Exercise: Evading growth suppressors

1) Concepts:

a) Heterozygous heritable mutations in tumor suppressors can predispose entire families to an increased cancer risk. Why does not every family member always develop cancer?

A single wild-type copy of a tumor suppressor gene (TSG) is usually sufficient for its function (though *not absolutely* always, see question 3). Since loss of heterozygosity (LOH) of the remaining WT allele occurs only at a limited frequency, it may not occur in premalignant cells of all individuals within their lifetime.

Furthermore, as predicted by Carl Nordling's epidemiological data, additional mutations in other genes are necessary to develop multiple hallmark capabilities.

b) Restoring a growth *suppressor* that was deleted is even more difficult than inhibiting a factor such as EGFR or KRAS that acquire gain-of-function mutations to become oncogenic. What therapeutic strategies might still be worth considering:

i. if a tumor suppressor has been *deleted*?

Gene therapy (e.g. by viral delivery): Conceivable in principle, but extremely challenging in reality. Achieving complete transduction of all cells of a given cancer is unrealistic even with viral vectors. In addition, cancer cells tend to quickly delete a transgene whenever it does not provide a selective growth advantage.

Probably more realistic: Instead of restoring the TSG, one could try to manipulate one of its essential targets (e.g. inhibit E2F in case of loss of RB1)

ii. if a tumor suppressor has been silenced *epigenetically* (e.g. by promoter methylation, or by upregulation of specific miRNAs)?

E.g. pharmacological inhibitors of DNMTs or HDACs to induce promoter demethylation. Emerging industry: Modified oligonucleotide drugs such as "antimirs" that block specific miRNAs.

iii. if a tumor suppressor is inactivated by opposing signals (e.g. Akt → Mdm2 –| p53)?

Insights into molecular mechanisms that regulate a specific TSG are exploited to interfere with negative regulators. Example: MDM2 inhibitors or Sirt1 inhibitors to resurrect p53. Limitations: This can only work in tumors that have *not* deleted p53 already. And applying a p53-activating drug will increase the selective pressure on cancer cells to quickly evolve drug resistance.

iv. What complications would you predict to arise from drug treatments that target MDM2 (s. slide 33)?

MDM2 inhibitors should be expected to interfere with (wild-type) p53 degradation in any tissues where p53 is induced, not only in cancer cells, thereby putting those normal cells at risk of entering apoptosis. Furthermore, MDM2 inhibitors exert great selective pressure on p53 wild-type tumors to mutate p53. Indeed, mutations in the DNA-binding domain drive the rapid development of resistance to nutlins.

v. Other drugs under development seek to restore tumor-suppressive activity of *mutant* p53 protein, or increase its degradation (slide 35). What advantages or risks/disadvantages would you predict for either of these approaches?

Drugs to rescue a wild-type conformation in mutant p53 to rescue DNA binding would be ideal because they could restore the proper feedback inhibition of p53 mediated by upregulation of one of its target genes, *MDM2*.

By contrast, drugs that aim to increase the degradation of *mutant* p53 (and associated oncogenic functions that remain incompletely understood) will also destabilize wild-type p53, thereby increasing the cancer risk in healthy tissues independently of p53 mutations.

2) Former exam MCQ: Tumor suppression can be compromised by any of the following, ***except***:

A. by mutations in SMAD2 or SMAD3.
B. binding of SMAD2 or SMAD3 to SMAD4.
C. Hyperphosphorylation of the retinoblastoma protein RB1.
D. Loss of heterozygosity of APC.
E. mutations in the TGF-beta receptor type II.

B: All of these events interfere with tumor-suppressive signaling and thus are tumorigenic, except B. By contrast, binding of receptor-activated SMADs to the co-SMAD (SMAD4) is required to induce a cytostatic (tumor-suppressive) response, including the expression of p21 and/or other CDK inhibitors.

3) Reasoning, deduction:

Knudson's 2-hit hypothesis states that both alleles of a tumor suppressor gene (TSG) must be mutated to disrupt its protective function. Mouse Rb1 (also known as pRb) and the human homolog RB1 fulfill this prediction. However, there are important exceptions: For some TSGs, haploidy reduces the dosage of the corresponding protein beyond a critical level that is needed for proper functioning. Such a gene is called "**haploinsufficient**" and the resulting phenomenon is "haploinsufficiency". In some cases, mutations can even have **"dominant negative"** effects if the mutant protein blocks the residual wild-type form in heterozygous cells.

a) Considering what is known about p53 feedback regulation by MDM2, do you expect p53 deletions to be haploinsufficient? Why or why not?

