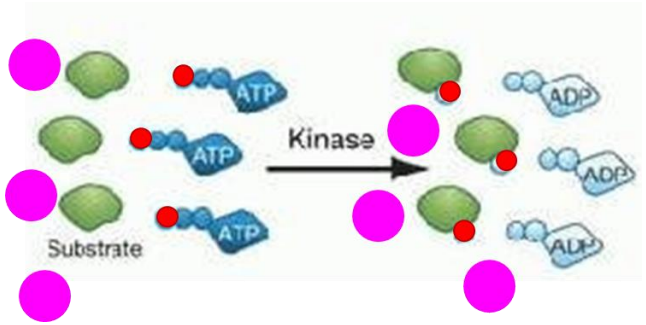
Advanced Cell Biology and Biotechnology

ACBB 2021/22

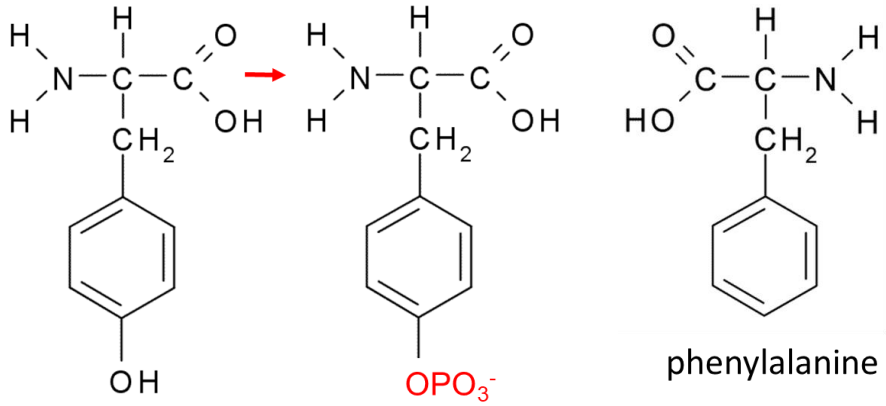
…the lecture will start soon…

Summary of the previous lesson



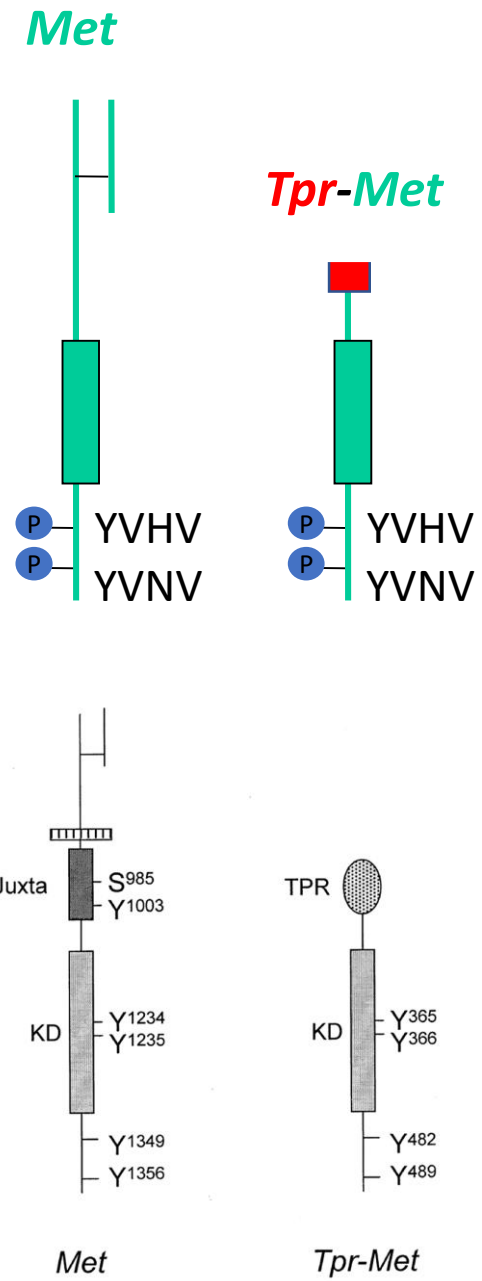
- kinase assay
- Phosphomymetic
- point mutation
- planning of an experiment to investigate the role the phosphorylation of a specific tyrosine in the interaction between two proteins

