StarCellBio

StarCellBio Exercise 3 – EGFR Function & Signaling

Goal

In this exercise, you will use StarCellBio, a cell and molecular biology experiment simulator, to better understand the function of the epidermal growth factor receptor (EGFR) in signaling and cancer. To do this you will characterize expression levels, subcellular localization, signaling activity, transmembrane orientation and internalization dynamics of two EGFR mutant proteins using western blotting, flow cytometry, and microscopy.

Learning Objectives

After completing this exercise, you will be able to:

1. Use StarCellBio to perform simulated western blot, microscopy, and flow cytometry experiments.
2. Design and implement experiments in StarCellBio using appropriate experimental conditions and relevant positive and/or negative controls.
3. Analyze the results of several different experimental techniques to determine how a protein's characteristics can be altered by a genetic mutation.
4. Hypothesize how genetic mutations alter protein function by assessing specific results gathered from a variety of experimental techniques.

Accessing StarCellBio

To begin:

1. Using **Google Chrome**, navigate to: http://starcellbio.mit.edu.
2. Sign in to your StarCellBio student account. If you need to set up a student account, use the course code **SCB_SampleExercises**. Note: while you can complete these exercises as a guest by clicking on **Try an Experiment** on the right side of the homepage, your work will not be saved.
3. Select "**Exercise 3**" from the **Assignments** window.

Introduction

You are doing an undergraduate summer research project in a lab that studies the role of the epidermal growth factor receptor (EGFR) in cancer. EGFR is the cell surface receptor for the epidermal growth factor (EGF), a small protein ligand that stimulates cell growth, proliferation, and differentiation (Figure 1). Mutations in EGFR or other proteins in the EGFR pathway often result in abnormal cell growth and proliferation and are associated with various cancers, including some types of lung, breast, and brain tumors.

A graduate student in the lab, who is your mentor for this project, previously identified a series of mutations in the human EGFR gene that affect cell growth through two different genetic approaches. She used site directed mutagenesis to separately introduce two of these specific mutations (M1 and M2) into a human EGFR gene construct, and engineered mouse cell lines to stably express the human EGFR mutant variants. These stably expressing cell lines were created using an EGFR-null mouse cell line, which means that they lack endogenous, wild-type EGFR protein and exclusively express one of the mutated EGFR versions.

Your graduate student mentor first confirms that each of the EGFR mutations she identified confers a growth phenotype by comparing the proliferation rate of mutant EGFRs and wild-type EGFR expressing cell

lines in growth media lacking serum, which contains a combination of essential amino acids and salts, but no growth factors. The following are the results of her proliferation analysis for these two cell lines, EGFR-M1 and EGFR-M2, and the wild-type EGFR expressing cell line, WT-EGFR:

Table 1: Doubling time for cell lines expressing wild-type and mutant EGFR proteins.

Cell line	Average doubling time in serum free media ± standard deviation (hours)
WT-EGFR	32.2 ± 1.5
EGFR-M1	60.5 ± 2.3
EGFR-M2	17.8 ± 0.9

Note: a cell line's "doubling time" is the duration of time that it takes for a starting population of cells in the exponential growth phase to double in number.

The goal of your summer research project is to analyze the effects that the M1 and M2 mutations have on EGFR function.

