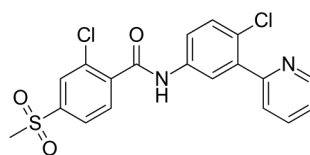


cyclops lamb born of
a sheep that ate corn lily
(Idaho farm, 1957)

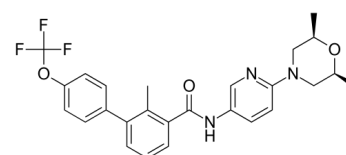
Adult cancers - basal cell carcinoma, medulloblastoma, prostate, breast, pancreas



saridegib



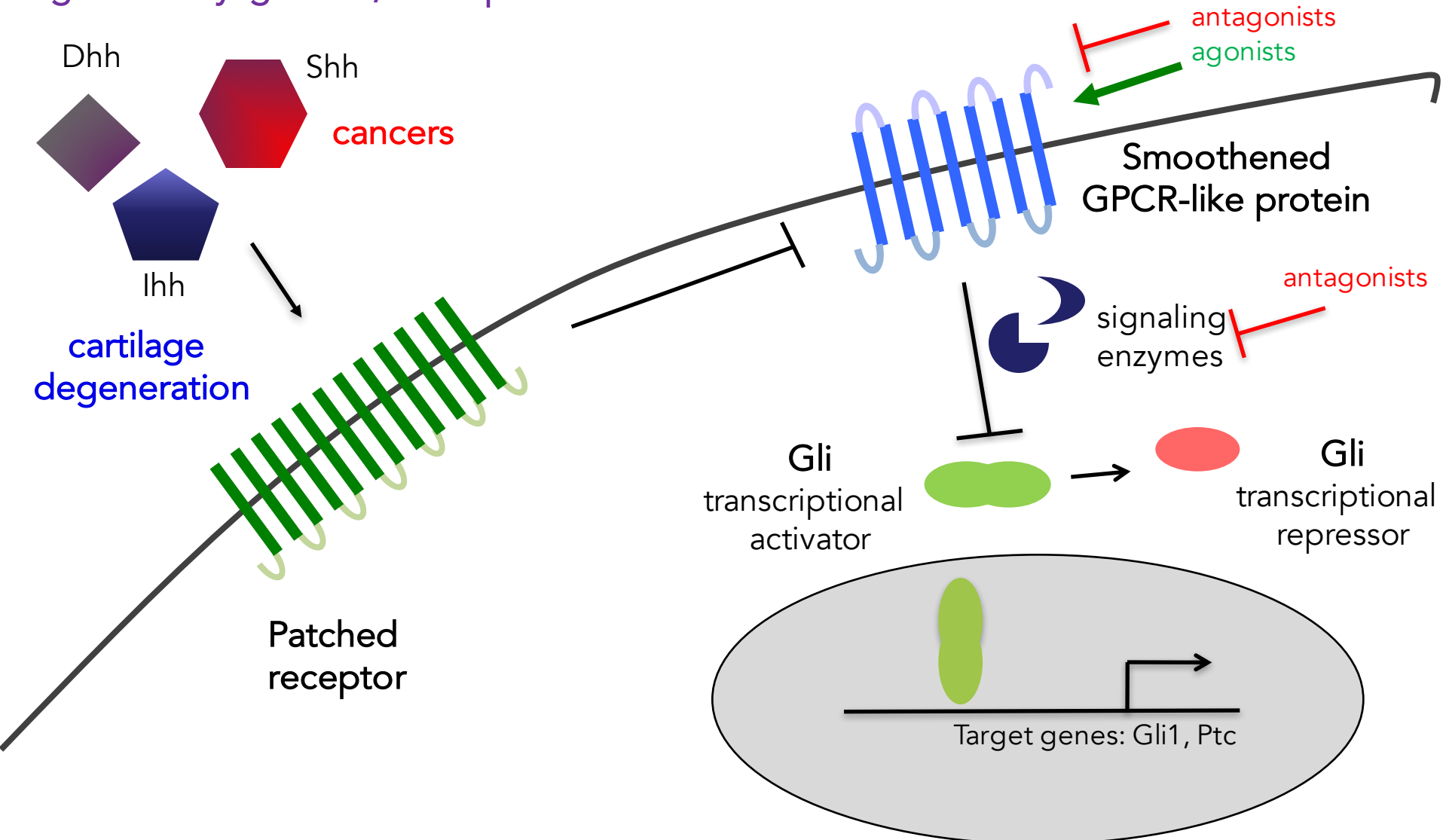
vismodegib



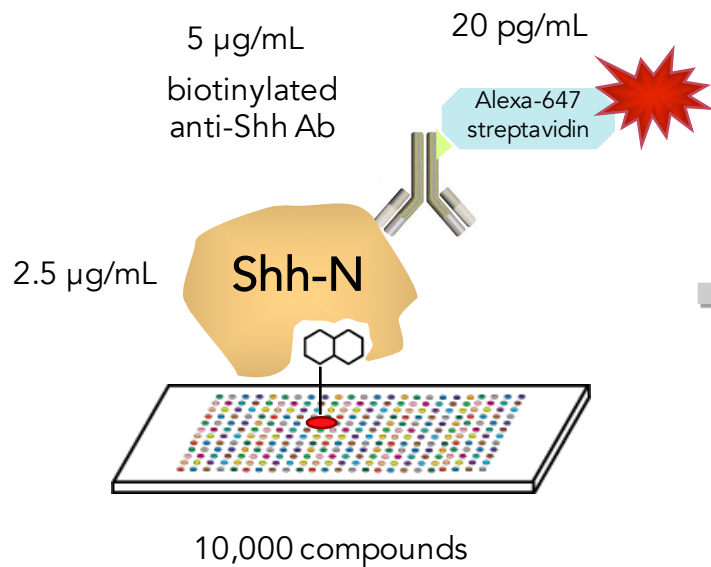
sonidegib

Selective targeting of Hh signaling upstream of Smo

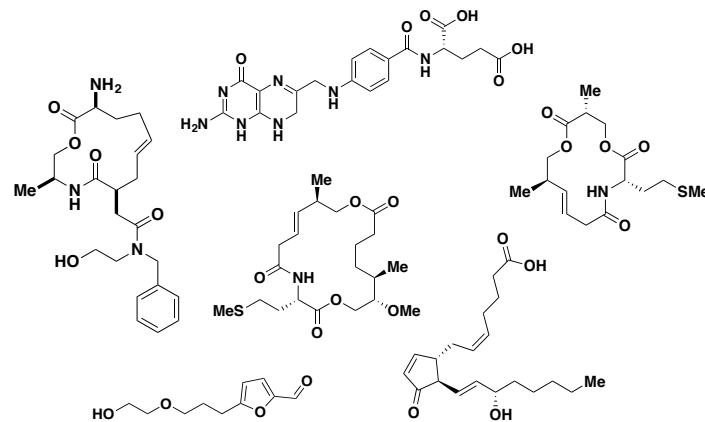
gonadal dysgenesis, neuropathies



Shh-N SMM assay



19 SMM hits



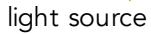
Angela, Broad Fellow

Lee Peng, MGH