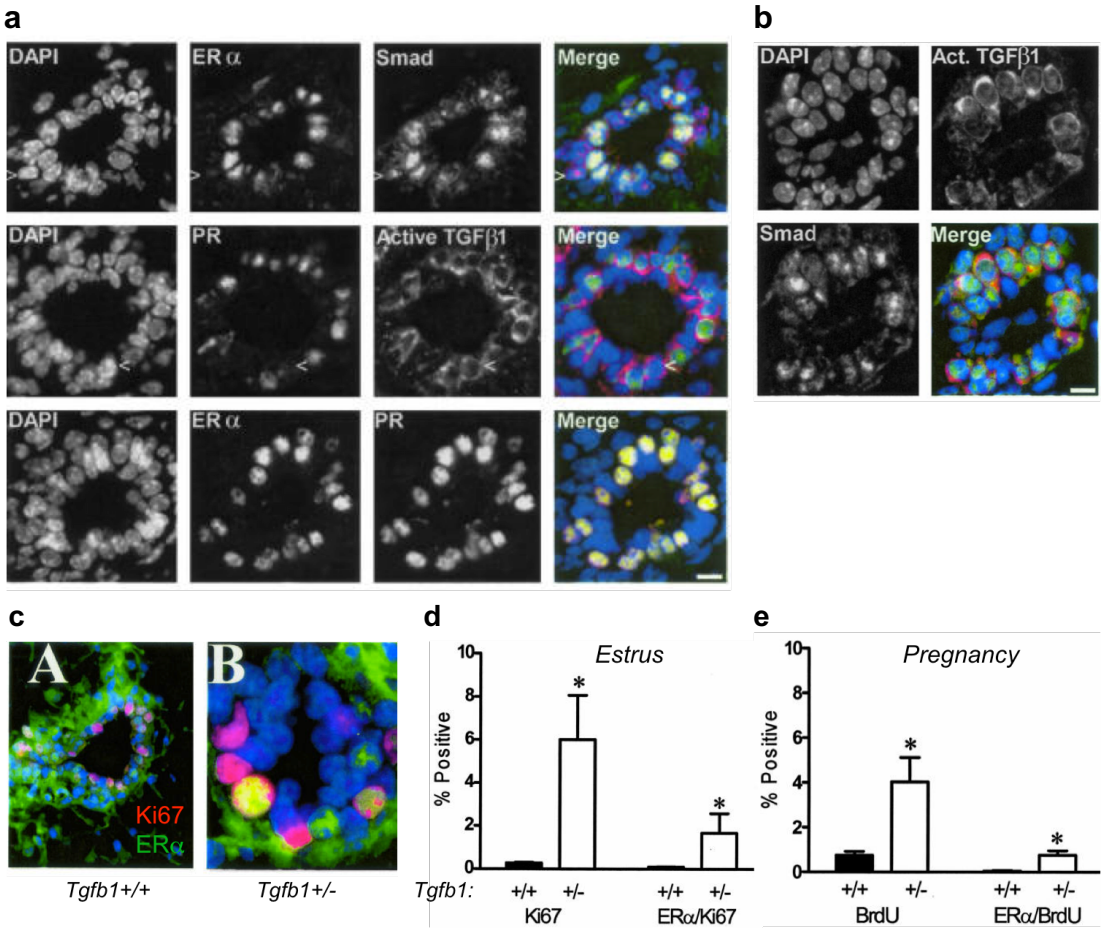
No. Reduction of the gene dosage of p53 by half in heterozygous cells that have only one copy of p53 should be compensated by a corresponding reduction in the expression of the p53 target gene MDM2 which encodes a ubiquitin ligase to target p53 for degradation. Because of feedback regulation, many proteins still accumulate at normal levels even if one copy of the gene is missing.

b) 96% of the p53 mutant cancers delete one copy of p53 whereas the other acquires a point mutation. Should we expect these point mutants to act as dominant negatives? Why or why not?

p53 binds to itself and functions as a tetramer. Residual wild-type p53 will be inhibited in tetramers containing transcriptionally inactive mutant subunits. However, one wild-type copy of *TP53* remains sufficiently active to induce MDM2 thereby keeping the levels of mutant p53 low enough so that a dominant negative effect on wild-type p53 remains too weak to manifest phenotypically.

