Exercise week 4:
Proliferative signaling II (JAK/STAT, MYC, WNT/β-catenin)

1) For memorization:

a) Which cyclin is rate-limiting for cell proliferation and why, and what strategies do cancer cells use to upregulate its expression?
Answer: Cyclin D, because it is essential to put CDK4 and CDK6 into an active conformation (note that there are in reality several CCND genes, including D1, D2, and D3). Cancer cells upregulate Cyclin D expression by hyperactivating upstream growth factor receptors (RTKs, and/or cytokine receptors) or specific downstream signal transduction components such as AP-1, STATs, TCFs, and MYC.

b) What kind of protein is MYC, and why is it so central for multiple hallmarks, including sustained proliferative signaling?
Answer: MYC is transcription factor of the basic helix-loop-helix (bHLH) family (here too, there are as many as 3 genes, including C-, N-, and L-MYC, where the letters stand for Cellular, Neural, and Lung carcinoma-derived). It binds to thousands of cancer-relevant target genes to either dampen or enhance their baseline expression, including CCND1 and several other cell cycle genes.

c) Which enzyme controls the degradation of cytoplasmic β-catenin?
Answer: The glycogen synthase kinase 3β. Phosphorylation by GSK3 primes β-catenin for subsequent degradation by the 26S proteasome.

2) Reasoning/deduction

Proteins of the JAK family are cytoplasmic tyrosine kinases. How do they become activated, and why does this mechanism make it more complicated and more challenging to find *safe* inhibitory drugs than in the case of RTKs?
Answer: JAKs are activated by autophosphorylation upon binding to ligand-activated cytokine receptors. Since a *plethora* of cytokine receptors all activate and signal through only a handful of JAKs, systemic delivery of any inhibitor of JAK/STAT signal transduction is bound to cause immune-related problems by messing up the delicate balance among pro- and anti-inflammatory functions in multiple immune cell types.

3) Exam-style MCQ:
Which one of the following statements about oncogenic mutations is ***correct***:

A. Over 90% of oncogenic mutations in APC increase its affinity for β-catenin
B. Mutations in β-catenin are oncogenic if they promote its degradation
C. Oncogenic mutations in β-catenin are not randomly distributed across the protein
D. Small molecule inhibitors of the kinase GSK3β are used to inhibit β-catenin
E. *LGR5* encodes a trans-membrane protein that binds frizzled receptors of WNT to mediate negative feedback

Answer: C. For E, note that LGR5 does the opposite: It is induced by WNT/β-cat signaling to mediate *positive* feedback by forming a complex with R-spondin that stabilizes frizzled at the plasma membrane.

4) Role of Wnt signaling in colorectal cancer

a) What findings in humans and in mouse models that we discussed in class speak for or against the notion that hyperactivation of canonical Wnt signaling mediates an *early* step in tumorigenesis?

Sustained WNT signaling after APC loss in FAP patients initiates the growth of benign adenomas (polyps). Additional cancer hallmarks have to be acquired before these benign lesions can progress to full-blown cancer. Loss of APC either by conditional knockout specifically in CBCs, or after deletion of the remaining wild-type allele in $APC^{Min/+}$ mice similarly gave rise to adenomas also in mice. Together, these observations strongly suggest that APC inactivation and the resulting sustained proliferation signaling are likely an early event in colorectal cancer formation.

b) You conducted an immunostaining of β-catenin on a histological section of mouse intestine and obtained the result shown on the right.

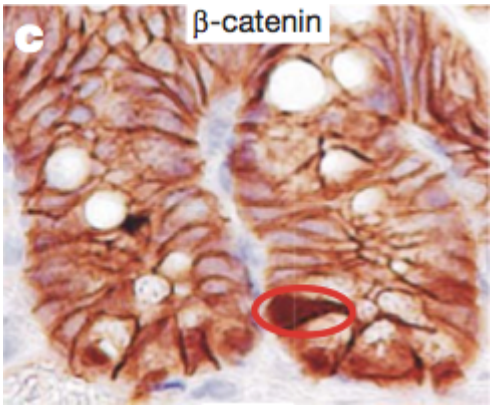

Figure 1. Immunihistochemical staining of β-catenin in two crypts of Lieberkühn.

What would be the most likely explanation(s) for the increased staining in the cell marked by a red circle?

A. downregulation of a diffusible Wnt antagonist
B. increased transcription of Tcf
C. somatic mutation of the β-catenin destruction complex
D. Germ line mutation of GSK3β
E. increased expression of a Wnt protein

Answer: Only C is correct. Accumulation of excess β-catenin in a single cell indicates a cell autonomous defect in its turnover (degradation).

A is incorrect because a soluble factor such as sFRP would inhibit Wnt not in only one cell.

B: No, because Tcf is not limiting for β-catenin stability.

