Excercise: Anti-apoptotic survival signaling

1) Distinctive features of apoptosis

a. Several assays exist to distinguish apoptotic cells from other dying cells. What characteristic feature is detected by each of the following techniques:

- Agarose gel electrophoresis: Non-random DNA fragmentation (180 bp ladder)
- Annexin V staining: Externalization of phosphatidylserine to the outer leaflet of the plasma membrane
- Caspase-3 immunostaining: Specifically stains the mature (cleaved) form that is derived from procaspase-3 by initiator caspases (Casp8, 9)
- TUNEL assay: Accumulation of free 3' ends due to DNA fragmentation

b. Why is it advantageous for multicellular organisms to eliminate supernumerary cells by apoptosis rather than by specific necrosis-inducing factors?
Cells that die by apoptosis do not release their content into the surrounding tissue, but instead retain it in membrane blebs. This prevents the activation of macrophages and inflammation-induced tissue damage.

c. What factors regulate the clearance of debris from apoptotic cells by phagocytes?
The surface of apoptotic cells displays specific "eat-me" signals such as phosphatidylserine at the surface, whereas they down-regulate "don't-eat-me" signals such as CD31.

2) Which of these statements about survival signaling and its deregulation in cancer cells is ***true***:

A. It involves numerous effects of AKT, including the downregulation of mTORC2
B. No cell survival signals mediated by mTORC1 are altered in cancer cells treated by rapalogs
C. It plays a key role in tumor recurrence after chemotherapy
D. It is facilitated by the loss of cell attachment to the ECM
E. It relies on the ability of cancer cells to adhere to one another via specific integrins

3) Role of apoptosis and its suppression in cancer

a. At what stage(s) of tumor progression or during the invasion & metastasis cascade are oncogenic cell survival signals essential?
Apoptotic signaling pathways typically remain functional and limit tumor growth in early primary lesions (disguising the true extent of hyperproliferation and the associated increased potential for acquiring new mutations during DNA replication).
By contrast, suppression of apoptosis is essential for metastasis at an early step, i.e. at least from the moment where they enter the circulation (intravasation). This is because once the cancer cells enter into the circulation, they lack survival signals mediated by integrin adhesion to the ECM of their initial niche. And during subsequent extravasation and seeding, they also have to remodel and adapt to their new microenvironment to

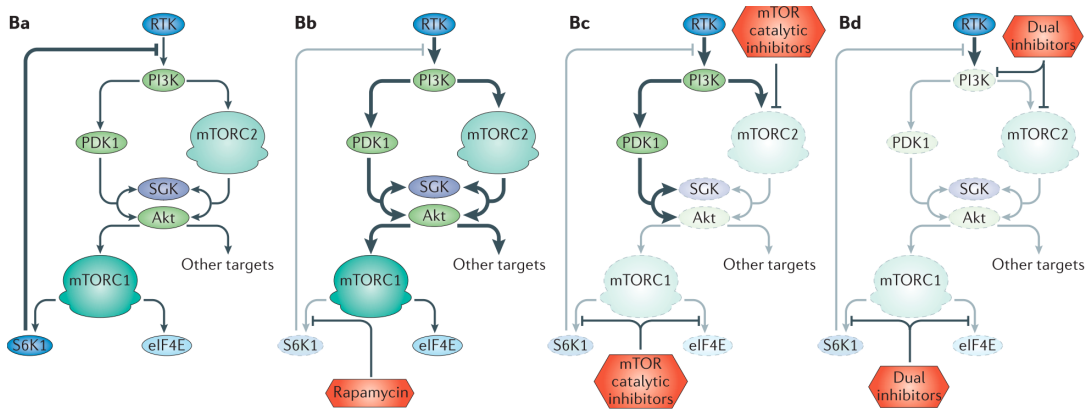
mobilize the support that they need to survive (growth factors, re-adhesion to ECM at the metastatic site, nutrients,…).

b. How does apoptosis and its evasion by oncogenic cell survival signals influence the response to chemo- or radiotherapy?
The ability of chemo- and radiotherapies to induce apoptosis is critical for therapy response/efficacy. The hallmark capability 'Evasion of apoptosis' at this stage promotes tumor recurrence and subsequent drug resistance.

c. From the Weinberg textbook: In a study of 35 primary lung and colon tumors, half of the tumors amplified and overexpressed a gene for a secreted protein that sequesters the ligand of Fas. How do you expect this protein to affect tumor surveillance by the immune system?
Secretion of Fas ligand enables cytotoxic lymphocytes (CTL) to induce apoptosis in tumor cells that express the proapoptotic receptor Fas. Cancer cells that manage to inactivate the Fas-ligand by an antagonistic secreted factor thus can evade CTL-induced apoptosis even if they still express Fas and tumor antigens.