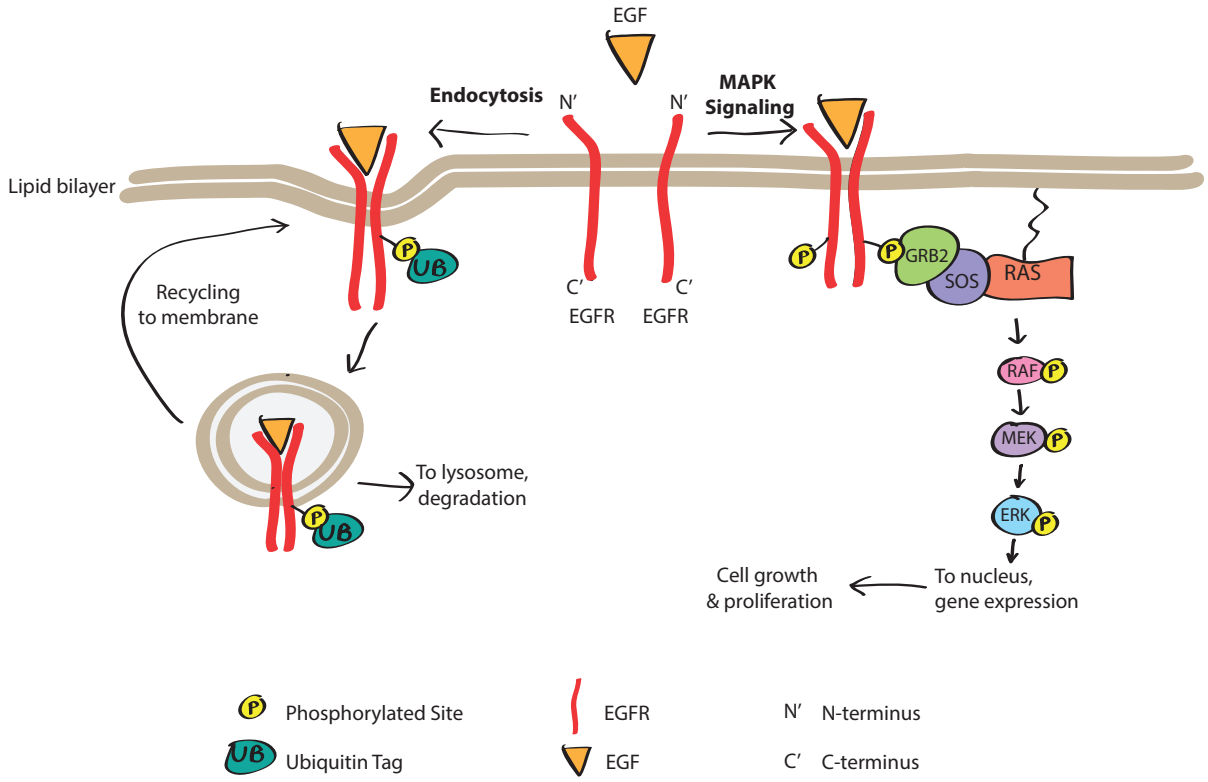

Figure 1. Activation of the EGFR signaling pathway upon EGF binding. Binding of EGF to EGFR results in EGFR dimerization and autophosphorylation (EGFR phosphorylates itself) at various sites on the receptor's intracellular domain. Depending on the specific sites phosphorylated, the downstream effects will be different. Autophosphorylation of specific tyrosine residues results in the activation of the MAPK (MEK/ERK) signaling pathway through the phosphorylation of its downstream components, leading to increased expression of genes that promote cell proliferation and growth. Autophosphorylation of certain tyrosine and serine residues leads to the ubiquitination and internalization of EGFR, followed by either recycling of the receptor back to the cell membrane or lysosome-mediated degradation. EGFR can bind to other ligands besides EGF, and the fate of EGFR after internalization depends on the ligand that induces EGFR signaling, the concentration of ligand present, and the length of time EGFR is stimulated. Internalization and degradation decrease the number of activated receptors on the cell membrane at a given point and attenuate signaling. Several details about EGFR signaling, such as the other pathways, for example the AKT/PKB and STAT5b pathways, which are activated in the presence of EGF, have been omitted in this figure for simplicity.

Your graduate student mentor suggests that you perform experiments to characterize the following properties and functions of the EGFR-M1 and EGFR-M2 mutant proteins:

1. Protein size and relative expression levels
2. Subcellular localization
3. Activation of downstream signaling molecules
4. Orientation within the membrane
5. Internalization/degradation of the receptor upon EGF stimulation

Background Information

Cell Lines

You are provided with the following cell lines:

Name	Description
WT-EGFR	A cell line that expresses the wild-type EGFR protein.
EGFR-Null	A cell line that does not express EGFR protein (neither wild-type or

	mutant).
EGFR-M1	A cell line stably expressing a mutant version of EGFR, EGFR-M1, without endogenous expression of the wild-type protein.
EGFR-M2	A cell line stably expressing a mutant version of EGFR, EGFR-M2, without endogenous expression of the wild-type protein.
NoUB	A cell line stably expressing a mutant version of EGFR where four serine residues that mediate EGFR degradation have been replaced by alanine residues to inhibit degradation upon EGF ligand binding. This cell line does not express the wild-type EGFR protein.
ConstActive	A cell line stably expressing a mutant version of EGFR that is constitutively active and does not require EGF binding to initiate MAPK signaling. In this cell line, the kinase domain of EGFR is always in its 'active' state. However, the mutant protein is internalized and degraded normally upon EGF binding. This cell line does not express wild-type EGFR protein.
His-EGFR-FLAG	A cell line stably expressing an epitope-tagged version of the wild-type EGFR with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.
His-EGFR-M1-FLAG	A cell line stably expressing an epitope-tagged version of the EGFR-M1 protein with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.
His-EGFR-M2-FLAG	A cell line stably expressing an epitope-tagged version of the EGFR-M2 protein with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.