Ben Stanton, Harvard



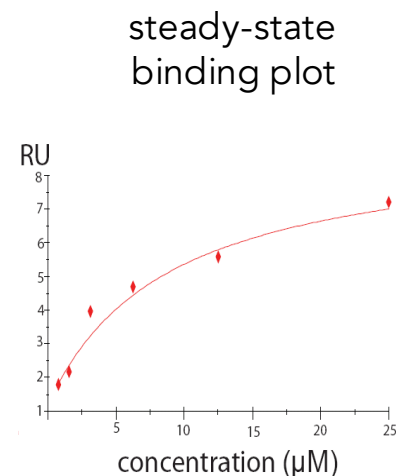
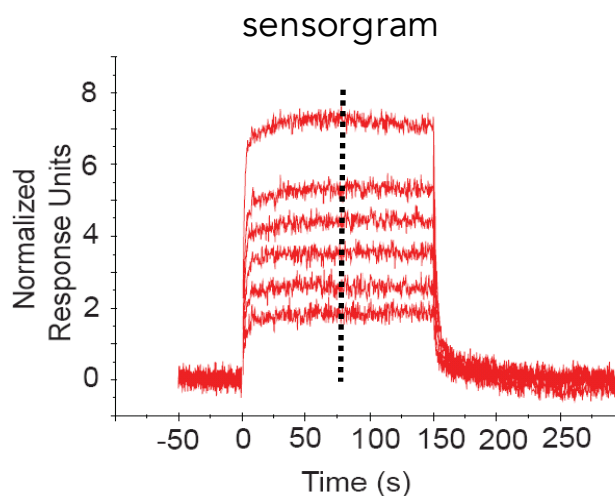
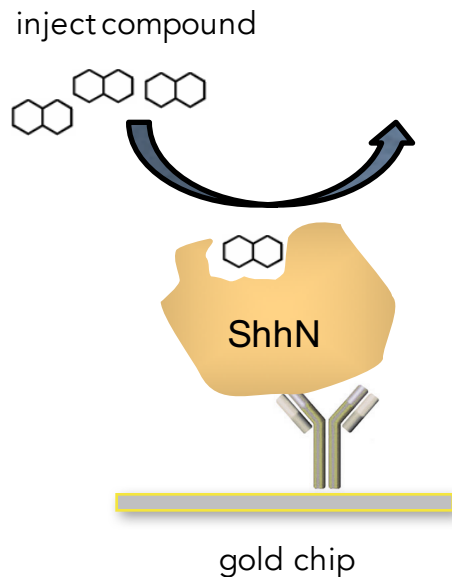
'mass sensing' by Surface Plasmon Resonance (SPR)



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

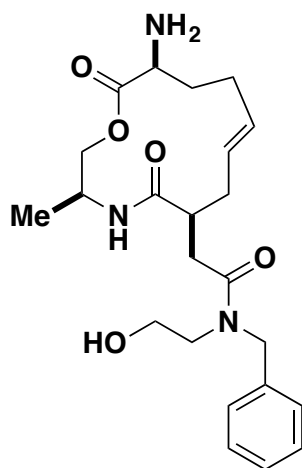


$$K_D = 9 \mu\text{M}$$

- reverses direction 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

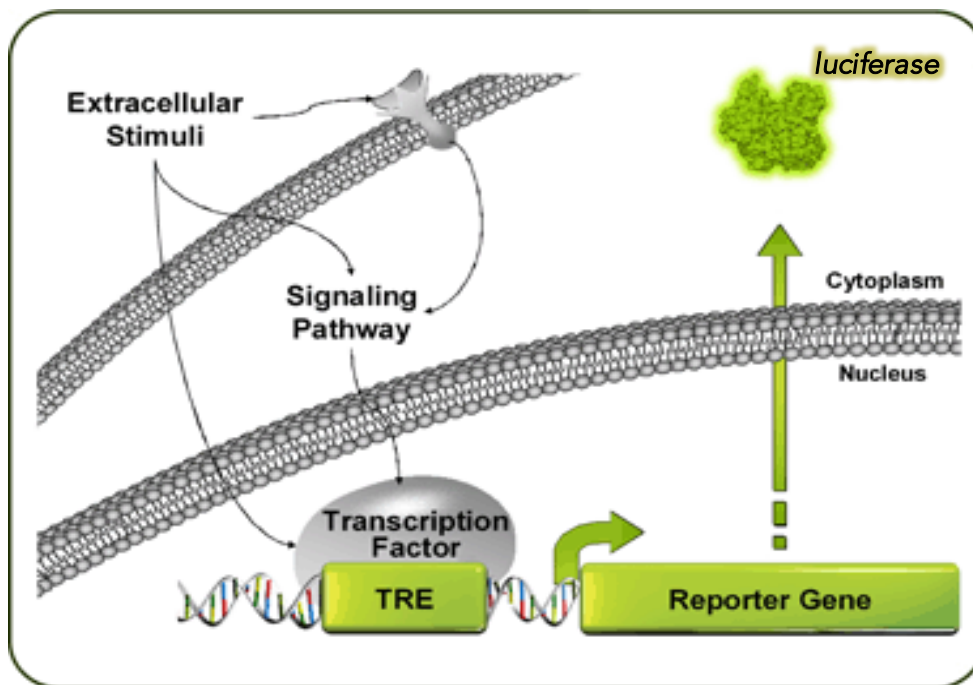
Measuring *GLI*-dependent transcriptional activity

quantitative assay for hedgehog signaling

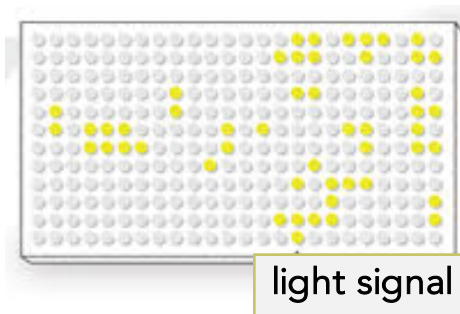
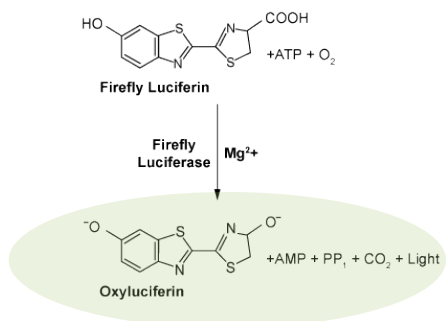