UAU	UUU
UAC →	UUC
Tyr/Y	Phe/F



thyrosine phospho-thyrosine phenylalanine

- identification of pathways involved in transformation and metastasis



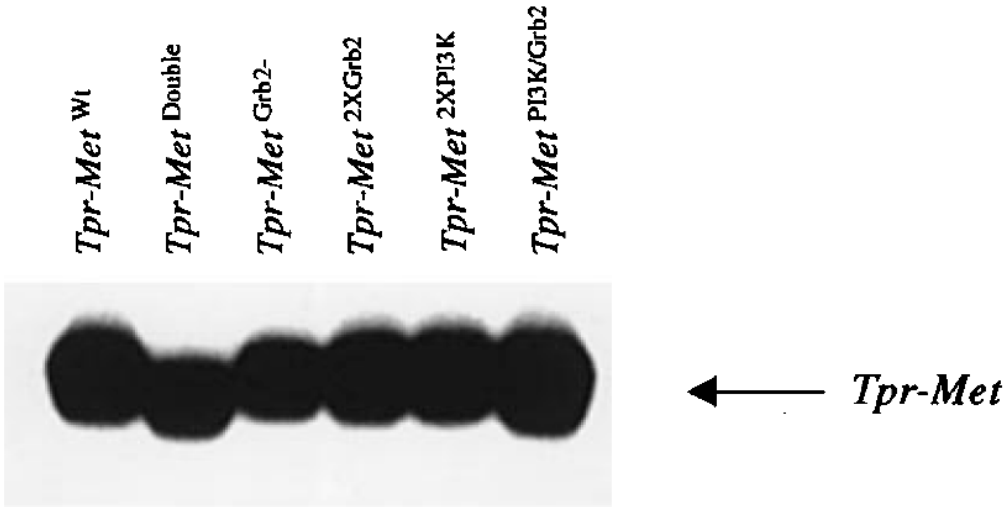
Docking site mutants

Table 1 *Tpr–Met* signaling mutants designed to preferentially bind Grb2 or PI 3-kinase

$Tpr-Met^{Wt}$	YVHVNATYVNV
$Tpr-Met^{Double}$	FVHVNATFVNV
$Tpr-Met^{Grb2-}$	YVHVNATYVHV
$Tpr-Met^{2\times Grb2}$	YVNVNATYVNV
$Tpr-Met^{2\times PI3K}$	YMPMNATYMDM
$Tpr-Met^{PI3K/Grb2}$	YMPMNATYVNV

Signalling mutants of the oncogenic form of the Met receptor (Tpr-Met) were generated by site-directed mutagenesis. The consensus sequences for the SH2 domains of Grb2 and p85 (the regulatory subunit of PI 3-kinase) were designed according to Songyang et al. (1993). Mutagenized residues are underlined.

Kinase activity of Tpr-Met mutants

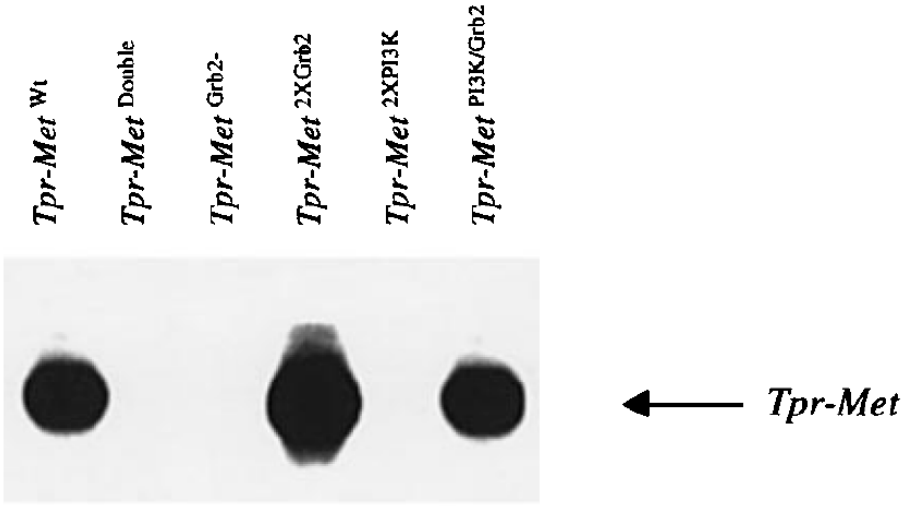


- transient trasfection of COS cells with different Tpr-Met constructs
- protein extraction
- IP anti Met
- kinase assay in vitro (with γ-^{32}P ATP)
- SDS-PAGE
- autoradiography

- the bands are radioactive, because they are phosphorylated with radioactively labeled γ-^{32}P ATP

- wild type and mutant Tpr - Met proteins were immunoprecipitated from COS-1 cells transfected with the corresponding constructs, using antibodies specific for human *Met*
- immunoprecipitated proteins were subjected to *in vitro* kinase assay with [γ-^{32}P]ATP
- labeled proteins were separated on 8% SDS-PAGE

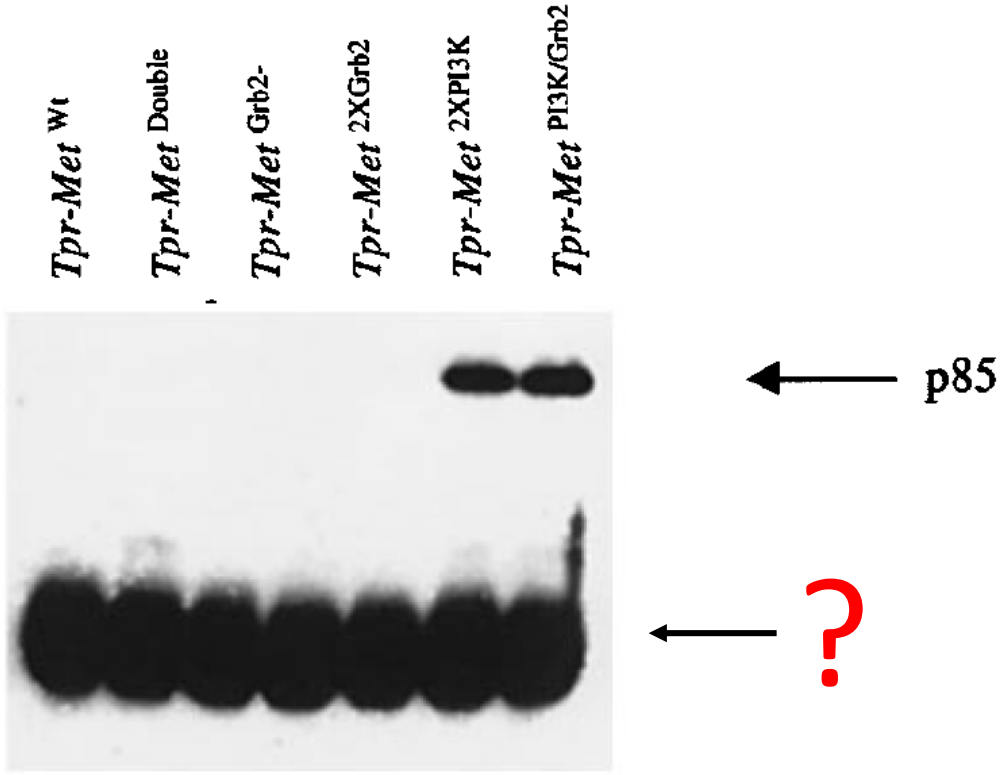
Association of Tpr-Met mutants with Grb2