D: GSK-3β does indeed prime β-catenin for degradation, but its mutation in the germ line would not only affect a single cell

E: Increased levels of WNT would be expected to affect β-catenin not in only one cell.

5) Data interpretation: Newly emerging anti-cancer drugs targeting WNT signaling.

Phosphorylation by GSK3β normally is essential for β-catenin to bind its E3 ubiquitin ligase β-TrCP (E3 ligases covalently attach specific lysine residues of their substrates to a polymer of the 7 kDa protein ubiquitin (polyUb) which marks them for proteasomal degradation). As shown in **figure 2a**, GSK3β sequentially phosphorylates several residues on β-catenin after phosphorylation of the nearby threonine 41 sites by casein kinase 1δ or 1ε (not covered in class). In a screen for small molecules that might be able to "glue" even the GSK3β-resistant mutant oncogenic β-catenin to its E3 ligase, a recent study identified several candidates, including NRX-1933, which was then crystallized in a ternary complex with purified β-cat and β-TrCp (**Fig. 2b**). Subsequent modifications based on the crystal structure data identified higher affinity binders, including NRX-252114 (**Fig. 2c**).

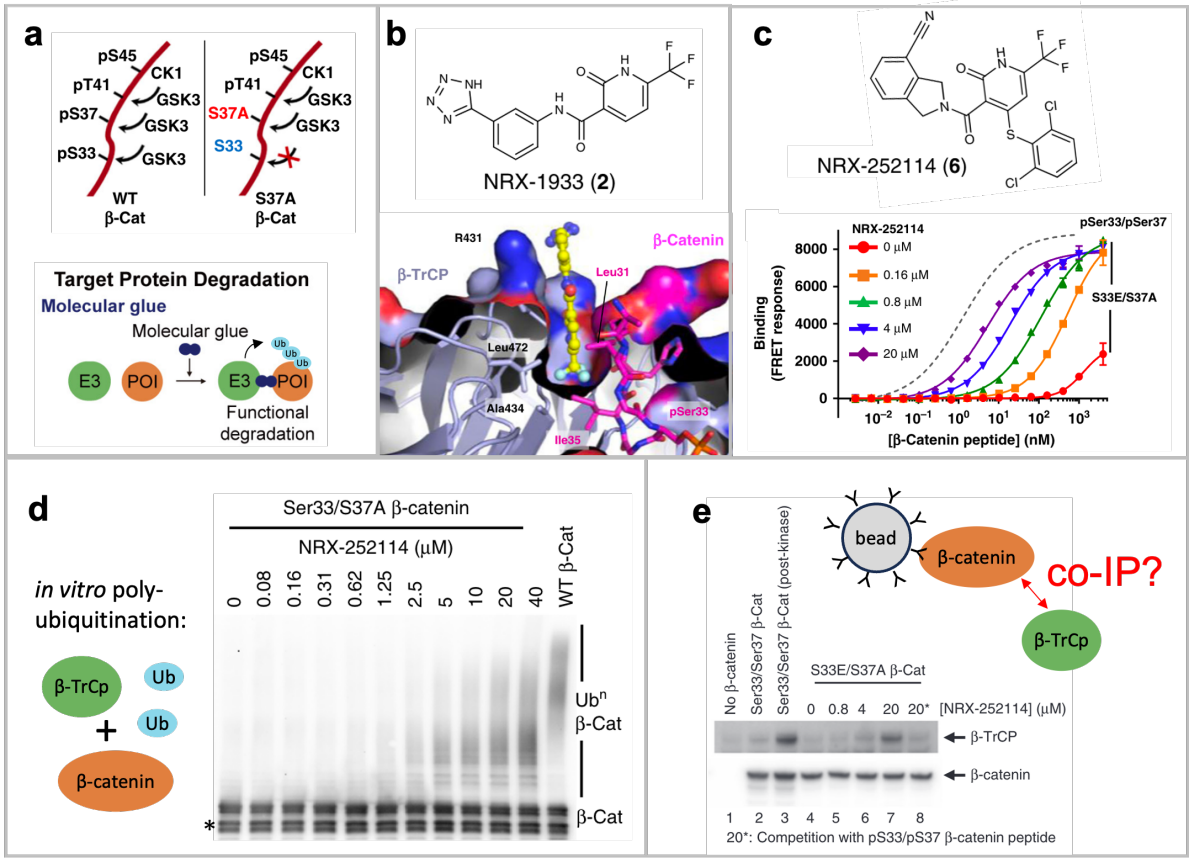

Figure 2. *Analysis of NRX-25114 as a molecular glue to target oncogenic mutant β-catenin for degradation.*

a) *Schematic of the proposed phosphorylation cascade for WT and S37A, S33E/S37A mutant β-catenin. Below: Strategy to restore the proteasomal degradation of oncogenic mutant β-catenin by linking it via a small molecule (molecular glue) to the Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TrCp) that normally binds and polyubiquitinates only wild-type β-catenin.*