1

$$K_D = 9 \mu\text{M}$$

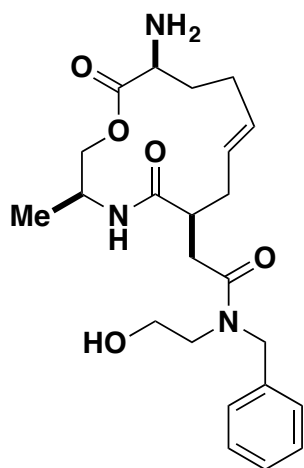


Luciferase reaction

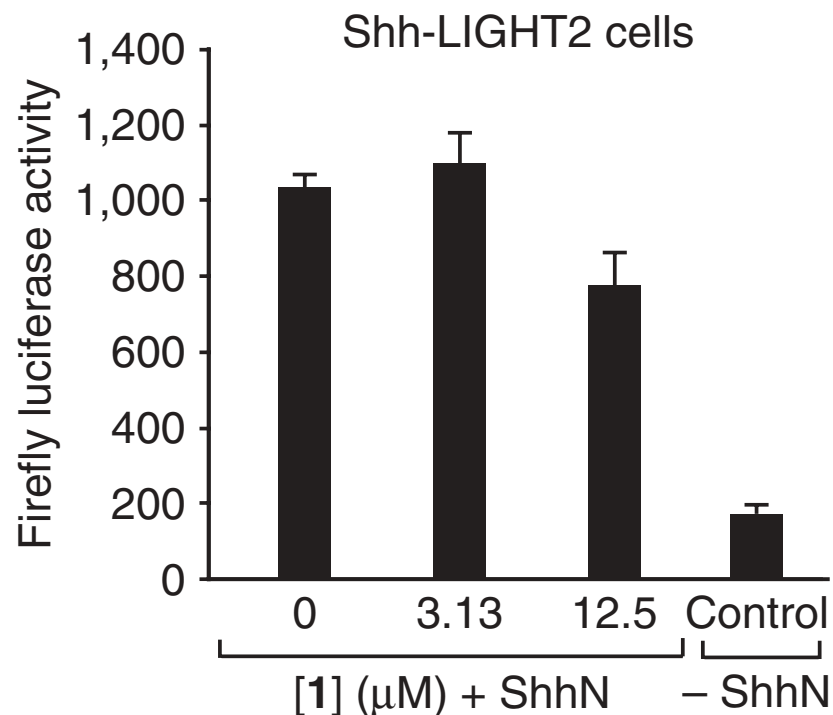


Measuring *GLI*-dependent transcriptional activity

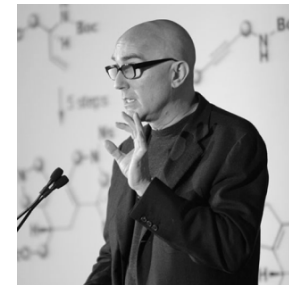
SMM hit modulates transcriptional output



1

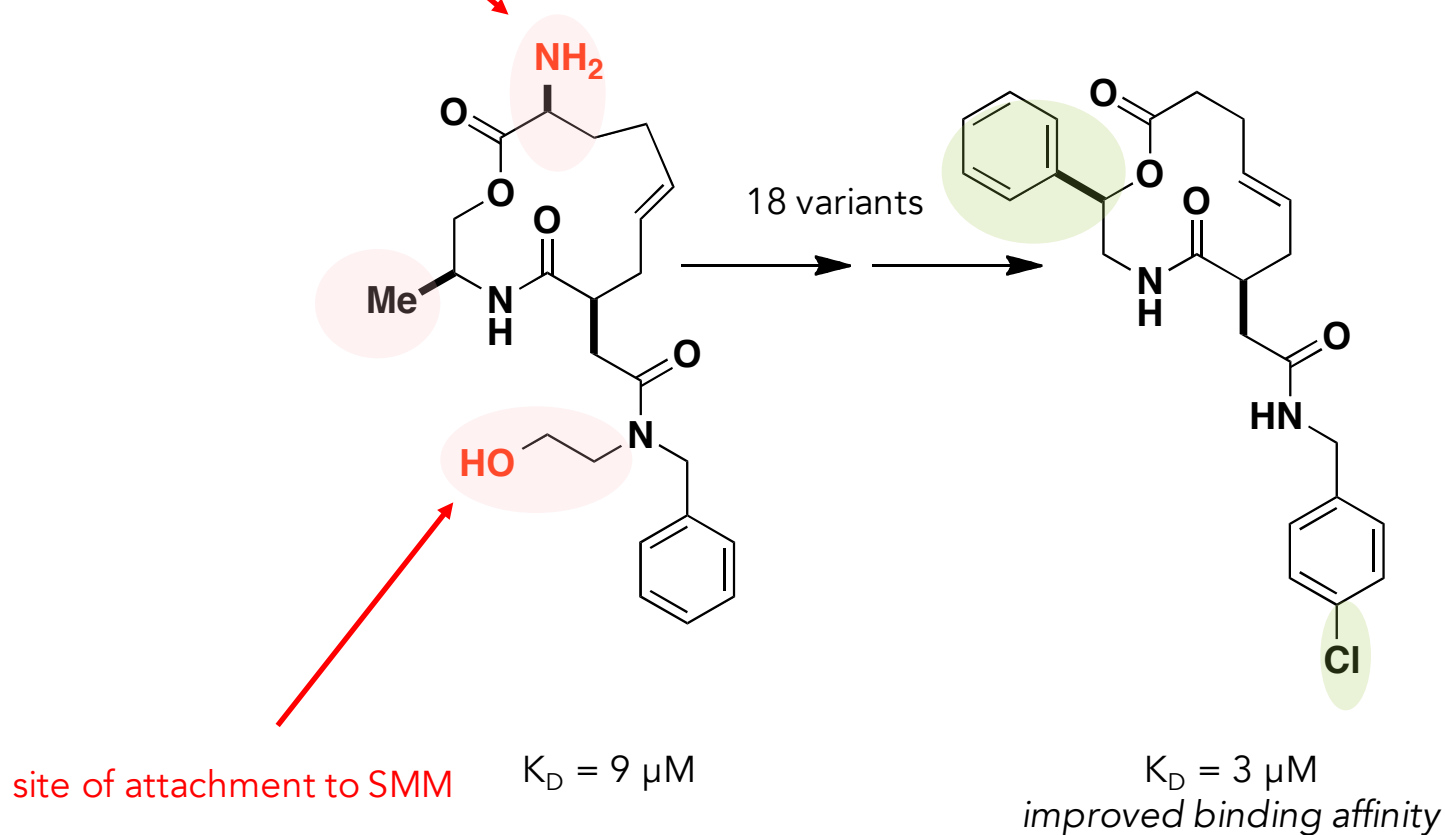
$$K_D = 9 \mu M$$


each value represents 5 technical replicates
error bars denote standard deviation