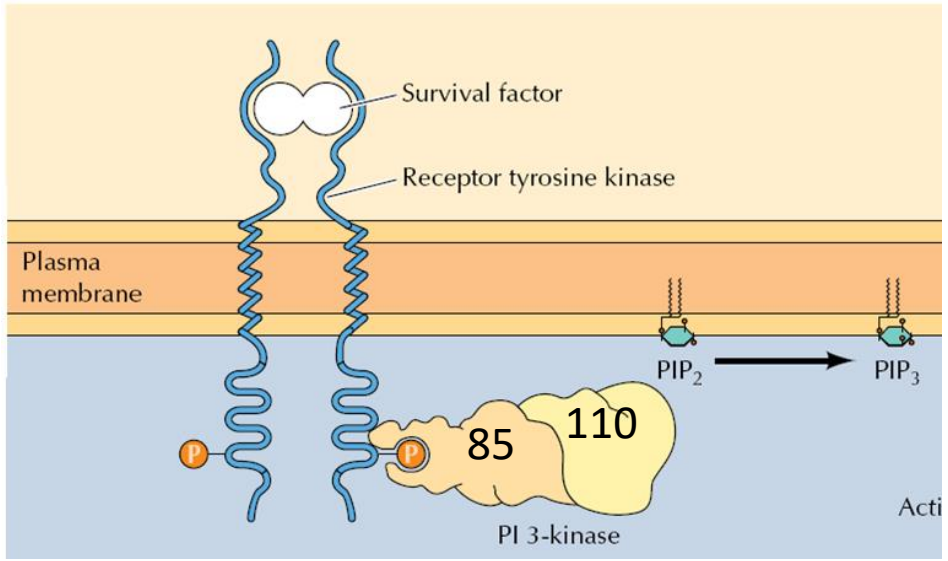
- fused protein: **Grb2-glutathione-transferase,** immobilized on **sepharose-glutathione beads**
- transient transfection of COS cells with various Tpr-Met constructs
- protein extraction
- "**pull down**": extracted proteins incubated together with the beads: some proteins bind to Grb2 and precipitate together with the beads
- kinase assay in vitro on precipitated proteins (with γ-^{32}P ATP)
- SDS-PAGE
- X-ray film exposure
- bands are proteins precipitated together with Grb2 and are radioactive because they are phosphorylated with radioactively labeled ATP

Grb2 fusion protein (approximately 500 ng/point) was immobilized on Glutathione-Sepharose beads and incubated with lysates of COS-1 cells containing comparable amounts of Tpr-Met mutants. Complexes were washed and the amount of Tpr-Met bound to Grb2 was visualized by in vitro kinase assay with [[γ-^{32}P]ATP. Labeled proteins were separated on 8% SDS± PAGE.

Association of Tpr-Met signaling mutants with PI 3-kinase



- transient transfection of COS cells with various Tpr-Met constructs
- protein extraction
- immunoprecipitation with anti-Met antibody
- SDS-PAGE
- western blotting with anti p85 antibody
- chemoluminescent reaction (enzyme associated with the secondary antibody, ECL substrate)
- the high band is p85



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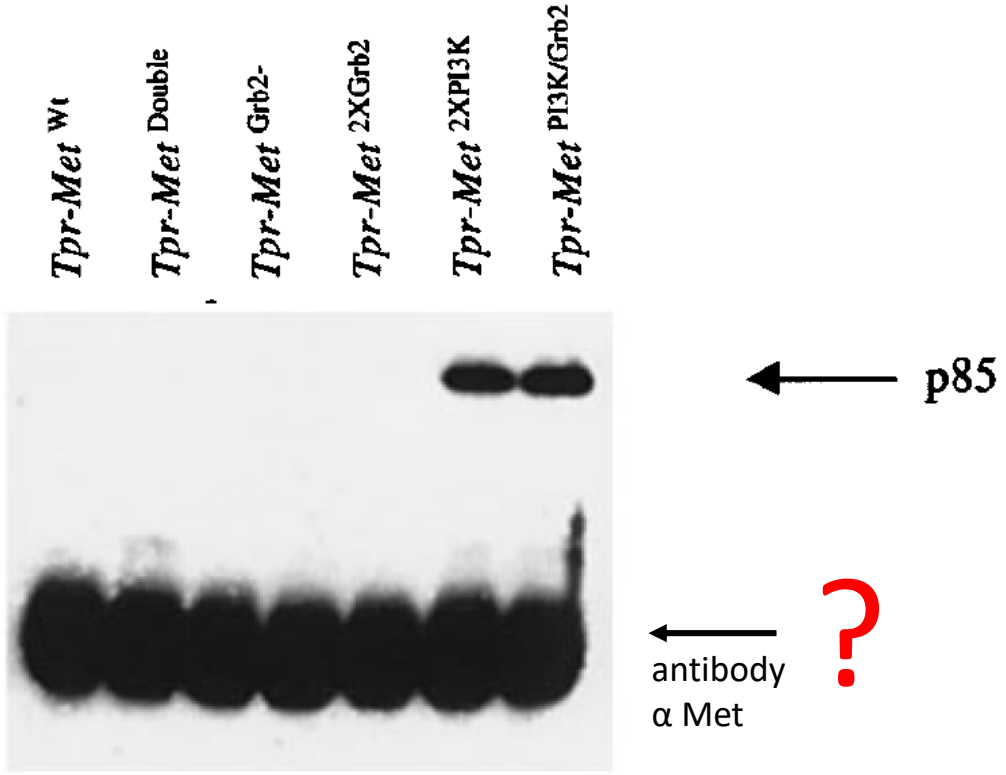

