

Characterization of corrector ARN23765 mechanism of action via Photo-Affinity Labeling (PAL) approach

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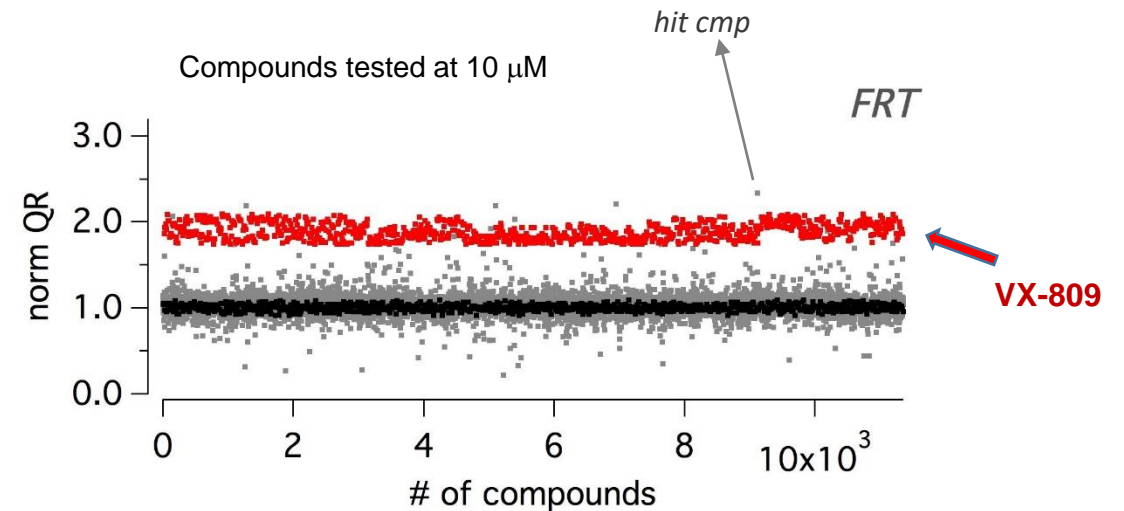
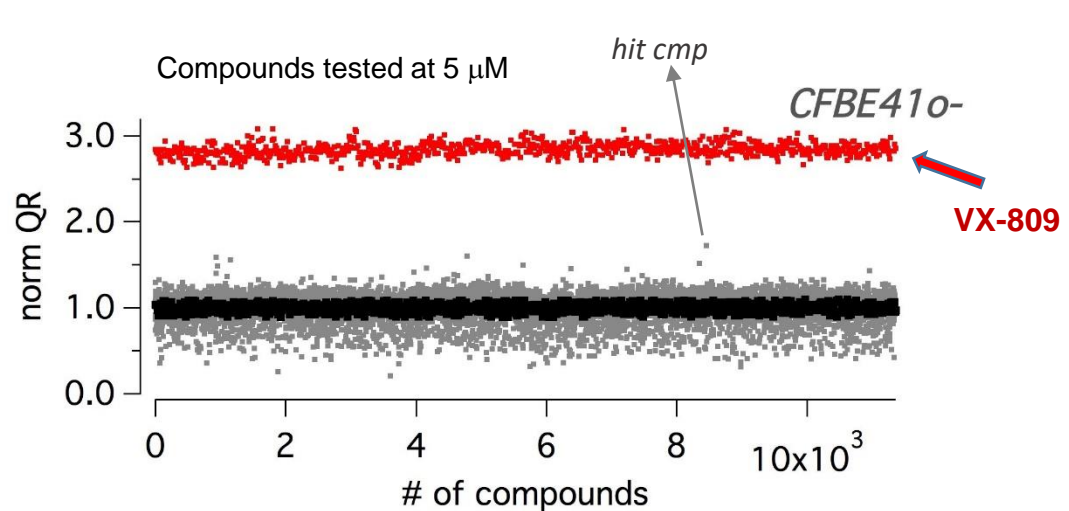
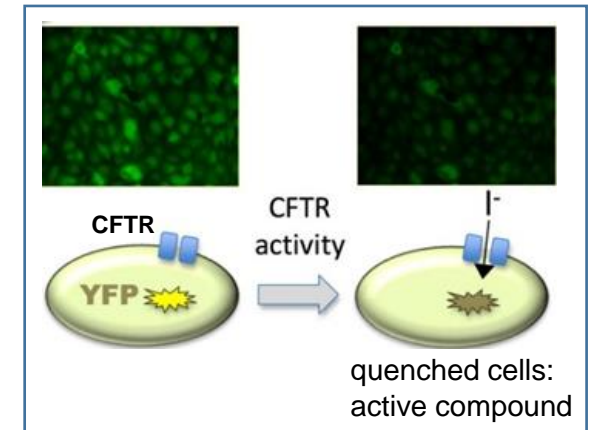
May 27th, 2024