b) *Chemical structure of one of the initial candidate molecular glue compounds, NRX-1933. Below: Cutaway view of a hydrophobic pocket occupied by NRX-1933 in the crystal structure of a complex of β-TrCP with β-catenin.*

c) *Chemical structure of an optimized molecular glue compound (NRX-252114). Below: A FRET-based assay to measure dose-dependent binding of a S33E/S37A mutant β-catenin peptide to purified β-TrCP in presence of varying NRX-252114 concentrations. The binding curve for the correctly phosphorylated control peptide pSer33/pSer37 from wild-type β-catenin is shown as a stippled grey line. FRET: Förster resonance energy transfer.*

d) *Western blot of purified wild-type (WT) or S37A mutant β-catenin that were treated with β-TrCp with or without NRX-25114. Asterisk: A non-specific degradation product.*

e) *Co-immunoprecipitation analysis of β-TrCp binding (red arrow) to WT or S33E, S37A mutant β-catenin on beads coated with anti-β-catenin antibodies. To estimate binding, bead eluates were analyzed by immunoblotting for the presence of β-catenin (bottom) and of co-immunoprecipitated β-TrCp (top). Where indicated, β-catenin was phosphorylated on S33 and S37 by a mix of GSK3, CK1 and Axin prior to loading on beads.*

i) To test NRX-252114 efficacy, the authors assessed its impact on the *in vitro* binding of a fixed amount of β-TrCp to increasing concentrations of a peptide comprising residues 17-48 of β-catenin that was either phosphorylated or mutated on the indicated residues (**Fig. 2c**). If you compare the colored curves in Fig. 2c with the stippled grey line, what can you conclude about the efficacy of this modified NRX compound?

Answer: As expected, NRX-252114 dose-dependently enhanced the binding affinity of the S33E/S37A β-catenin peptide for β-TrCP. At the highest NRX dosage (20 μM, purple curve), binding of the phosphomutant β-cat peptide was still below that of doubly-phosphorylated WT peptide (stippled line), but not very much (Kd 6 nM vs 2 nM). Therefore, this compound holds considerable potential as a novel inhibitor of oncogenic mutant β-catenin. However, serious caveats remain (see below).

ii) Figure 2c does not show if NRX also influences the binding of β-TrCp to *phosphorylated* WT peptide. If it does, would this increase or diminish the therapeutic potential or safety of NRX as an anti-cancer drug? Why or why not?

Answer: To avoid devastating side effects of existing WNT inhibitors on healthy stem cells during normal tissue homeostasis, ongoing efforts focus on drugs which ideally will inhibit canonical WNT/β-catenin signaling specifically in cancer cells but not in healthy tissues. If NRX would further enhance binding of β-TrCp to even the phosphorylated β-catenin, one would not expect any adverse effects, because the phosphorylated β-catenin will still be ubiquitinated and degraded anyway, regardless of NRX.

However, the chief safety concern of all WNT-targeting drugs remains unaddressed in this study, namely whether NRX can be administered *in vivo* without unduly depleting even the *wild-type* β-catenin in healthy tissues.

iii) Treatment with increasing concentrations of NRX was essential for the indicated phosphosite mutant β-catenin to become polyubiquitinated by purified β-TrCP in cell-free assays (Fig. 2d), correlating with significant binding of these proteins to each other (Fig. 2e). In lane 8 of panel (e), the indicated phosphopeptide was added at a molar ratio of 3:1 relative to the phosphomutant full-length β-catenin protein.

Describe the purpose of each of the various specificity controls in lanes 1-3 and 8 what they are supposed to estimate:

Lane 1: Estimates the background of non-specific binding of…

Lane 2: Estimates…

Lane 3:

Lane 8:

Answer: This question serves primarily to remind you of coIP assays, or to introduce them in case you have not yet heard of it.
Lane 1: Estimates the background of non-specific binding of β-TrCp to “empty” anti-β-Cat beads.
Lane 2: Estimates the amount of non-specific binding of β-TrCp to *un*phosphorylated β-Cat
Lane 3: Estimates the increase of specific β-TrCp binding induced by β-Cat phosphorylation
Lane 8: Inhibition of the β-TrCp co-IP by excess phosphorylated peptide suggests that the

corresponding region in full-length β-Cat is essential for NRX to stabilize the ternary complex.

iv) Unlike traditional small molecule inhibitors, molecular glues and PROTACs have to bind not only one but *two* specific target molecules to link those to each other. In terms of thermodynamics, how do you expect this to enhance or diminish drug efficacy?

Answer. The binding affinity of the drug (or its concentration) will have to be even higher than for conventional targeted drugs to compensate for the increased entropic penalty associated with ternary complex formation.