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- SDS-PAGE
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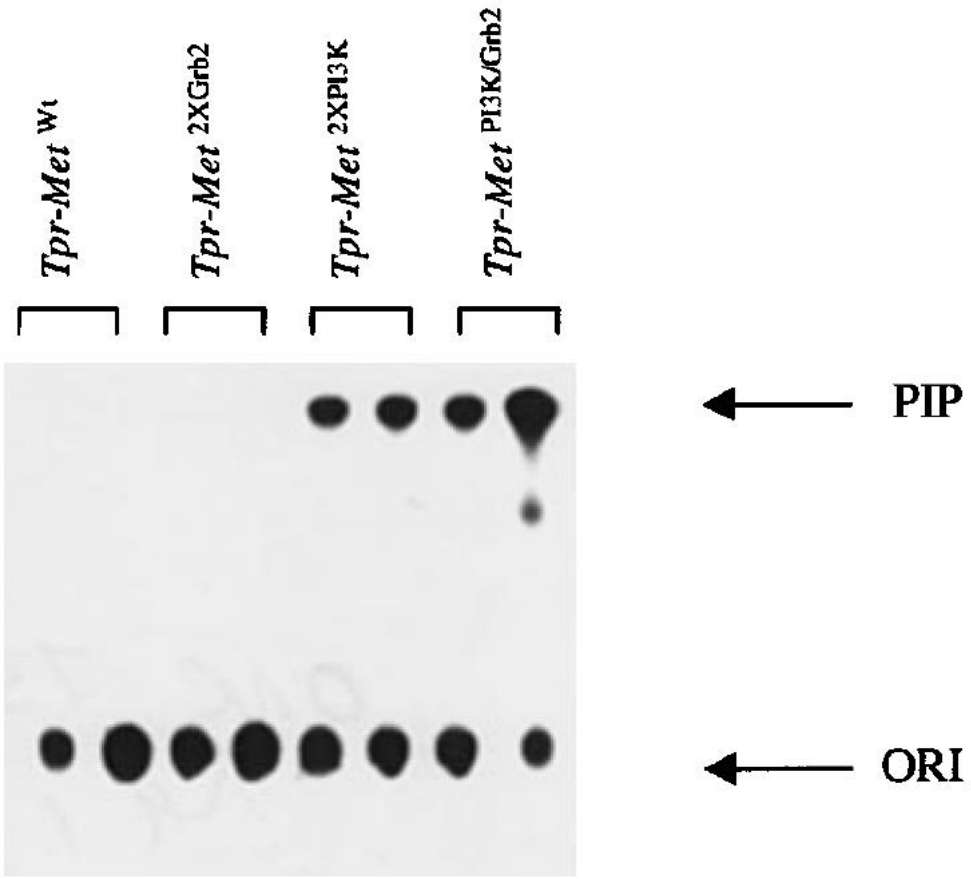
- the high band is p85

- the low bands correspond to the antibody used to immunoprecipitate, which is recognized by the secondary antibody

Tpr-Met mutants were immunoprecipitated with anti-Met antibodies from lysates of transfected COS-1 cells. Associated p85 was visualized by Western blotting with anti-p85 antibodies. Exposure was optimized to show the amount of p85 associated to the Tpr-Met ^{2xPI3K} and Tpr- Met $^{PI3K/Grb2}$ mutants. Upon longer exposure a faint p85 band can also be detected in the Tpr-Met Wt lane, as previously shown.