site of attachment to SMM

Hit to probe chemical editing



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



Doctor Ivo "Eggman" Robotnik

SEGA®



Sonic the Hedgehog

Robotnikinin

Shh binder and antagonist

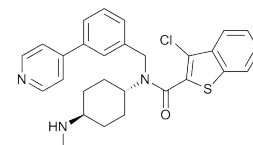
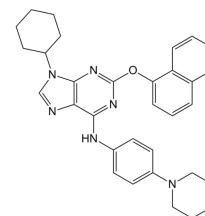
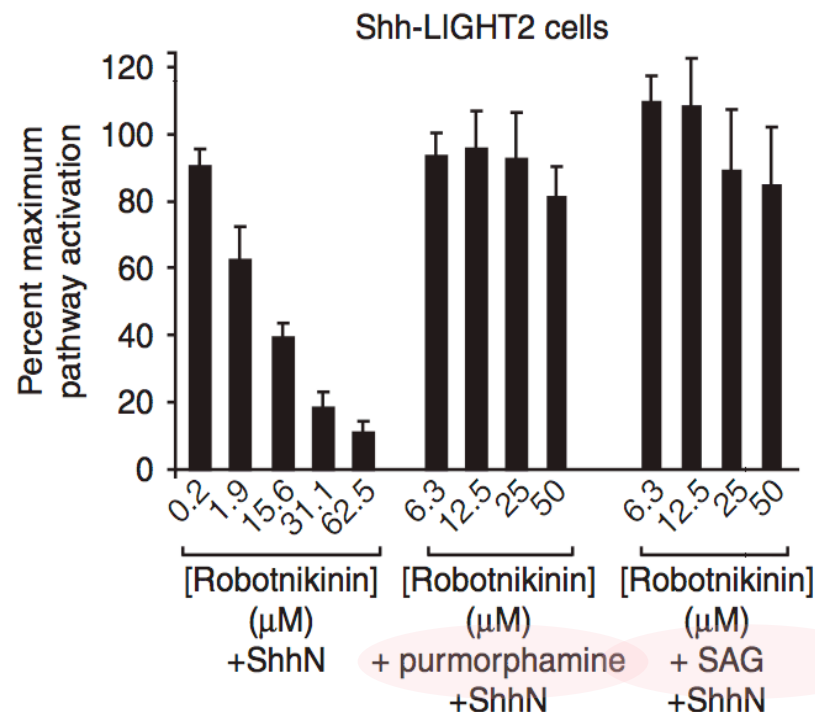
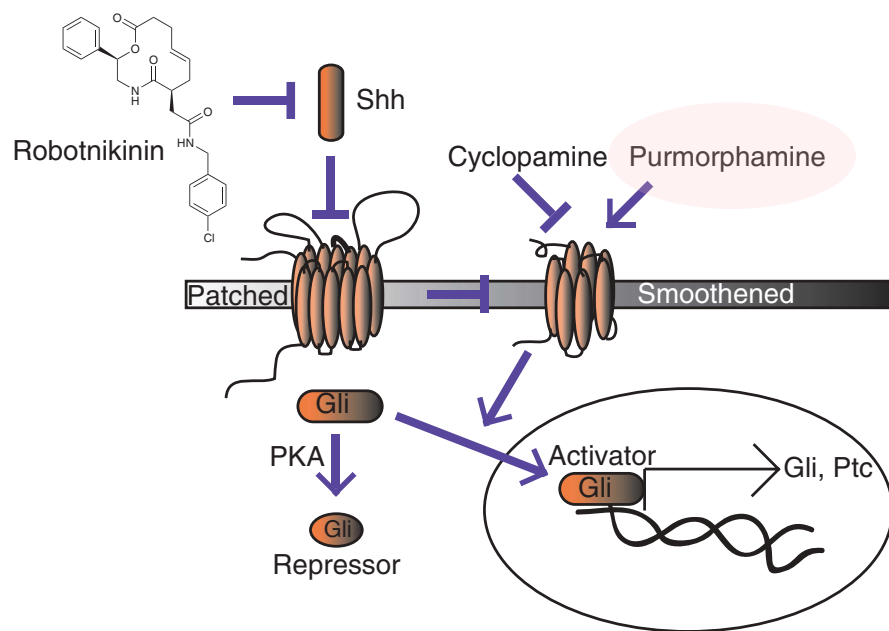
nature
chemical biology

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 Maloof¹,
Kazuo Nakai², Xiang Wang¹, Jay L Duffner¹, Kennedy M Taveras¹,
Joel M Hyman⁴, Sam W Lee⁵, Angela N Koehler¹, James K Chen⁴,
Julia L Fox⁶, Anna Mandinova⁵ & Stuart L Schreiber^{1,2}

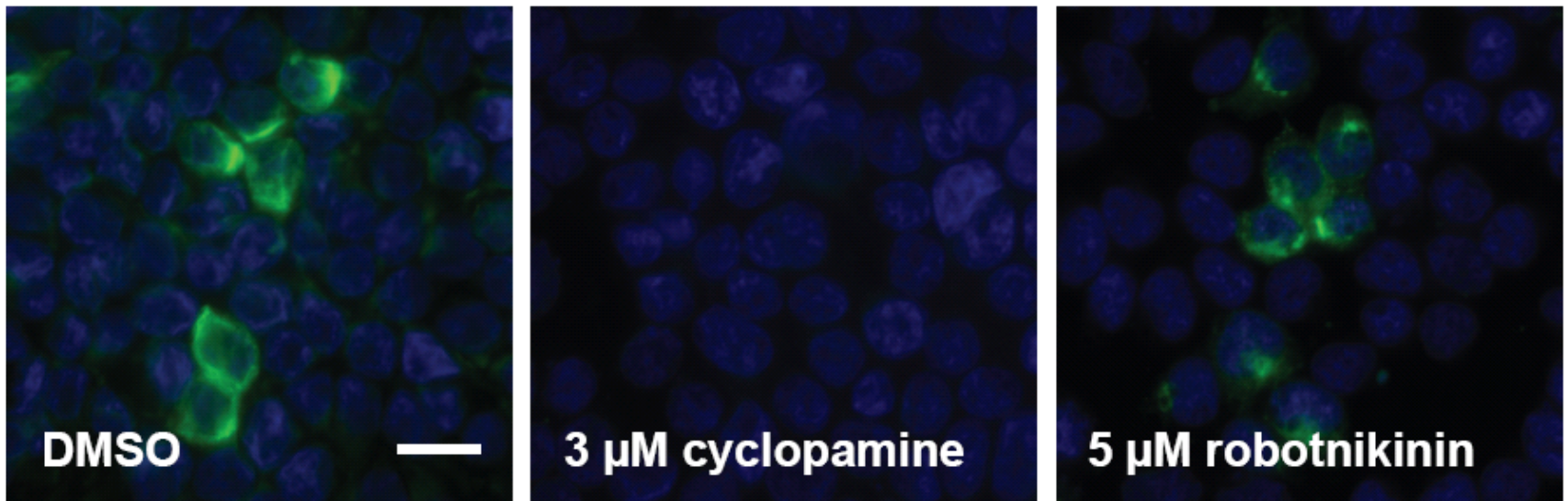
Small-molecule inhibition of extracellular proteins that activate membrane receptors has proven to be extremely challenging. Diversity-oriented synthesis and small-molecule microarrays enabled the discovery of robotnikinin, a small molecule that binds the extracellular Sonic hedgehog (Shh) protein and 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.