4) Data interpretation & testing hypotheses

Background information: Mammals have three TGFβ genes (isoforms 1, 2 and 3) that signal through the same receptors, but most cancer research has been conducted on TGF-β1. Epithelial cells store secreted TGF-β1 in their extracellular matrix as a latent complex. Dissociation from an inhibitory prodomain in this latent complex is tightly regulated to control when and where the active form is released to bind and stimulate TGF-β receptors, e.g. to thereby inhibit the cell cycle. On the other hand, entry into the cell cycle requires specific proliferation signals, mediated e.g. by RTKs. In normal mammary glands, the production of important proliferation signals is governed by the hormones progesterone and estrogen and their "nuclear receptors" ER and PR that function as transcription factors upon arrival in the nucleus.



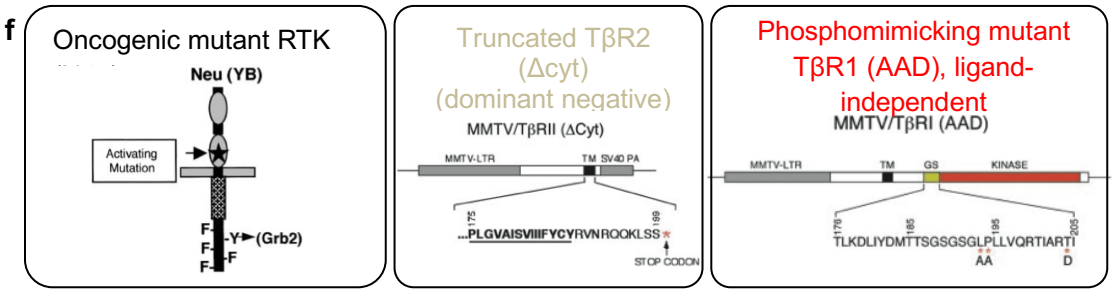


Figure 1. TGF-β signaling in adult mouse mammary epithelium. a, b) Double immunostainings of the indicated proteins, superimposed to nuclear staining of DNA by DAPI in transverse sections through mammary ducts. ERα: Estrogen receptor α; PR: progesterone receptor; Smad: Antibody that binds phosphorylated Smad2 and Smad3; Active TGF-β1: Antibody that stains the active growth factor. **c)** Co-immunostaining of ERα and Ki67, a marker of proliferating cells in S-phase. **d)** Quantification of Ki67 single positive and Ki67/ERα double positive mammary epithelial cells at the time of ovulation (Estrus). **e)** Quantification of ERα-negative and ERα-positive cells in S-phase, marked by incorporation of the thymidine analog BrdU during pregnancy. **f)** Transgenes introduced in mice to evaluate a tumor-suppressive role of TGF-β signaling in mammary epithelial cells.

a) Immunofluorescent labelling by antibodies that specifically bind active TGF-β1 but not the latent form stain only a subpopulation of cells in the mammary epithelium. What distinguishes these TGF-$\beta1^{+}$ cells from their neighbors in the histological sections shown in **figure 1a, b**?

"Active TGF-β1" represents only a fraction of the total TGF-β, because the bulk of secreted TGF-β remains in a latent form that cannot bind receptors. In panel B, the cells stained by an "active TGF-β1"-specific antibody were found to be the same as the ones stained by an antibody that reacts with phospho-Smad2 and phospho-Smad3. Interestingly, the same cells also express estrogen receptor α (panel A, top row), the receptor for the main hormonal stimulus of cell proliferation in the mammary gland. They also include the PR+ subset of cells.

b) Some sections in **figure 1a, b** were stained by antibodies against phospho-Smad2&3 (p-Smad), together with anti-ERα or with anti-TGF-β. Based on what we discussed in the lecture (and considering that TGF-β is a *secreted* factor), which cells would you have predicted to stain positive for p-Smad?

If secreted TGF-β were freely soluble, one would expect it to signal both in and around the cells producing it. In that case, nuclear p-Smad staining would be relatively uniform. Moreover, anti-proliferative TGF-β signaling was predicted to inhibit all cells, except perhaps those that receive a hormonal growth stimulus (i.e. ERα/PR positive cells).