Treatments

You are provided with the following treatment options:

Treatment	Treatment Duration	Description[1]
Growth media only	30 sec	Cells are cultured in serum-free growth media[1] for various periods of time.
	1 min	
	6 hrs	
Growth media + EGF	30 sec	Cells cultured in serum-free growth media[1] are incubated with a high concentration of EGF for 30 seconds, washed to remove EGF, and collected.
	1 min	Cells cultured in serum-free growth media[1] are incubated with a high concentration EGF for 1 minute, washed to remove EGF, and collected.
	6 hrs	Cells cultured in serum-free growth media[1] are incubated with a high concentration EGF for 6 hours, washed to remove EGF, and collected.
Growth media + buffer	30 min	Cells cultured in serum-free growth media[1] are collected. Intact cells are then incubated with Proteinase K buffer only for 30 min.
Growth media + ProK	30 min	Cells cultured in serum-free growth media[1] are collected. Intact cells are then incubated with the Proteinase K (ProK) enzyme for 30 min to digest any extracellular peptides[2].

Western Blotting

You are provided with the following antibodies for western blotting experiments:

Antibody	Description	Expected molecular weight of detected band(s) (kDa)
Mouse anti-EGFR	Primary antibody recognizing both the	120 – 150 → depending on the

	phosphorylated and unphosphorylated forms of EGFR. Note: This antibody recognizes an epitope on the N-terminal end of the protein, and can recognize this epitope in wild-type and mutant forms of EGFR in all cell lines.	extent of phosphorylation and whether the protein is tagged or untagged.
Mouse anti-pEGFR	Primary antibody recognizing EGFR phosphorylated on tyrosine 1088, an indicator that EGFR is 'active'. Note: Tyrosine 1088 is on the C- terminal end of the protein. This antibody can recognize this epitope in wild-type and mutant forms of EGFR in all cell lines.	140 – 150 → depending on the extent of phosphorylation and whether the protein is tagged or untagged.
Mouse anti-pMEK	Primary antibody recognizing the phosphorylated (active) form of MEK.	45
Mouse anti-pRAF	Primary antibody recognizing the phosphorylated (active) form of RAF.	74
Mouse anti-PGK1	Primary antibody recognizing PGK1, a housekeeping protein expressed in all cell types at relatively equal levels.	44
Rabbit anti-6xHis	Primary antibody recognizing the 6xHis epitope tag.	Varies depending on the molecular weight of the 6xHis tagged protein. The 6xHis tag adds about 1 kDa to the molecular weight of the tagged protein.
Rabbit anti-FLAG	Primary antibody recognizing the FLAG epitope tag.	Varies depending on the molecular weight of the FLAG tagged protein. The FLAG tag adds about 1 kDa to the molecular weight of the tagged protein.
Rabbit anti-mouse HRP	Secondary antibody recognizing mouse primary antibodies, conjugated to horseradish peroxidase (HRP)[3].	Varies, depending on primary antibody used.
Goat anti-rabbit HRP	Secondary antibody recognizing rabbit primary antibodies, conjugated to horseradish peroxidase (HRP)[3].	Varies, depending on primary antibody used.

Flow Cytometry

You are provided with the following conditions for flow cytometry experiments:

Antibody conditions	Description
EGFR A488	Incubation with mouse anti-EGFR primary antibody recognizing the EGFR protein, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4]. Note: The anti-EGFR primary antibody recognizes an epitope on the N-terminal end of the protein, and can recognize the wild-type and mutant forms of EGFR in all cell lines.