Gli inhibition by Robotnikinin is rescued by a Smoothened agonist



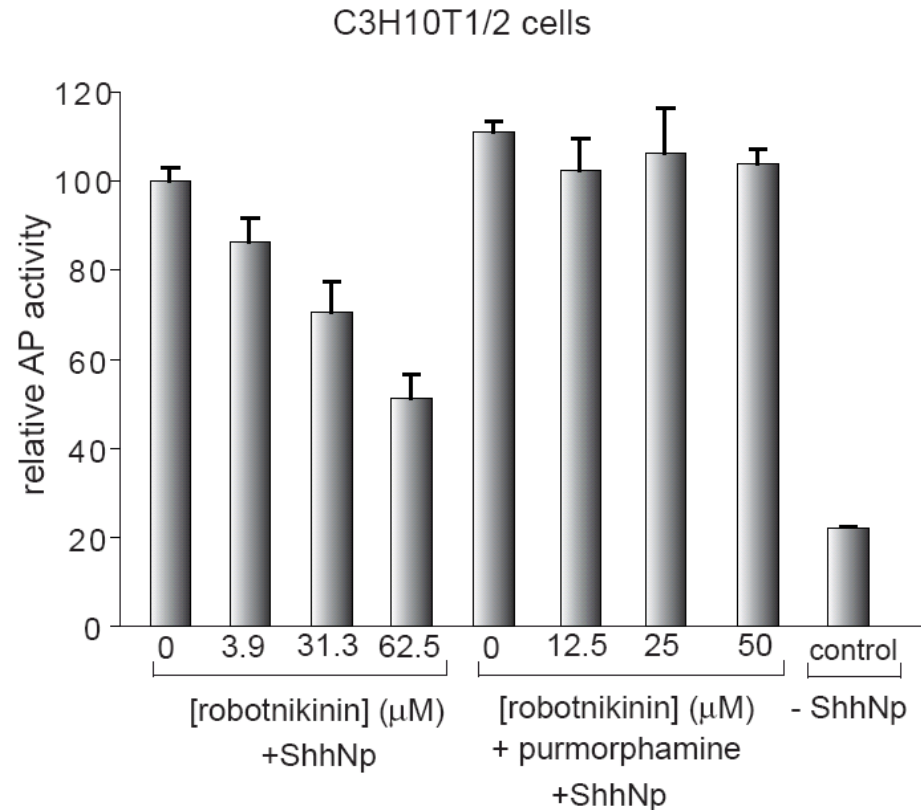
Ligand competition assays

BODIPY-cyclopamine binds to Smoothed at cell surface



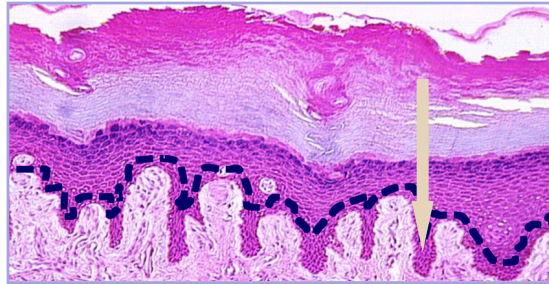
Smoothed-overexpressing human embryonic kidney cells

Inhibition of stem cell differentiation



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

Robotnikinin blocks lowers levels of *Gli2* mRNA in primary human keratinocyte cells

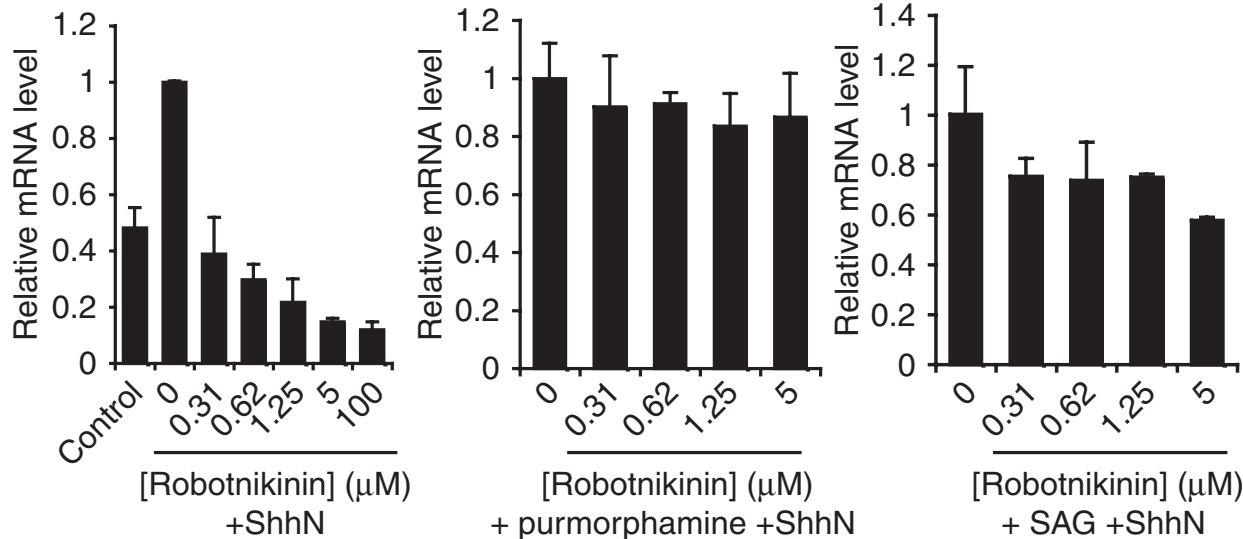


primary human keratinocytes
isolated from the **basal cell layer**

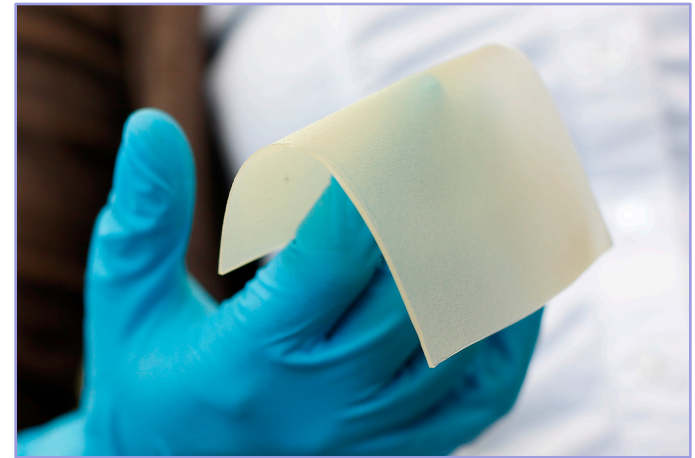
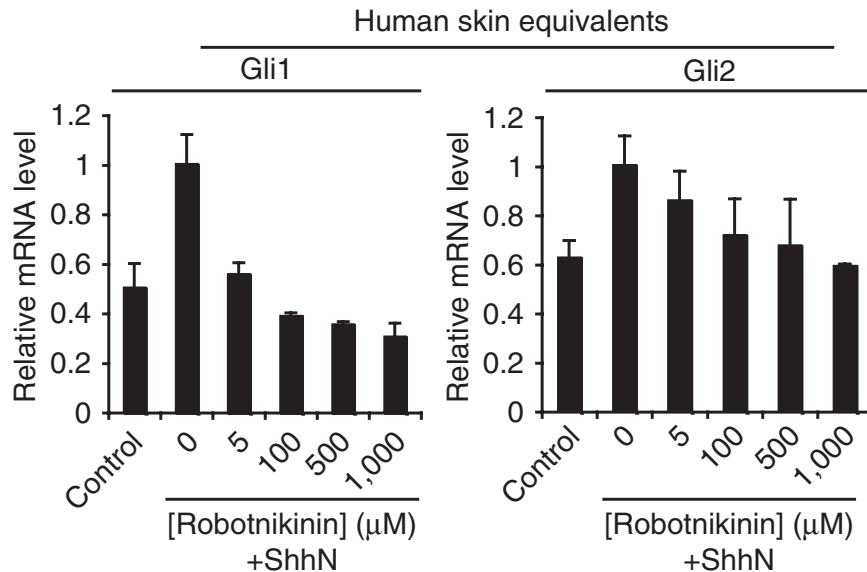


