QUESTION 1 (25 points)

Epidermal growth factor (EGF), a peptide hormone, binds to the epidermal growth factor receptor (EGF-R), which is expressed on the cell surface of particular cell types. EGF-R is a member of the receptor tyrosine kinase family, and the subsequent signal transduction cascade is summarized in a schematic diagram below.

Ras in turn activates Raf (MAPKKK), which phosphorylates MEK (MAPKK), which phosphorylates MAPK, which phosphorylates effectors.

A. (5 pts) Describe the expected effect that the following alterations in the system would have on Ras-mediated MAPK signaling output.

1. a mutation in Ras that locks it in the GTP-bound form (that is unable to change back to the GDP-bound form)
   - Hyperactivated signaling (more activated Ras)

2) overexpression of Ras-GAP (GTPase-activating protein)
   - less signaling (less activated Ras)

3) overexpression of Sos, Ras-GNEF (guanine nucleotide exchange factor)
   - hyperactivated (more chance that Sos encounters Ras)

- full credit for "no effect, because Sos needs to be recruited by activated Grb2"

-- 1/2 pt for "no effect" with no explanation

4) introduction of a truncated EGF-R which lacks the extracellular domain and is constitutively autophosphorylated
   - constitutively activated (regardless of EGF addition)

5) overexpression of mutant Grb2 protein that lacks the SH3 domain
   - mutant acts as dominant negative -> less signaling

B. (3 pts) Describe briefly the experiment demonstrating that receptor dimerization is the mechanism by which EGF-R autophosphorylation and activation occurs.

Two acceptable answers:

1) Add antibody to cross-link/dimerize EGF-R in absence of EGF and check phosphorylation of EGF-R -> receptors are phosphorylated

2) Express extracellular domain of EGF-R without the kinase domain (along with the wild-type EGF-R) -> check phosphorylation or wild-type EGF-R -> kinase domain - mutant should act as competitive inhibitor of phosphorylation

- "block dimerization and see receptor is no longer phosphorylated" got 1 pt, because, while that data correlates dimerization with autophosphorylation, it does not demonstrate that
dimerization is the mechanism (i.e., is sufficient) for receptor phosphorylation.

- "delete a copy of EGF-R such that it exists only as a monomer" received no credit, because the EGF-R is a homodimer.

C. (6 pts) Examination of Grb2 protein sequence reveals that it contains an SH2 domain; previous research indicates that SH2 domain can mediate binding to proteins that are phosphorylated on tyrosine.

1. (3 pts) Design an experiment that addresses whether the SH2 domain of Grb2 is necessary for its interaction with the activated EGF receptor. What is the expected experimental data?
   - experiment:
     Delete/mutate SH2 domain of Grb2 (1 pt); add EGF;
     Immunoprecipitate/affinity column with EGF-R antibody (1 pt),
     Western blot with Grb2 antibody as probe.
   - expected experimental data:
     wild-type Grb2 -> is detected in EGF-R Immunoprecipitate
     SH2 - Grb2 -> not detected in EGF-R Immunoprecipitate (1 pt)
   - gel shift assay (retarded mobility upon complex formation) was accepted
   - co-immunofluorescence received 1/2 pt because it is not direct (locates two proteins in the same part of cell)

2. (3 pts) Using a similar experiment, how can you address that a particular tyrosine residue of the EGF-R, Y709, is required for interaction between EGF-R and Grb2?
   - Mutate Y709 of EGF-R to F709 (Phenylalanine) (2 pts); "delete Y709" was accepted>
   - and do IP (with Grb2 antibody);
   - interaction should be lost, i.e., EGF-R not detected in Grb2 IP (1 pt)

D. (6 pts) From Part C you determine that both the SH2 domain of Grb2 and Y709 of EGF-R are necessary for the interaction between the two proteins. You are interested to know whether the Grb2 SH2 domain has any sequence requirements in addition to Y709 in recognizing the EGF-R protein.