Microscopy

You are provided with the following conditions for immunofluorescence microscopy experiments:

Antibody	Description

conditions	
EGFR A488	Incubation with mouse anti-EGFR primary antibody recognizing the EGFR protein, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5]. Note: The anti-EGFR primary antibody recognizes an epitope on the N-terminal end of the protein, and can recognize the wild-type and mutant forms of EGFR in all cell lines.
PM A488	Incubation with primary antibody recognizing LCK, a protein that localizes to the **plasma membrane** (PM), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
Cyto A488	Incubation with primary antibody recognizing RPS20, a protein that localizes to the **cytoplasm** (Cyto), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
Nuc A488	Incubation with primary antibody recognizing histone H2B, a protein that localizes to the **nucleus** (Nuc), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
ER A488	Incubation with primary antibody recognizing calnexin, a protein that localizes to the **endoplasmic reticulum** (ER), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
NM A488	Incubation with primary antibody recognizing lamin B1, a protein that localizes to the **nuclear membrane** (NM), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].

Notes:

[1] Serum-free growth media does not contain growth factors, including EGF.

[2] Proteinase K is an enzyme that digests proteins. It cannot penetrate the plasma membrane when the plasma membrane is intact, which means that the cell membrane has not been disrupted or permeabilized. As a result, incubating intact cells with Proteinase K results in the digestion of extracellular peptides only.

[3] These secondary antibodies are conjugated to horseradish peroxidase (HRP). Horseradish peroxidase catalyzes a reaction that produces light as a by-product, which is detected using photographic film

[4] The secondary antibodies used for these experiments are conjugated to fluorescent molecules, or fluorophores. In this case, the fluorophore is Alexa Fluor 488 (A488), which fluoresces or emits green light when excited by light with a wavelength of 488 nm.

[5] When the green channel of the microscope is chosen, the fluorescence emitted by a sample is captured as an image taken with a black and white camera.

Question 1

As a first step, your graduate student mentor advises you to look at whether the M1 and M2 mutations in the EGFR gene affect the expression levels and/or size of the corresponding EGFR-M1 and EGFR-M2 proteins.

To do this, you perform a western blotting experiment to analyze the protein expression levels and molecular weight of the mutant EGFR-M1 and -M2 proteins in the **absence** of EGF (Growth media only treatment), making sure to include any relevant controls and using the appropriate primary and secondary antibody combinations. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. How does the molecular weight of the EGFR-M1 and EGFR-M2 proteins compare with the wild-type EGFR protein? Explain how you arrived at your answer using your experimental results.

EGFR-M1 and EGFR-M2 are the same size (120 kDa) as the wild-type EGFR protein. On the western blot film, the bands corresponding to EGFR-M1 and EGFR-M2 are in the same location as the band corresponding to the wild-type EGFR protein, which aligns with a molecular weight of 120 kDa.

*** EGFR-M1 is not phosphorylated upon EGF addition, but both EGFR-M2 and WT-EGFR are phosphorylated upon EGF addition. As a result, if the "Growth media + EGF" treatment is included in this experiment, two bands will be detected in the WT-EGFR and EGFR-M2 expressing cells when blotting with the anti-EGFR antibody, corresponding to the phosphorylated and unphosphorylated forms of each protein. In contrast, only one band would be detected in EGFR-M1 expressing cells, corresponding to the unphosphorylated form. However, the sizes of the unphosphorylated forms for each mutated protein are the same as that of the wild-type protein.**

b. How does the expression of the EGFR-M1 and EGFR-M2 proteins compare to that of wild-type EGFR? Explain how you arrived at your answer using your experimental results.

The EGFR-M1 and EGFR-M2 proteins are expressed at approximately the same levels as wild-type EGFR. The intensity of the bands corresponding to the mutant and wild-type EGFR proteins are roughly the same. The relative intensity of a band on a western blot film correlates with the average amount of protein present in these cells.

c. Can the growth phenotypes observed in EGFR-M1 and EGFR-M2 expressing cells (Table 1) be explained by increased or decreased levels of mutant EGFR transcription or translation rates? Explain your answer using your experimental results.

No, increased or decreased rates of transcription and/or translation in the EGFR-M1 and EGFR-M2 expressing cells cannot explain the growth phenotypes observed in these cells. Decreased rates of transcription or translation would cause a reduction in the amount of EGFR protein expressed. Increased rates of transcription or translation would result in an increase in the amount of EGFR protein expressed. However, we can see by western blotting, that EGFR-M1 and EGFR-M2 protein expression is comparable to that of wild-type EGFR.