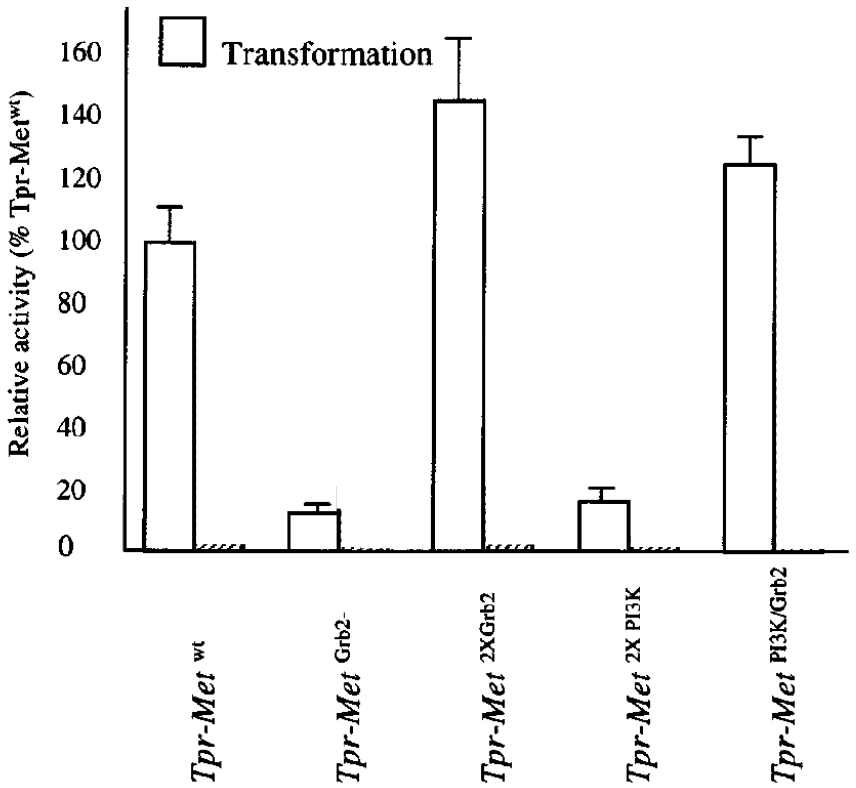
Association of Tpr-Met signaling mutants with Grb2 and PI3K



- transient transfection of COS cells with various Tpr-Met constructs
- protein extraction
- immunoprecipitation with anti-Met antibody
- PI3-kinase assay (with radioactive substrate)
- THIN LAYER CHROMATOGRAPHY: PIP, i.e. PI phosphorylated by PI3-kinase, is negatively charged, has affinity for the solvent and migrates faster than PI, which remains at the point of origin

Tpr-Met mutants were immunoprecipitated with anti-Met antibodies from lysates of transfected COS-1 cells and the amount of Tpr-Met associated PI 3-kinase activity was determined by PI 3-kinase assay. The position of the phosphatidylinositol-3-phosphate (PIP) product of the PI 3-kinase reaction is indicated

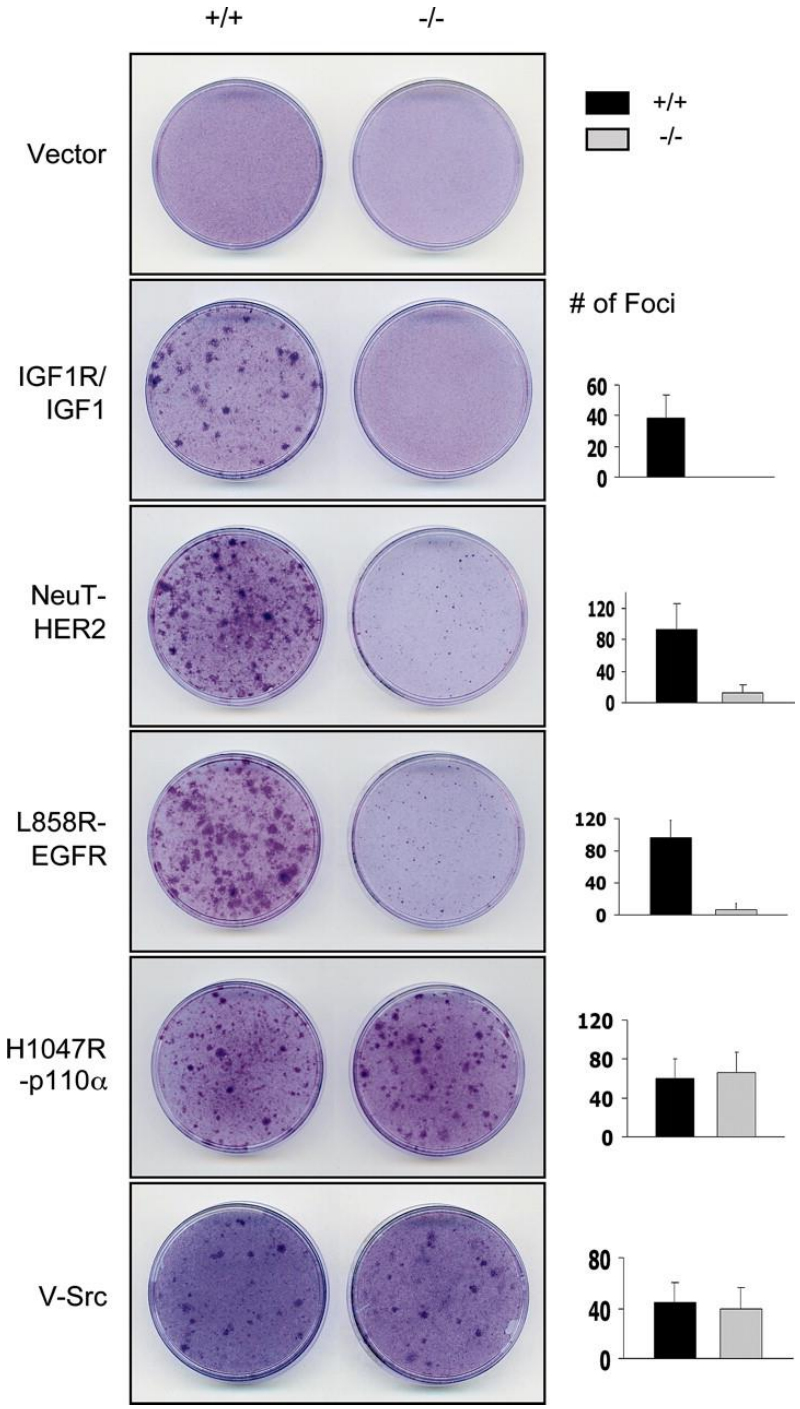
Grb2 coupling is necessary for transformation