To address this question you make a synthetic peptide library including the wild-type sequence around Y709, YVPML, and sequences that are slightly altered from the wild-type sequence. You introduce an excess of the peptide library into the cell and ask whether the peptide can compete with binding between the EGF-R and Grb2 proteins. The results are shown in the table below:

Synthetic peptides competing for EGF-R <-> Grb2 binding

<table>
<thead>
<tr>
<th>peptide sequence</th>
<th>ability to block EGF-R/Grb2 interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>*p-YVPML</td>
<td>+</td>
</tr>
<tr>
<td>YVPML (unphosphorylated Y)</td>
<td>-</td>
</tr>
<tr>
<td>*p-YGPML</td>
<td>-</td>
</tr>
<tr>
<td>*p-YVPAL</td>
<td>-</td>
</tr>
<tr>
<td>*p-YVAML</td>
<td>+</td>
</tr>
<tr>
<td>*p-YVGML</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: *p- represents phosphorylation on the tyrosine.

What preliminary conclusions can you make about the sequence requirements on the EGF-R to mediate Grb2 binding? Specifically, what can you conclude about the status of the tyrosine and the identity of the amino acids around the tyrosine?

To bind to bind Grb2/SH2 domain:
Y709 of EGF-R needs to be phosphorylated (2 pts)
The flanking amino acids sequences contribute in recognition by Grb2 (1 pt).
Specifically, it seems: V required; M required; (2 pts)
P somewhat required (1 pt) (can be replaced with certain a.a. is but not with others)
- Those that said "L is required": -1 pt (don't know from this experiment)
- Those that said "the flanking amino acids are important" without specific description: -2 pts

E. (5 pts) Previous research has shown that upon addition of EGF, the MEK (MAPKK) is phosphorylated on two serine residues: S218 and S222. Design an experiment to demonstrate that the phosphorylation of S218 and S222 are necessary for the activation of MEK kinase activity. Include the expected experimental result in your answer.

Hint: You know the in vivo substrate of MEK!
Mutate the serines of MEK: S218-, S222- , S218- S222- (2 pts);
introduce to system, add EGF, IP with MEK antibody, do in vitro kinase assay (1 pt);
specifically see if substrate MAPK (1 pt) can be phosphorylated
if serine phosphorylation is required, then the assay with the mutants should display no MAPK phosphorylation (1 pt).
Alternatively, treat MEK protein with specific phosphatases before kinase assay.
- Saying "use phosphorylated and unphosphorylated MEK" without specific method: - 1 pt
- Saying "look for MEK-MAPK interaction by IP" instead of kinase assay received no credit because the question asks for an experiment about MEK kinase activity.

QUESTION 2 (25 points)

I.
A. (1 pt) Label the subunits of the trimeric G protein. Don't forget to indicate which nucleotide is used in this reaction and where it binds.
B. (1 pt) On the same diagram, label the ON and OFF states for both Ras and the trimeric G protein. In both cases, State A (left side of reaction) is the OFF state. State B is the ON state.

C. (6 pts)

i. What chemical processes are diagramed in reactions 1, 2, 3, 4? (2 pts)

Reactions 1 and 3 are GTP exchange. Reactions 2 and 4 are GTP hydrolysis.

ii. For which reactions are additional protein factors required (2pts)?

What are these proteins (be as specific as possible) (2pts)?

- Reaction 1 requires a guanine exchange factor.
- Reaction 2 requires a GTP activating protein.
- Reaction 3 requires a GEF internal to the receptor.
- Reaction 4 requires a GAP intrinsic to Gas.

D. (1 pt) You have isolated a mutant in state "A" of both proteins. What is the effect on the downstream signaling pathway for Ras- "A"? For trimeric G protein - "A"?

Downstream signaling is turned off.

E.(1 pt) You have isolated a mutant in state "B" of both proteins. What is the effect on the downstream signaling pathway for Ras- "B"? For trimeric G protein - "B"?
II.

F. What receptor is involved in the activation of Ras?
   Receptor tyrosine kinase

G. (1pt) What receptor is involved in the activation of trimeric G protein?
   7 Transmembrane Receptor

H. (3 pts) Draw the pathway that leads to the activation of Ras. Label both extracellular and intracellular signals. Sketch the downstream signaling pathway and effectors.