Question 2

To further characterize the mutant cell lines, your mentor suggests you determine whether the mutant EGFR proteins localize correctly to the plasma membrane since the correct localization of the receptor is essential for EGF binding and proper EGFR signaling.

To determine if the EGFR-M1 and EGFR-M2 proteins localize to the plasma membrane, you perform an immunofluorescence (IF) microscopy experiment, making sure to include any relevant controls. First, you fix and permeabilize cells mounted on microscope slides to allow antibodies to cross the plasma membrane and have access to intracellular components. Then, you incubate them with the appropriate primary and secondary antibody combinations. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. What are the subcellular localizations of the EGFR-M1 and EGFR-M2 proteins? Justify your answer using your experimental results.

EGFR-M1 and EGFR-M2 both localize to the plasma membrane. The visual pattern observed with the fluorescent EGFR antibody (EGFR A488) in both mutant cell lines is characteristic of localization to the plasma membrane as seen in the PM A488 antibody-treated control samples (either WT-EGFR or EGFR mutant cell lines).

b. Does the localization of either mutant EGFR protein differ from that of wild-type protein?

No, all forms of EGFR localize to the plasma membrane.

Question 3

Next you would like to examine MAPK signaling in EGFR-M1 and EGFR-M2 expressing cells upon EGF stimulation.

To do this, you perform a western blotting experiment to examine the expression and activation of proteins in the MAPK signaling pathway following EGF stimulation (see Figure 1), making sure to include any relevant controls. Your laboratory has antibodies against several MAPK signaling components, including: 1) phosphorylated (active) EGFR, 2) phosphorylated (active) RAF, and 3) phosphorylated (active) MEK. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. How does EGF-induced MAPK signaling in the EGFR-M1 cell line compare with the WT-EGFR cell line? Explain using your experimental results. If there are differences, include which signaling components are different and how.

The MAPK signaling pathway is not activated in EGFR-M1 cells but it is activated in WT-EGFR cells. We do not detect phosphorylated EGFR-M1, RAF or MEK in EGFR-M1 upon EGF stimulation (at any treatment duration), as seen by the absence of bands on the western blot film when the blot is probed with antibodies against the phosphorylated forms of these 3 proteins and the lack of a mobility shift due to phosphorylation when probed with the anti-EGFR antibody. In contrast, we can detect the phosphorylated form of EGFR-WT, RAF, or MEK after EGF stimulation in the WT-EGFR cells.

b. How does EGF-induced MAPK signaling in the EGFR-M2 cell line compare with the WT-EGFR cell line? Explain using your experimental results. If there are differences, include which signaling components are different and how.

Signaling through the MAPK pathway in EGFR-M2 expressing cells does not differ from WT-EGFR cells. We detect phosphorylated EGFR-M2, RAF and MEK upon EGF stimulation as seen by the presence of bands on the western blot film when the blot is probed with antibodies against the phosphorylated forms of these 3 proteins and by the presence of a mobility shift due to phosphorylation when probed with the anti-EGFR antibody. This is also what we see in WT-EGFR cells.

** Answer Questions 4 and 5 for any mutant EGFR proteins that localize to the plasma membrane. For proteins that are not localized to the plasma membrane, skip to Question 6. **

Question 4

The orientation of EGFR is important for its proper functioning and activation of downstream signaling pathways. EGFR is a single-pass transmembrane protein with the N-terminus, responsible for ligand binding (EGF), outside the cell and the C-terminus, responsible for initiating signaling, inside the cell. Your graduate student mentor advises you to perform a western blot experiment to determine the orientation of the mutant EGFR proteins that localize to the plasma membrane.

Before performing the western blotting procedure, you collect His-EGFR-M1-FLAG and/or His-EGFR-M2-FLAG cells and incubate intact cells with Proteinase K (ProK), an enzyme that digests proteins. Because Proteinase K cannot penetrate the membrane of intact, non-permeablized cells, only intracellular proteins and peptides will remain after incubation with Proteinase K. After Proteinase K digestion, you lyse the cells and perform a western blot analysis to determine the presence or absence of each epitope tag using the appropriate primary and secondary antibody combinations, while ensuring to include any relevant controls. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. What is the orientation of the mutant EGFR protein(s) in the plasma membrane with respect to the N- and C-terminus? Justify your answer using your experimental results.