FOCUS FORMING ASSAY

- transfection of Fisher rat fibroblast cells with 10 µg of various Tpr-Met constructs
- count of the foci that are formed for each construct (focus forming assay)
- transformed cells forming foci were recovered for metastasis assay

The transforming potential of the indicated Tpr-Met mutants was evaluated by focus forming assay using Fisher rat fibroblasts. The values reported are expressed as percentage of the Tpr-Met^{Wt} transforming activity (150+12 foci/10 µg of DNA) and represent the average of three independent experiments.

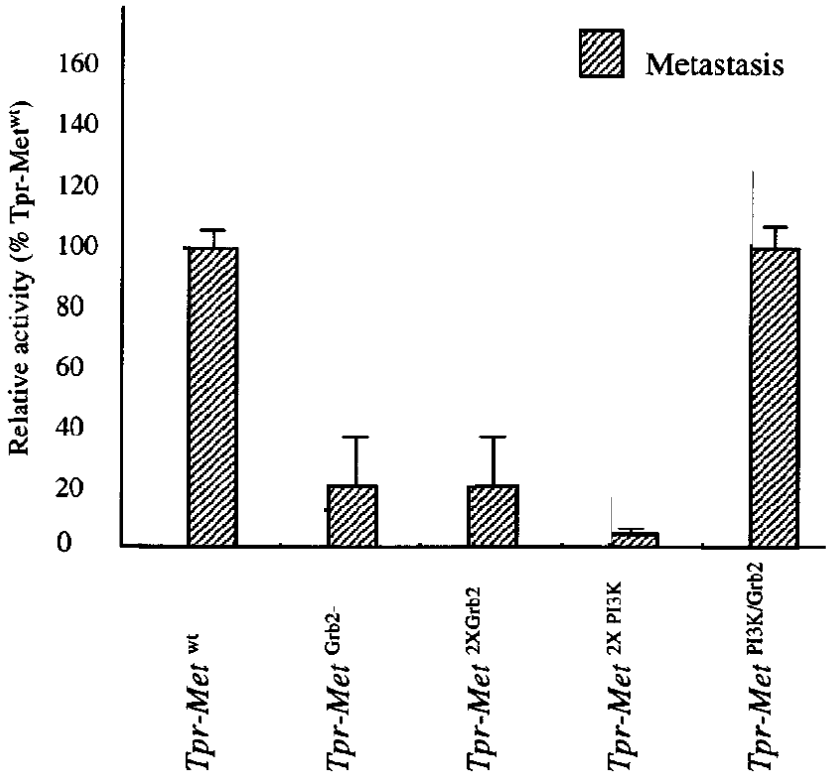


Example of focus forming assay

The p110α isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation

The ablation of p110α impairs transformation induced by various oncogenic signals. Immortalized p110α (+/+) and (−/−) MEFs were infected with a control empty-vector virus, a virus overexpressing wild-type IGF1R, or viruses carrying various oncogenes: mutant alleles of EGFR, L858R-EGFR, NeuT-HER2, or the tumor mutant allele of p110α, H1047R, as indicated. Cells were transfected with pSG5 expressing v-src. Cells were then cultured in the medium containing 5% FBS or containing 2% FBS but with addition of insulin (30 μg/ml) or IGF1 (50 ng/ml) in the case of cells infected with IGF1R. Foci were scored when wild-type MEFs were cultured for 3 weeks, p110α-knockout (−/−) cells for 4.5 weeks.

The metastatic potential of Tpr-Met needs coupling to both Grb2 & PI3-kinase



METASTASIS ASSAY

- recovered the transformed cells that form the foci
- injection of transformed cells into the tail vein
- after two weeks, evaluation of the number of mice that died from lung metastases

The metastatic ability of Tpr-Met signaling mutants was tested in an experimental metastasis assay. Bars indicate the percentage of mice dead because of lung metastases 2 weeks after injection into the tail vein of cells transformed by the various Tpr-Met signaling mutants. The values reported are expressed as percentage of the lethality caused by Tpr-Met^{Wt} and represent the average of three independent experiments (18 mice/group)