How do the results in **figure 1a, b** compare to your prediction, and what does it reveal about which mammary epithelial cells might depend on TGF-β to limit their proliferation?

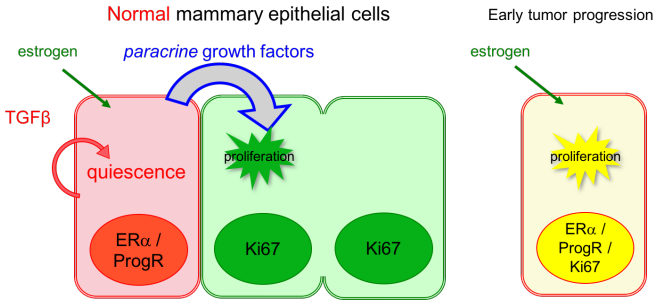
In contrast to what is predicted for a freely diffusible factor, TGF-β only induced Smad2,3 phosphorylation (pSmad2,3) in those mammary epithelial cells that activated the latent form. This result suggests that in healthy mammary epithelium, TGF-β1 does not freely diffuse, but rather signals in an autocrine manner in only those cells that can activate it.

Furthermore, the fact that p-Smad is induced in the hormone receptor-positive cells suggests that it may selectively inhibit the proliferation of these but not other cells.

c) To evaluate the influence of impaired TGF-β signaling on cell proliferation, mouse mammary glands were stained for the S-phase markers Ki67 (**Fig. 1c, d**) or bromo-deoxyuridine (BrdU), a thymidine analog that can be injected into mice and stained after incorporation into DNA of dividing cells using anti-BrdU antibodies (**Fig. 1e**). What do these data reveal about the role of TGF-β signaling in mammary epithelial cells?

Reducing the gene dosage of TGF-β1 increases the proliferation of estrogen receptor (ERα)-positive mammary gland epithelial cells, as determined by Ki67 staining and BrdU incoporation both during estrus phase and pregnancy (i.e. when estrogen levels rise). This result indicates that TGF-β1 expression is required in the mammary gland to prevent excessive hormone-induced cell divisions.

By limiting the number of ERα+ cells, TGF-β1 *indirectly* also reduces the proliferation of other cells (myoepithelial stem cells *and* hormone receptor-negative luminal progenitors), because the ERα+ cells are a major source of growth factors that signal in a paracrine manner to their neighbors when stimulated by estrogen. Escape from TGF-β-induced growth arrest thus is thought to be a critical step for the growth of ERα+ breast cancers:



d) To test the role of TGF-β signaling in a breast cancer model, researchers crossed into MMTV-Neu transgenic tumor mice a second transgene encoding either C-terminally truncated TGF-β type II receptor (Δcyt), or AAD mutant TGF-β type I receptor where the threonine residue that is subject to phosphorylation by type II receptors was deliberately substituted by aspartic acid (D) to mimic the structure of phospho-threonine (**Fig. 1f**).

- How do you predict each of these mutant type II or type I receptors to alter endogenous TGF-β signaling strength?
  TGFBR2_Δcyt competes with wild-type TGFBR2 for its ligands but lacks the cytosolic Ser/Thr kinase domain that normally would trans-phosphorylate the type I receptor. Therefore, the Δcyt mutant cannot signal and instead blocks the activation of wild-type TGFBR2 in a dominant negative manner. By contrast, the Thr>Asp mutation (AAD) in TGFBR1 serves to structurally mimic the threonine phosphorylation by TGFBR2. Thus, even in cells that receive no active TGF-β, the AAD mutant TGFBR1 will phosphorylate SMAD2 and SMAD3 and other substrates "constitutively", i.e. independently of ligand.

- What experiment would you propose to quickly validate your predictions?
  You could express each mutant in breast cancer and other cell lines *in vitro* and in transgenic mouse models or tumor grafts. Then test in Western blots and immunostainings whether they can inhibit or stimulate, respectively, the

phosphorylation of SMAD2 and SMAD3, and the expression of CDK inhibitors, and whether they influence cell proliferation (e.g. Ki67 staining, or BrdU incorporation).

- Predict for each of these TGF-β receptor transgenes whether they will accelerate or slow the growth of MMTV::Neu-induced breast tumors.
  TGFBR2_Δcyt has been shown to accelerate tumor growth (by inhibiting endogenous cytostatic TGF-β signals), whereas TGFBR1_AAD slowed it.