EGFR-M1's N-terminus is intracellular while its C-terminus is extracellular. I know this because after Proteinase K treatment, the FLAG epitope on the C-terminal end of EGFR-M1 is not intact (band not visible on western blot when probing sample with anti-FLAG antibody, so the C-terminal end must have been outside the cell and digested by Proteinase K). Also, the His epitope on the N-terminal end of EGFR-M1 is intact (band visible on western blot when probing sample with anti-His antibody, so the C-terminal end must have been inside the cell and protected from Proteinase K). This means that the N- terminal end, which is responsible for binding ligand, is intracellular, and the C-terminal end, which is responsible for phosphorylation and initiating the signaling cascades is extracellular.

In contrast, EGFR-M2 has an intracellular N-terminus and an intracellular C-terminus. I know this because after Proteinase K treatment, the FLAG epitope on the C-terminal end of EGFR-M2 is intact (band visible on western blot when probing sample with anti-FLAG antibody, so the C-terminal end must have been inside the cell and protected from Proteinase K). Also, the His epitope on the N-terminal end of EGFR-M2 is not detected after Proteinase K treatment (band not visible on western blot when probing sample with anti-His antibody, so the N-terminal end must have been outside the cell and digested by Proteinase K).

b. Does the orientation of the mutant EGFR protein(s) differ from that of the wild-type protein? If so, how? Explain.

Yes, the orientation of the EGFR-M1 protein differs from that of the wild-type protein. As explained above, the EGFR-M1 protein has an extracellular C-terminus and an intracellular N-terminus, while the wild type is the opposite.

Both the EGFR-M2 protein and wild-type EGFR have the same orientation, as they both show the same patterns on the western blot (as explained for EGFR-M2 above).

Question 5

Internalization and degradation of EGFR initiated by EGF binding is an important mode of regulation and is essential for proper signaling (Figure 1). The fate of EGFR after internalization (whether it is recycled back to the membrane or degraded) depends on the ligand that induces EGFR signaling, the concentration of ligand present, and the length of time EGFR is stimulated. For example, the presence of high concentrations of EGF for prolonged periods of time (i.e. > 60 min) leads to ubiquitination of EGFR's intracellular domain, which in turn results in EGFR internalization and lysosome-mediated degradation.

To examine the internalization dynamics of EGFR-M1 and/or EGFR-M2, you perform flow cytometry experiments to measure the amount of mutant EGFR protein on the cell surface following stimulation with EGF, making sure to include any relevant controls. Prior to flow cytometry, you incubate live cells with EGF for different time periods. After EGF stimulation you collect and fix intact cells for flow cytometry analysis, followed by incubation with anti-EGFR primary antibody and the appropriate fluorescently-labeled secondary antibody. The primary antibody used in this experiment (mouse anti-EGFR) recognizes an epitope on the N-terminal end of the EGFR protein (note that the N-terminal end of WT-EGFR is extracellular). Since antibodies cannot cross the plasma membrane of intact, non-permeabilized cells, binding to the N-terminal EGFR epitope by the anti-EGFR antibody will only occur for receptors with an extracellular N-terminal domain that have not been internalized. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

Note: Assume that the conditions of the experiment, including EGF concentration and incubation time, result in most of the internalized EGFR being trafficked to the lysosome-mediated degradation pathway rather than being recycled to the membrane. Therefore, the rate of internalization will give you a good estimate of the rate of degradation in these mutants.

a. Do the internalization dynamics of the mutant EGFR protein(s) differ from that of the wild-type protein? Justify your answer using your experimental results.

Internalization is possibly affected in EGFR-M1. By flow cytometry analysis, we are not able to detect EGFR-M1 on the plasma membrane even in the absence of EGF. This is likely due to the incorrect orientation of this protein in the plasma membrane. The anti-EGFR antibody used in this experiment recognizes an N-terminal epitope, which in the wild-type protein is extracellular. However, in EGFR-M1, the N-terminal end is intracellular and not accessible to antibodies without permeabilization. To examine internalization in this case we would need to use a different EGFR antibody against an extracellular region of EGFR-M1, such as the C-terminal end. Therefore, we cannot determine if internalization is affected in the EGFR-M1 mutant with this approach.