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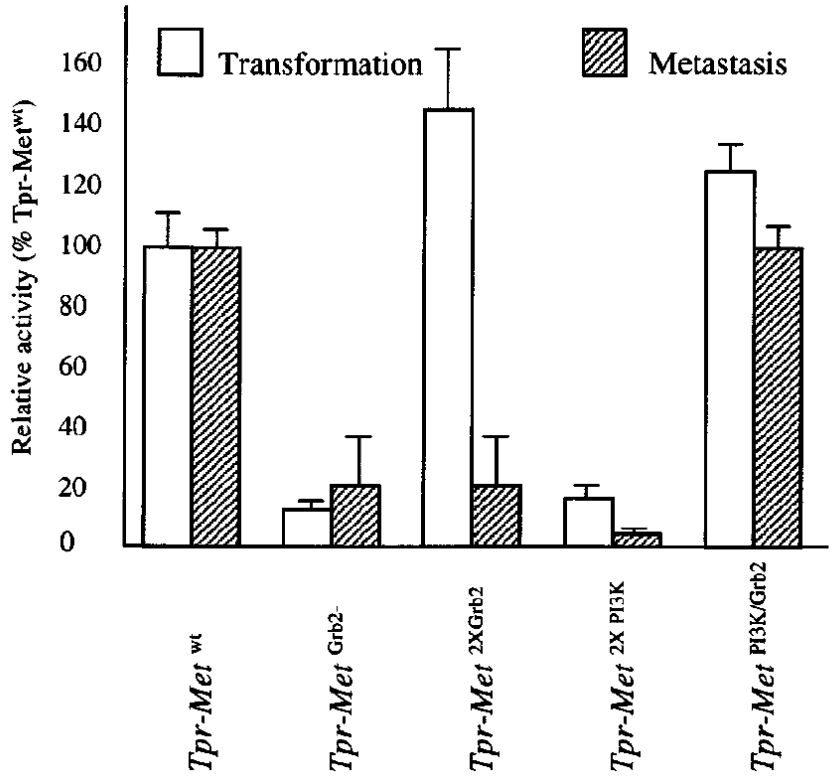

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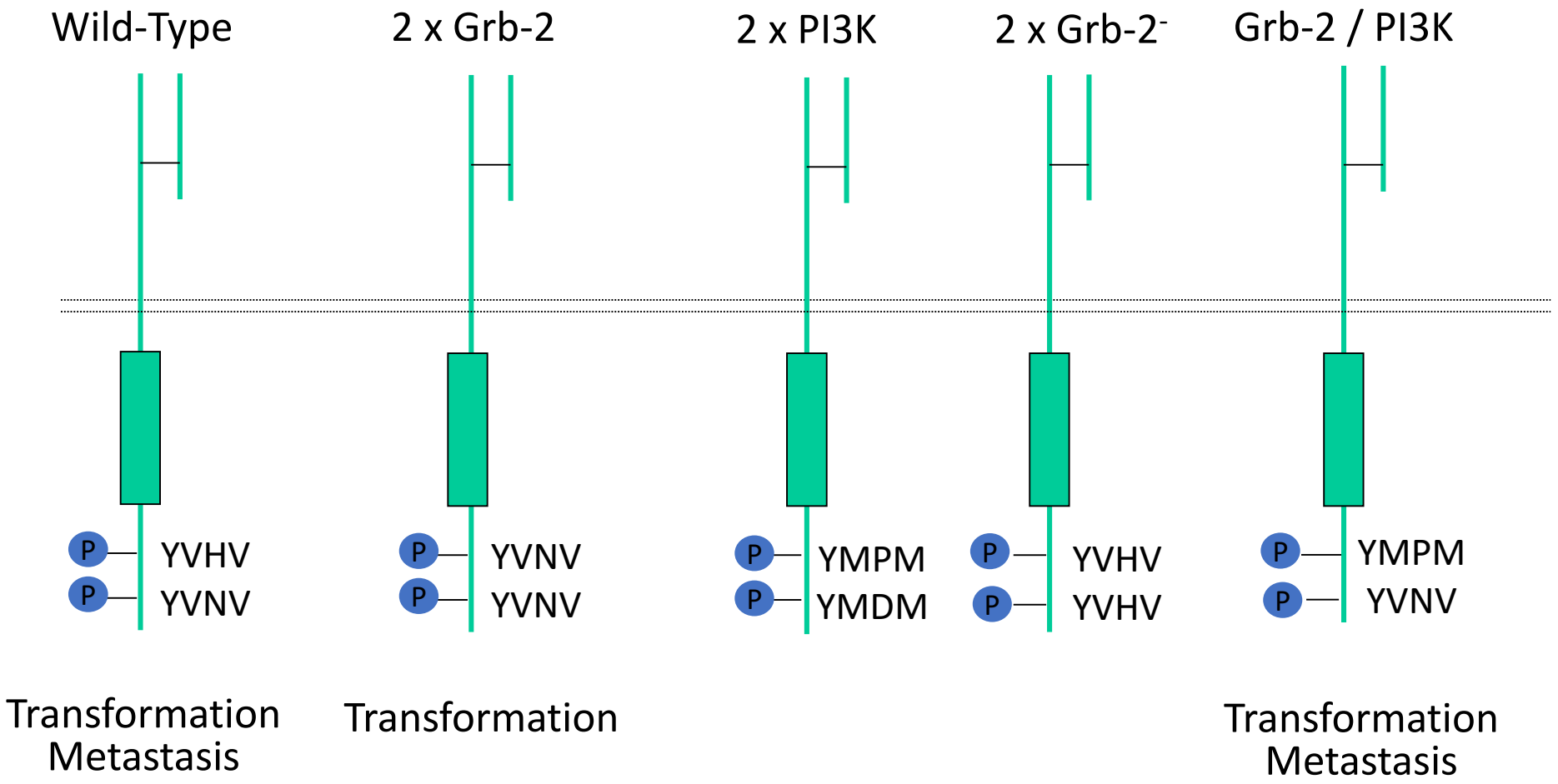
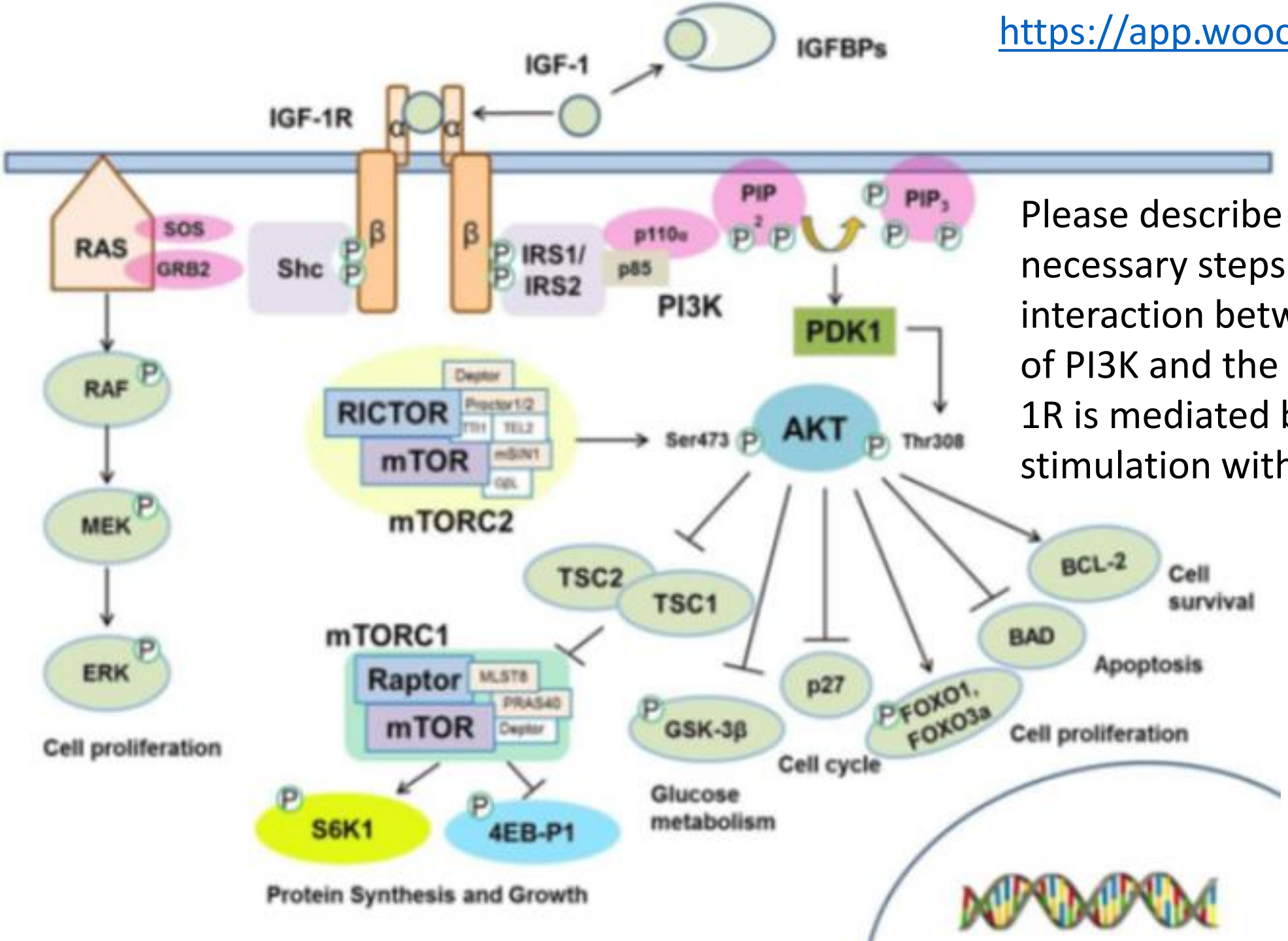
Dissection of MET signalling pathways