measure mRNA by quantitative
PCR after 30-hr treatments

Gli2



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

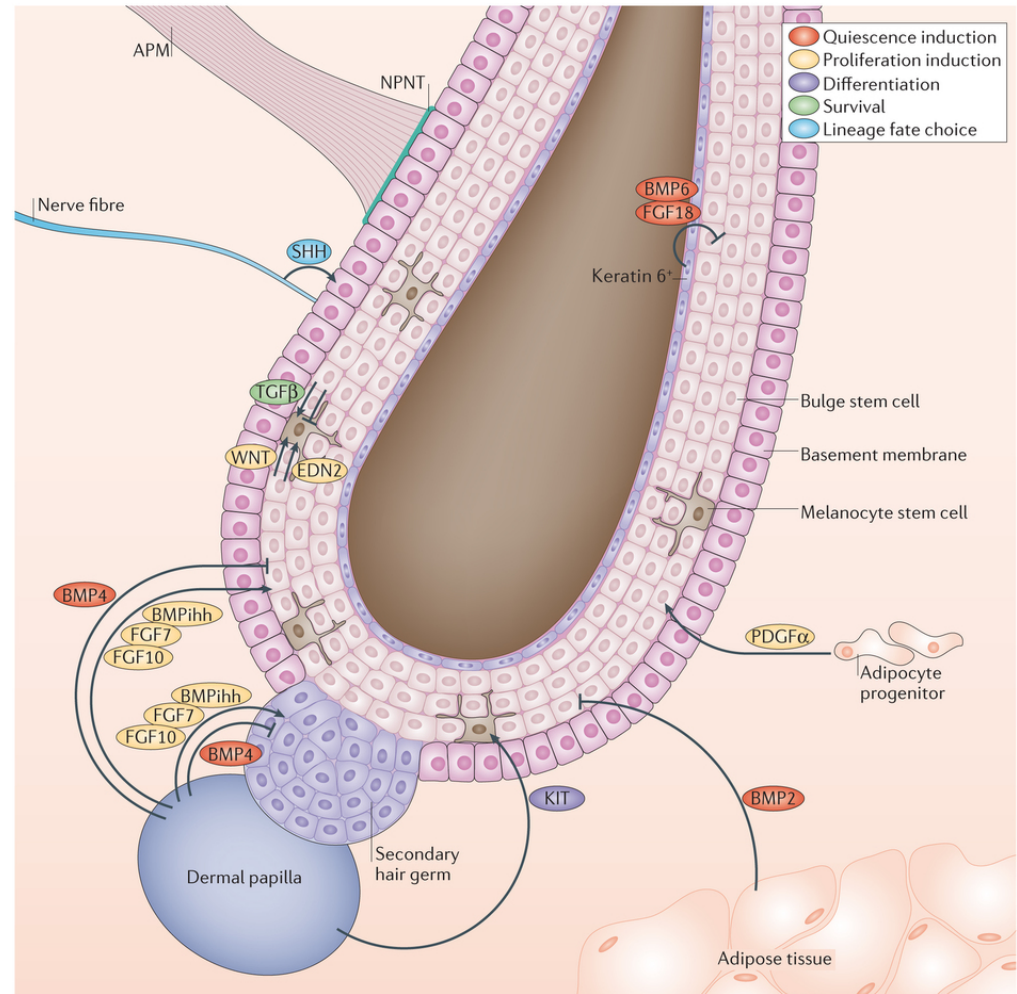


MGH synthetic human skin model:

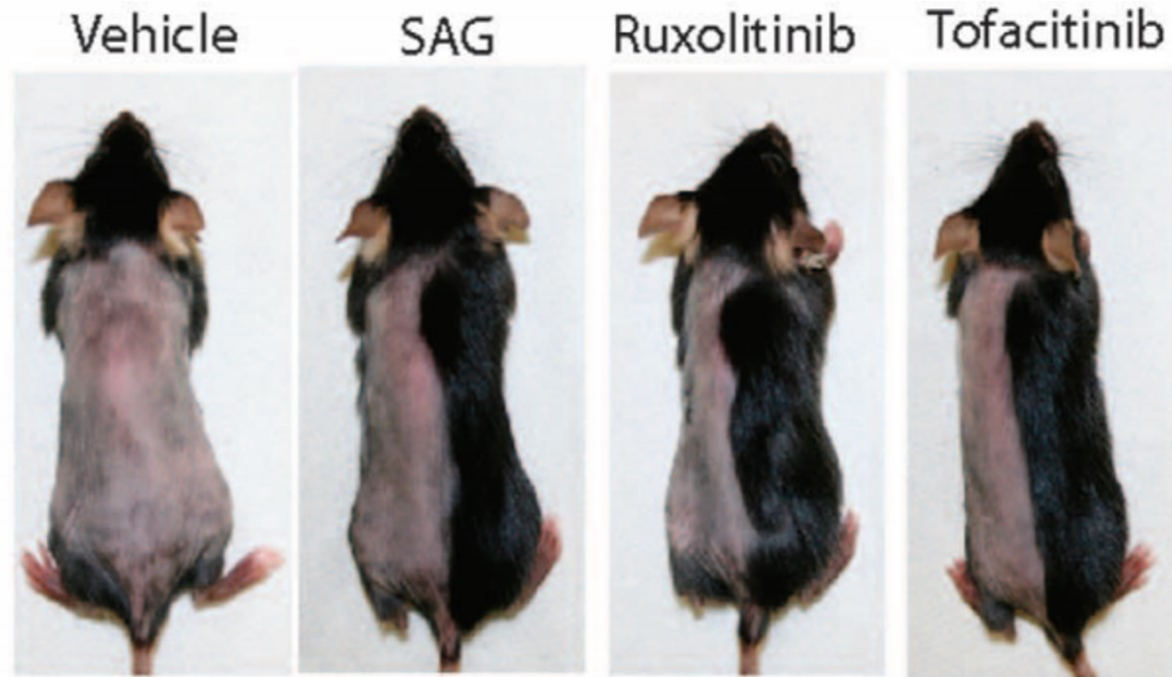
1. Extract dehydrated collagen matrix from skin grafts
2. Populate matrix with primary keratinocytes
3. Culture to form several dermal layers
4. Incubate with compound, analyze by qPCR and histology