EGFR-M2 exhibits an internalization defect. Even after 6 hours of incubation with EGF, we can still detect most of the EGFR on the plasma membrane as seen by the amount of fluorescence detected by the flow cytometer. In contrast, we are only able to detect low levels of wild-type EGFR on the plasma membrane after 6 hours of EGF incubation because EGFR is internalized and degraded after prolonged exposure to EGF.

b. After incubating cells with high concentrations of EGF for 6 hours, would you expect the EGFR-M1 and/or EGFR-2 cell lines to show higher, lower, or similar levels of EGFR degradation compared to the WT-EGFR cell line? What experiment(s) could you perform with the provided reagents to test your hypotheses? Explain.

EGFR-M2 expressing cells should exhibit lower levels of EGFR degradation relative to WT-EGFR, because in the conditions of the experiment, internalization is representative of degradation. We are not able to detect internalization of EGFR-M2 protein with this experiment, so we can assume that levels of degradation are low.

We can also hypothesize that the levels of EGFR-M1 degradation should be lower in comparison to wild-type because EGFR-M1 is not properly oriented in the plasma membrane, which could affect the ability of the mutant receptor to bind to EGF. However, we do not have sufficient evidence that this is the case and additional experiments would be needed to probe for EGF binding and proper internalization.

To test the above hypotheses, you can perform a western blot experiment using the anti-EGFR antibody provided upon various time points after addition of EGF. As the rate of degradation of EGFR mutant protein increases, the intensity of the band on the western blot when probed with the anti-EGFR antibody will decrease (there will be less protein for the antibody to bind to). Therefore, if there is less degradation of EGFR-M1 and M2 as hypothesized, the band on the western blot should be more intense when samples are prepared from the EGFR-M1 or M2 expressing cell lines than when samples are prepared from the WT-EGFR cell line.

Question 6

***Note: In the answers to this question, all possible experimental results are listed below, including those where the mutants are similar to those in WT-EGFR cells. Students need only discuss experimental results that are different from those in wild-type EGFR expressing cells.**

a. Using all of your experimental results and conclusions from Questions 1-5, propose a hypothesis that explains how the EGFR-M1 and EGFR-M2 mutations result in the abnormal growth phenotypes observed in the EGFR-M1 and EGFR-M2 expressing cell lines, respectively (Table 1). Make sure to include the results that allowed you to arrive at your conclusion.

i. EGFR-M1

The EGFR-M1 protein has the opposite plasma membrane orientation from wild-type EGFR (the N-terminus is intracellular while C-terminus is extracellular). This likely means that it cannot bind to EGF since EGF normally interacts and binds to the extracellular N-terminal end of the protein. This could explain why EGFR-M1 expressing cells do not respond to EGF stimulation – no EGFR, MEK, or RAF phosphorylation is visible on a western blot. It is also possible that there is no internalization/degradation of the receptor since autophosphorylation of EGFR is required for its internalization. However, without performing the internalization assay with a different antibody that can detect the extracellular C-terminal end, we cannot be sure of this.

The following experimental results support this hypothesis/conclusion:

1. **In western blotting experiments, we can observe that the EGFR-M1 protein is expressed at the same levels as that of wild type in the absence of EGF.**
2. **Based on microscopy experiments, we can see it is located in the plasma membrane.**
3. **In the presence of EGF, there is no visible activation (phosphorylation) of EGFR-M1, since we cannot detect a band on the western blot when M1 expressing cells are probed with pEGFR antibodies. Similarly, there is no activation of the RAF or MEK downstream signaling proteins, as no bands are detected on the western blot with pRAF or pMEK antibodies. This is because EGFR-M1 cannot bind EGF (the binding site is located inside the cell and EGF is outside the cell), preventing activation of the mutant EGFR protein and downstream proteins.**
4. **Next, looking at its orientation, we see that the EGFR-M1 protein has the opposite orientation in the plasma membrane, compared with its wild-type counterpart. I know this because after Proteinase K treatment, the FLAG epitope on the C-terminal end of EGFR-M1 is not detectable (the band is not visible on the western blot when probing the sample with an anti-FLAG antibody, which means that the C-terminal end must have been outside the cell and digested by Proteinase K). Also, the His epitope on the N-terminal end of EGFR-M1 is intact (the band is visible on the western blot when probing the sample with an anti-His antibody, which means that the C-terminal end must have been inside the cell and protected from Proteinase K digestion). This means that the N-terminal end, which is responsible for binding ligand, is intracellular, and the C-terminal end, which is responsible for phosphorylation and initiating the signaling cascades, is extracellular. Finally, internalization dynamics may be different between EGFR-M1 and the wild-type EGFR protein. Flow cytometry experiments show that no EGFR signal is detected in the cells treated with EGFR. This is because, if the orientation of the protein is flipped, the anti-EGFR antibody used in the flow cytometry experiments cannot bind to EGFR (the epitope for this antibody is on the N-terminal end which in this case is intracellular and antibodies cannot penetrate intact cells).**