I. (3 pts) Draw the pathway that leads to the activation of trimeric G proteins. Label both extracellular and intracellular signals. Sketch the downstream signaling pathway and effectors.
You have raised many different antibodies against both the receptor for Ras and against the receptor for a trimeric G protein. You find that none of the antibodies raised against the receptor for the trimeric G protein are able to activate the downstream signaling pathway. You also find that all of the antibodies raised against the receptor for Ras are able to activate the downstream signaling. Further studies suggest this to be indicative of the normal functioning of these receptors.

J. (3 pts) What are the similarities and differences in the mechanisms each receptor uses to transduce a signal?

The RTK is activated through dimerization, while the 7TMR acts as a monomer.

K. (3 pts) Why is an antibody against the receptor for the trimeric G protein less likely than an antibody against the receptor for Ras to be able to activate downstream signaling?

The antibody is more likely to activate the RTK than the 7TMR. The antibody can bind two RTK receptors and bring them into close proximity, facilitating the dimerization that leads to receptor phosphorylation in trans and activation. It would be more difficult to imagine how the binding of an antibody could facilitate an activation change in conformation of the 7 TMR.

QUESTION 3 (25 points)
Step 1 Step 2 Step 3 Step 4

A. (6 pts) Name a class of molecule and an example that could be mutated to cause the above progression of colon carcinogenesis.

Class of molecule

1 to 2 Anything having to do with proliferation or loss of the requirement for ECM
2 to 3 Anything to do with angiogenesis
3 to 4 Anything to do with cell morphology or an increase in genetic instability

B. (6 pts) Given that scientists believe that 6-8 steps (or changes) are required to form cancer, what single type of mutation could have the largest affect on the rate of cancer formation? Why?

Any mutation that affects genetic stability. This is due to the fact that one needs multiple mutations and any mutation that increases the chance for more mutations is favorable for the cancer cell.

There are two distinct types of inherited colon cancer in people that map to two distinct genes. Type I is characterized by young adults having hundreds of benign polyps (polyps in picture above), but a polypt only becomes cancerous only rarely, on average at middle age. Type II on the other hand is characterized by polypt formation being normal, but all polyps once formed become cancerous, on average by middle age.

C. (4 pts) What do you think is the fundamental difference between these two types of familial disease? (ie, what steps are affected?)

Type I has to do with initiation and Type II with progression. See diagram above.

D. (4 pts) Give an example of what types of genes could be mutated for Type I? Type II?

Type I Any gene involved in the first step above, so proliferation or ECM independence
Type II Any gene involved in genetic stability

E. (5 pts) How would you determine if these disease genes are oncogenes or tumor suppressors once you have cloned the genes?

There is more than one correct answer to this question:

1. You could introduce the cancerous form of the Type I and do the same for the Type II into 3T3 cells and see if you have colony formation. If so then the gene is an oncogene.
2. You could make a mouse knock out and determine if there is an increase in tumor incidence. If so then the gene is a tumor suppressor.
3. You could look at tumors from different people and determine if one or both alleles of the gene are mutated. If only one is affected the gene is likely an oncogene. Typically both alleles must be affected for a gene to be a tumor suppressor.

Question 4 (25 points)

You are studying a type of nasal tumor in the rare Norwegian man-eating aardvark. Alas for the aardvark, it is known that mutations in a single gene are sufficient to lead to this tumor. You culture cells of one such tumor from a particular animal and extract the DNA. You then transfect this DNA into 3T3 cells. Several days later, you note the formation of foci in the transfected cells but not the control cells.

A. (3 pts) What does this result tell you about the nature of the gene defective in this tumor?
This indicates that the gene defective in this tumor is a dominant-acting oncogene, since apparently putting the mutant copy into 3T3 cells is sufficient to transform these cells. Loss of function of both endogenous copies of a tumor supressor gene would be necessary to confer this phenotype.