Anna Mandinova, MGH





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

Robotnikinin inhibits hair growth in vitro

8 days post depilation



10 uM robotnikinin

DMSO

12 days post depilation

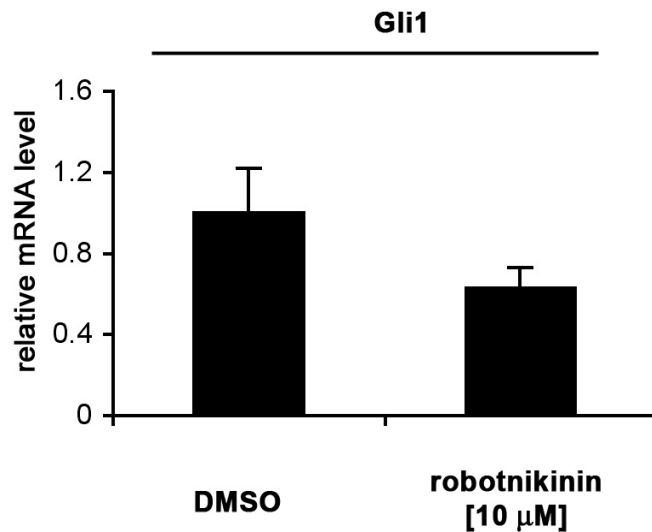
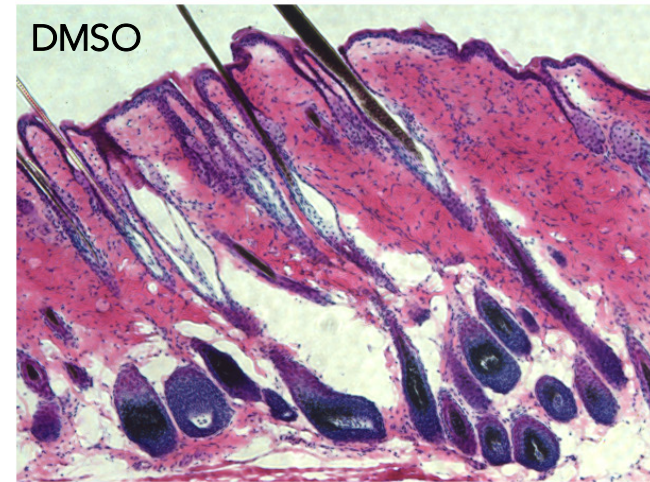


10 uM robotnikinin

DMSO

Robotnikinin inhibits hair growth in vitro

12 days post depilation



hair follicles fail to enter active growth phase (anagen) efficiently and reduce levels of Gli TFs, yet robotnikinin treatment shows no signs of inflammation or failed skin differentiation