ii. EGFR-M2

EGFR-M2 is correctly oriented in the plasma membrane, but is not internalized and degraded upon EGF stimulation. It is possible that the M2 mutation caused one of the tyrosine or serine phosphorylation sites necessary for ubiquitination to change to an amino acid that cannot be phosphorylated. MAPK signaling in response to EGF proceeds normally, but without ubiquitination, EGFR-M2 is not internalized or degraded, and the signal cannot be attenuated. Overall, this mutant cell line has increased MAPK signaling in the presence of EGF.

The following experimental results supporting this conclusion:

1. **In western blotting experiments, we can see that EGFR-M2 is expressed at normal levels.**
2. **Based on microscopy experiments, we can see that EGFR-M2 is located in the plasma membrane.**
3. **In the presence of EGF, there is activation (phosphorylation) of EGFR-M2 since we can see a band on the western blot when EGFR-M2 cell lysates are probed with pEGFR antibodies. Similarly, there is also activation of RAF or MEK downstream signaling proteins, as bands are seen on the western blot when M2 expressing cells are probed with pRAF or pMEK antibodies.**

4. **Next, looking at its orientation, we see that the EGFR-M2 protein has the correct orientation in the plasma membrane (same as the wild-type EGFR protein) with an extracellular N-terminal end. I know this because after Proteinase K treatment, the FLAG epitope on the C-terminal end of EGFR-M2 is intact (the band is visible on the western blot when probing the sample with an anti-FLAG antibody, which means that the C-terminal end must have been inside the cell and protected from Proteinase K digestion). Also, the His epitope on the N-terminal end of EGFR-M2 is not detected after Proteinase K treatment (the band is not visible on the western blot when probing the sample with an anti-His antibody, which means that the N-terminal end must have been outside the cell and digested by Proteinase K). This means that EGFR-M2 should be able to bind EGF normally and initiate downstream signaling cascades.**
5. **Finally, internalization dynamics are different for the EGFR-M2 and the wild type EGFR proteins. EGFR-M2 is not internalized when exposed to high EGF concentrations for prolonged periods of time. This suggests that there is also a lack of lysosome-mediated degradation of the protein. This is the first piece of evidence that gives us information about what is causing the M2 phenotype – there is less internalization (and degradation) of the mutant EGFR-M2 protein increasing the levels of this receptor on the plasma membrane.**

Considering observations 4 and 5, the data suggest that EGF can still initiate MAPK signaling leading to proliferation and growth, but since EGFR-M2 cannot be internalized/degraded, signaling continues. This contrasts with WT-EGFR cells, where the signal attenuates and the cell becomes less sensitive to EGF.

b. A member of your lab studying the same mutant cell lines discovers that when EGF is added to the EGFR-M2 cell line at high concentrations for a prolonged period of time, the levels of ubiquitinated EGFR-M2 protein are surprisingly low.

Does this observation lend support to your hypothesis regarding EGFR-M2 in Question 6a.ii? Explain why or why not. If necessary, use Figure 1 to help you answer this question.

Yes, this observation agrees with the hypothesis for how the M2 mutation affects EGFR function. Ubiquitination is dependent on the binding of EGF to EGFR through its N-terminal end, which then results in EGFR autophosphorylation at specific tyrosine and serine residues on EGFR's C-terminal end (normally intracellular). We hypothesized that the M2 mutation is somehow preventing autophosphorylation of residues necessary for ubiquitination, subsequently preventing internalization and degradation of EGFR-M2. Since the lack of autophosphorylation would prevent EGFR-M2 from being ubiquitinated, this is in agreement with the decreased levels of ubiquitin-bound EGFR-M2 observed.