https://app.wooclap.com/events/ACBB



Please describe schematically the necessary steps to investigate if the interaction between the p85 subunit of PI3K and the beta chain of the IGF-1R is mediated by IRS1/IRS2 following stimulation with IGF-1

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Two examples of correct answers from the students :

4 plates are required
1-2: WT (IGF-1 -/+)
3-4: RNAi IRS1/IRS2 (IGF-1 -/+)
In total cell lysate: WB: p85, IGF1-R, IRS1/IRS2
In IP anti IGF1-R: WB: p85, IGF1-R

The more complete, containing all necessary information; it considers that all proteins are endogenously expressed in the cell. I added the WB for IGF1-R in the IP samples. A final comment on the possible results could be added.

- 4 plates: 2 transfected with p85 of PI3K and IGF-1R, -/+ IGF-1 treatment
  2 transfected with p85 of PI3K and IGF-1R and IRS1/IRS2, -/+ IGF-1 treatment
- protein extraction
- IP against IGF-R1
- SDS-PAGE on total cell lysates and IP
- western blot for p85 PI3K and IGF-1R to verify if p85 PI3K interacts with IGF-1R and to investigate if they interact when IRS1/IRS2 is present.
- if they do not interact when IRS1/IRS2 is not present, it suggests that the presence of IRS1/IRS2 is necessary for the interaction between p85 PI3K and IGF-1R.

Another complete version, that considers that not all proteins are endogenously expressed in the cell. Nevertheless, it should be verified that IRS1/IRS2 are <u>really</u> not endogenously expressed.
It contains a useful comment to the possible results.