Hh homolog selectivity

BRD-K81967595

K_D (SHH) = 9500 nM

K_D (DHH) = 13 nM

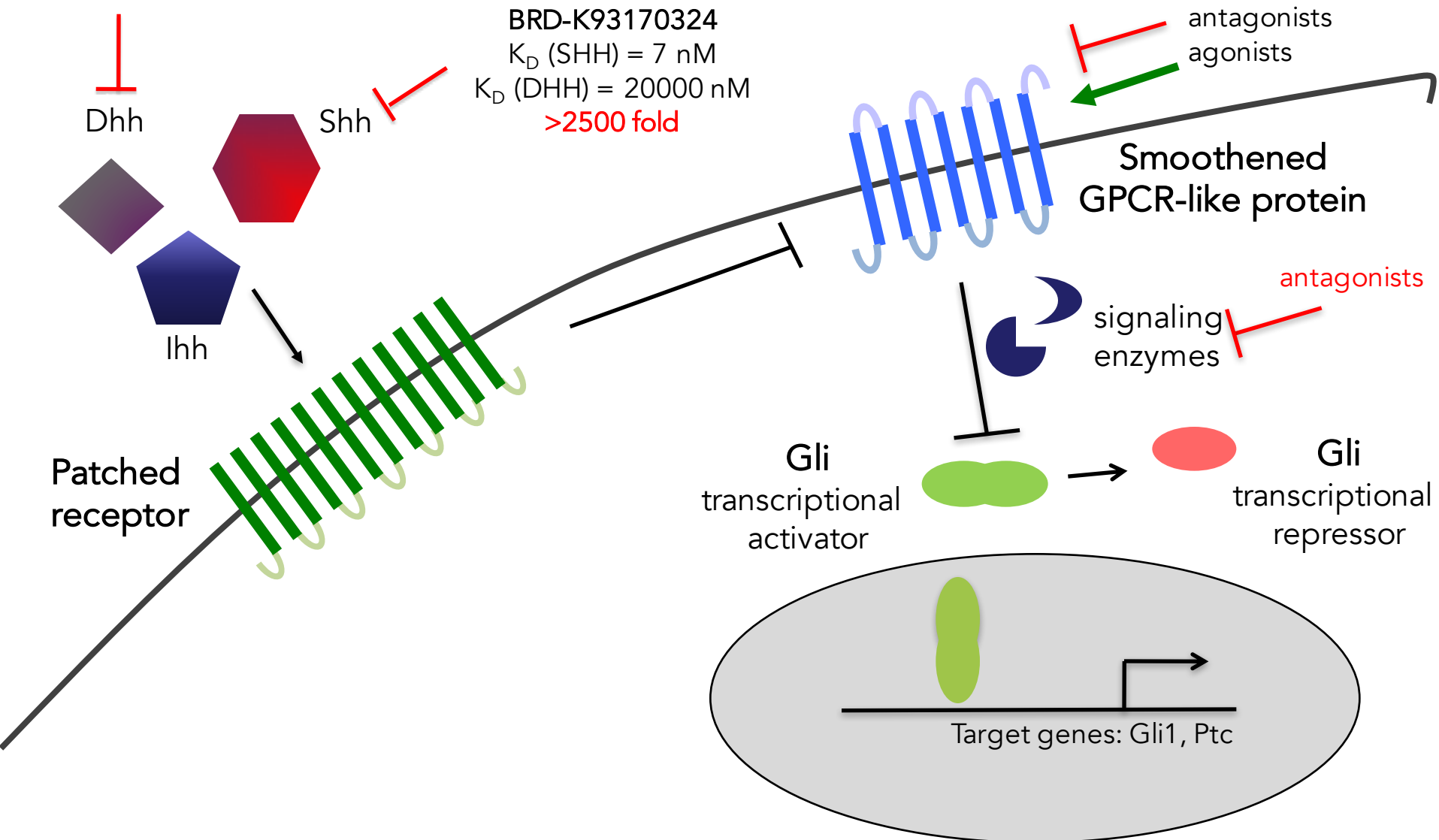
>500 fold

BRD-K93170324

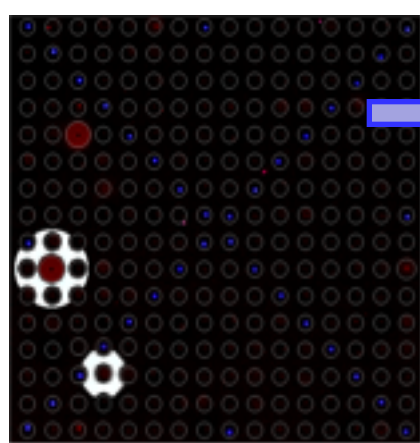
K_D (SHH) = 7 nM

K_D (DHH) = 20000 nM

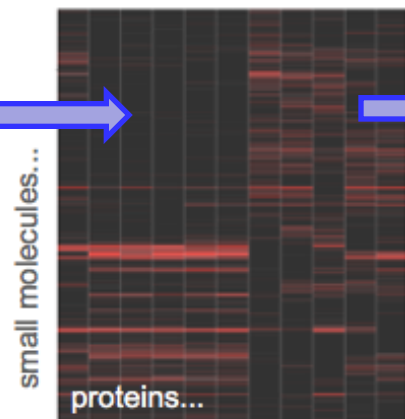
>2500 fold



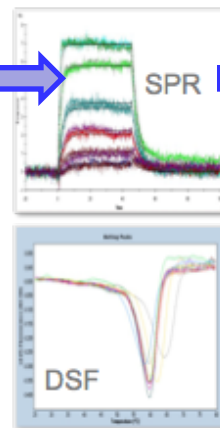
Path for probe discovery, validation, and development



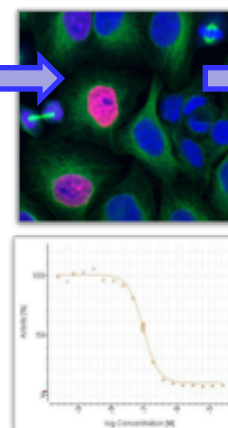
SMM



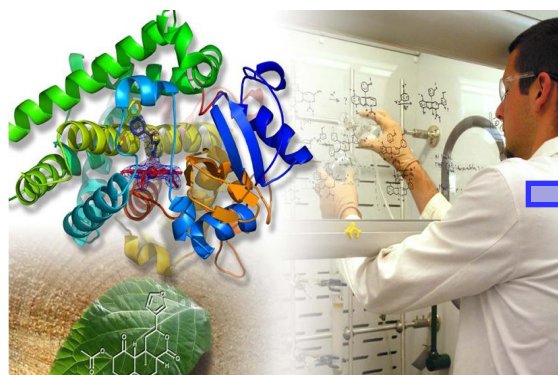
specificity analysis



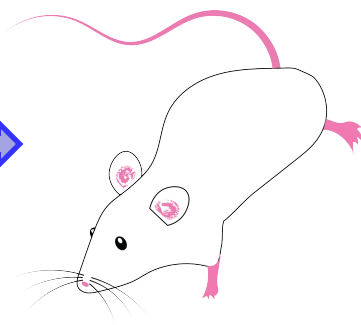
secondary
binding



cellular or
biochemical assays

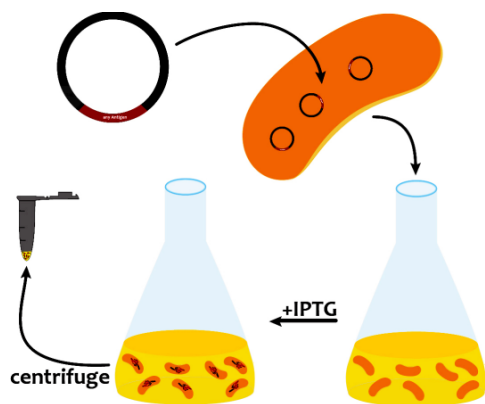


optimize molecules
using chemistry

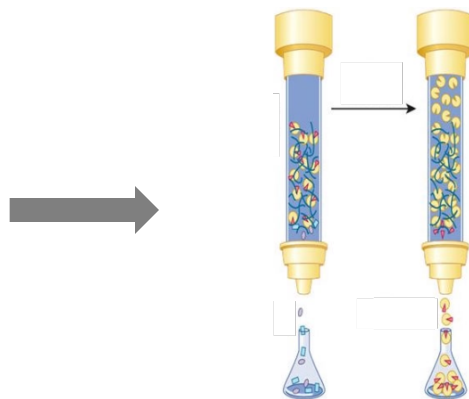


additional cell biology
animal models

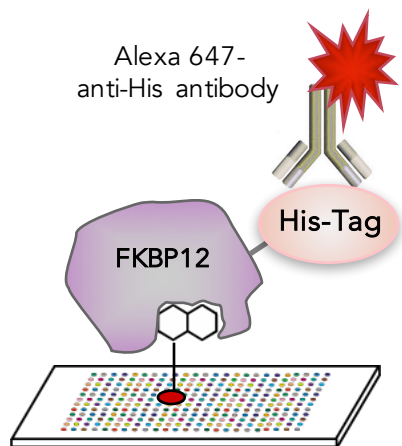
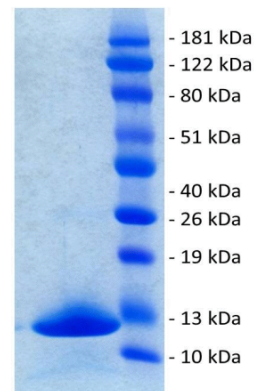
Our path to probe discovery



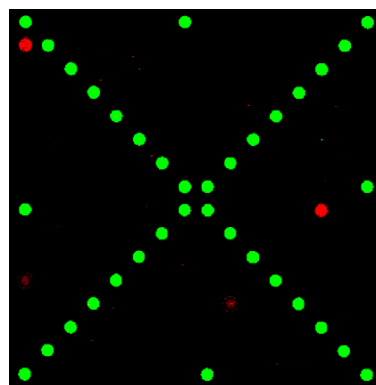
overexpress FKBP12
lab day 1



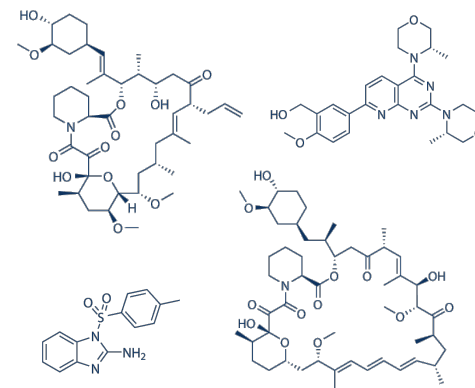
purify and characterize FKBP12
lab days 2 and 3



SMM screen
lab day 4



scan images and analyze data
lab days 5 and 6



compare hit lists for teams
lab day 7