4) "Application": Therapeutic inhibition of survival signaling

a. The first generation of mTOR inhibitors including Sirolimus (Rapamycin) and related "rapalogs" only partially inhibit mTORC1, but not mTORC2. Considering the schema of mTOR signaling regulation below, how can one explain that rapalogs nevertheless tend to increase cancer cell survival signaling by mTOR rather than blocking it?



Rapalogs block a negative feedback loop mediated by the inhibitory effect of S6K1 on PI3K. PI3K in turn stimulates mTORC2. When only mTORC1 is inhibited (Bb), both PI3K/Akt and mTORC2-mediated survival signals increase due to loss of negative feedback regulation. Thus, rapalogs do not inhibit but rather activate mTORC2 and its oncogenic function mediated by Akt and by the Akt-related Serum Glucocorticoid Kinase (SGK).

b. What would you expect to be the advantages and potential disadvantages of the second generation mTOR inhibitors in panels Bc and Bd?
Bc, advantage: 'Catalytic' mTOR inhibitors (compounds that compete with ATP for mTOR binding) also inhibit mTORC2 and block mTORC1 more completely than what is seen with the allosteric inhibitor Rapamycin.
Disadvantage: More complete mTOR inhibition comes at the cost of increased risk of side effects (e.g. in insulin signaling; impairment of anti-tumor immunity; stimulation of

unwanted autophagy).

Bd, advantage: Dual specificity PI3K/mTOR inhibitors (in development) simultaneously block mTOR as well as PI3K/Akt survival signals (Bd).
Disadvantage: This broader specificity comes at the cost of increased risk of side effects (s. above).

RapaLink-1 (3rd generation) can completely inhibit mTORC1, but the risk of the above side effects remains to be evaluated.

5) Regulation of apoptosis

Cultured skin cells from three patients (X, Y, Z) were exposed to strong UV radiation. Subsequent monitoring in culture revealed that these UV-irradiated cells behaved as follows:

Hours after UV	X	Y	Z
24	Alive	Alive	Apoptotic
48	Necrotic; cytosol contains cytochrome C and other mitochondrial proteins; no active Casp3	Alive; no mitochondrial proteins in the cytosol; no active caspases	
96		Alive	

a. What molecular defects would explain the transient and the complete resistance to UV radiation in X or Y cells, respectively? In other words, what alterations in apoptotic signal transduction proteins could account for the X and Y cell phenotypes? Hint: Use the maps of known cell death signaling pathways, e.g. on slides 16 and 42-46.
In X cells, cytosolic cytochrome C indicates that mitochondria leaked their content. Nevertheless, the executioner Caspase Casp3 remained inactive. Together, these observations imply that the release of mitochondrial content failed to activate the initiator caspase Casp9. This could result from lack of apoptosome formation (required for Casp9 dimerization, e.g. due to loss of Apaf1), or due to Akt phosphorylation of Casp9.
In Y cells, the mitochondrial membrane remained impermeable. This indicates that the proapoptic **BH3-only factors** (Puma, Bim, Noxa, …) remained inactive (e.g., loss of p53 function leads to failure of Puma induction). Alternatively, the action of BH3-only proteins may be blocked by elevated expression of anti-apoptotic Bcl-2 or Bcl-X_L. A third possibility is that Y cells lost the expression or function of the BH123 proteins Bax or Bak or their ability to heterooligomerize.

b. After UV treatment, the cells from patient X eventually still died, but by necrosis (i.e. independently of apoptosis) and only after a delay of 48 hrs. How do you explain this outcome?
Release of cytochrome C by cells from patient X indicates that their mitochondria are damaged. Impaired mitochondrial function will lead to ATP depletion and disruption of the redox balance, which explains why cells will eventually die.