B. (5 pts) In a CLEARLY and LEGIBLY labeled diagram, show how you would clone such the gene responsible for the tumors.

You could use a slight variation on the Weinberg focus-forming assay. Isolate DNA from the tumor and transfecit into 3T3 cells. Allow foci to form and grow up the cells from the foci. Isolate DNA from these foci and transfecit this into fresh 3T3 cells. Again culture the focus cells and isolate their genomic DNA. Repeating this procedure enriches for the original aardvark oncogene, since you select each time for the ability of the genomic DNA to promote focus formation. Eventually, most of the aardvark DNA still present consists of just the oncogene, and the genomic DNA from the last round of foci is cloned into lambda phage and plated on bacteria. The phage plaques are screened for the presence of aardvark DNA by probing for an aardvark repetitive element (the aardvark equivalent of ALU elements, for example). The vast majority of the phage clones will contain no aardvark DNA at all. Those phage clones that do hybridize to the aardvark repetitive probe can be isolated and their DNA inserts sequenced. This DNA can also be transfected into 3T3 cells to ensure that the transforming sequences have indeed been cloned.

Note that some variations on this answer were given full credit

The experiment in part B goes as planned and you isolate gene responsible, which you call MAT (for Man-eating Aardvark Tumor gene). You take this cloned gene and use it as probe to screen normal DNA as well as a panel of tumor DNA samples that you possess. To your surprise, you find that although the gene is present in the normal DNA sample, nearly every tumor sample lacks the MAT gene.

C. (4 pts) How does this change the model you presented in part A?

Oncogenes are dominantly-acting mutant forms of normal cellular genes that promote tumor formation. The fact that the MAT gene is absent from most tumor samples indicates that this gene cannot be acting dominantly to promote tumor formation in these samples, since it isn’t even present! Instead, this information argues that MAT acts as a tumor suppressor gene (TSG). TSGs normally act to prevent tumor formation, typically through stimulation of DNA repair, promoting cell death, or control of cell proliferation. Loss of function of both copies of a TSG can allow enhanced mutation rate, avoidance of apoptosis, or uncontrolled cell division and lead to cancer.

D. (2 pts) What would you expect to find if you compared the sequence of the gene you cloned in part B with the wild-type gene sequence?

The MAT copy you cloned in B must have contained a rare dominantly-acting mutation which allowed it to inhibit the function of the remaining wild-type MAT copy. Thus, cells which contain a MAT possessing such a dominant allele are functionally MAT- even though they may still posses a wild-type copy of this gene. Peter discussed this situation in class in the context of p53, where many mutations found in human cancers behave this way. For all this to be true, however, MAT must contain a mutation in the coding region which confers such dominant-negative behavior. Note that this mutation is NOT completely loss of function, since the mutant protein must still be able to interact with its target (either other molecules of MAT or another protein) to abrogate the effect of the wild-type MAT protein.

Nasal tumors are actually quite rare amongst the Norwegian man-eating aardvarks. However, careful laboratory studies have shown that in certain aardvark families, many animals develop these tumors and at a younger age. Among the progeny of an animal who develops such a tumor, approximately half develop tumors at an early age themselves.

E. (5 pts) Explain this observation.

This is the classic genetic behavior of a TSG. Affected individuals are born with one defective and one wild-type MAT copy. At some frequency, the wild-type copy of this gene is lost from a nasal cell, resulting in a cell with no good copies of MAT which goes on to form a tumor. Virtually every animal carrying such a MAT allele goes on to develop nasal cancer because while loss of the second MAT allele is rare on a cell-by-cell basis, given a large number of cells this event is a virtual certainty. Indeed, in humans bearing one defective copy of the Rb gene, multifocal retinoblastoma is often observed, even though the occurrence of even on of these tumors in a normal individual is extremely rare. In the case of our aardvarks, these mutant animals pass on the defective gene at a frequency of 50%
(as predicted by simple Mendelian genetics), and the progeny of these animals who inherit this allele will themselves show a high predisposition to tumors.

Note that vague statements about the genetic nature of cancer were given very little credit.

F. (6points) List three molecular mechanisms which can explain this finding.
   1)gene conversion
   2)whole chromosome loss
   3)de novo mutation of the allele

Note the first two will cause loss of genetic markers the third will not.