Phenotypic HTS of IIT compound collection

A collection of 11,334 maximally diverse compounds was screened on two cell lines:

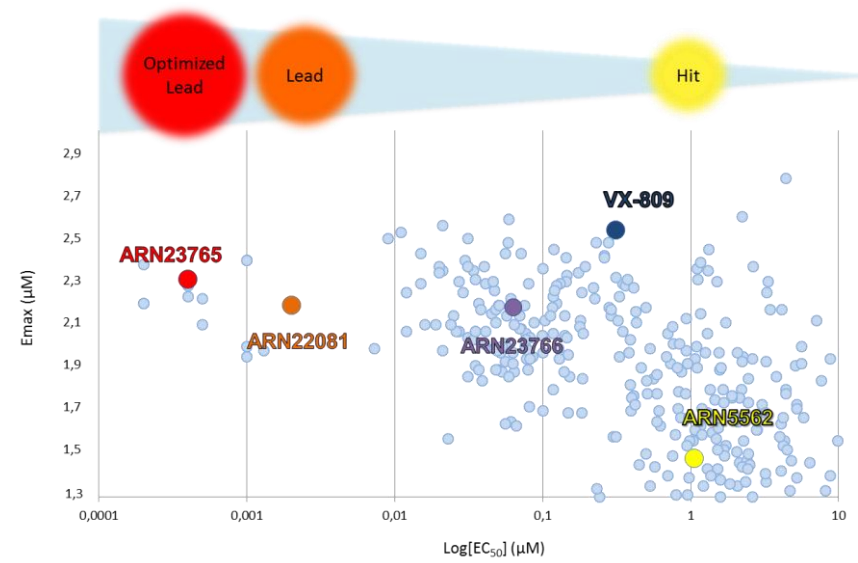
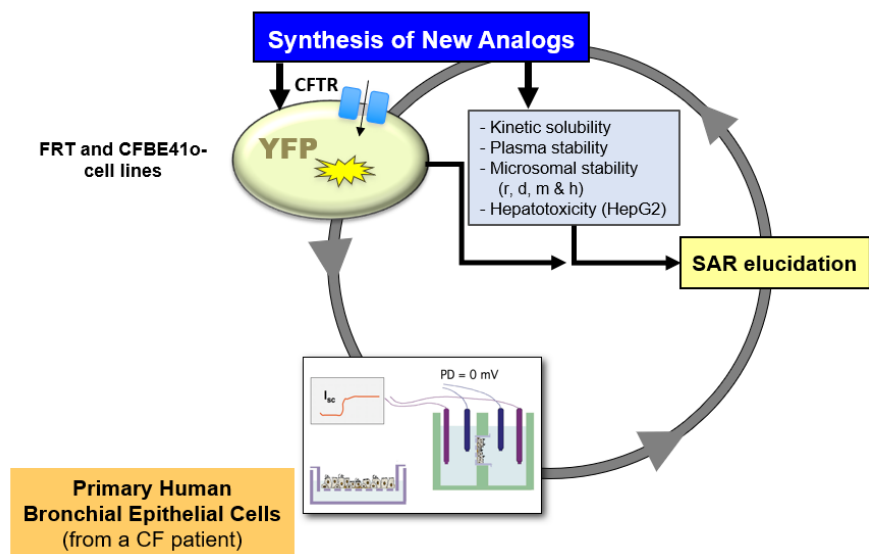
- **CFBE41o-** and
- **FRT**

Both cell lines stably express F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP)¹.



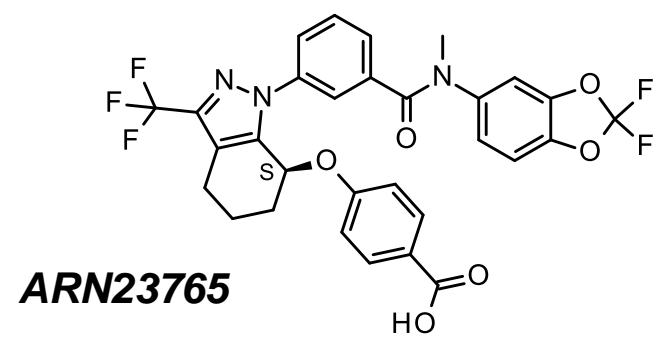
CFBE41o-: Cystic Fibrosis Bronchial Epithelial cells; **FRT**: Fisher Rat Thyroid cells

Discovery of ARN23765

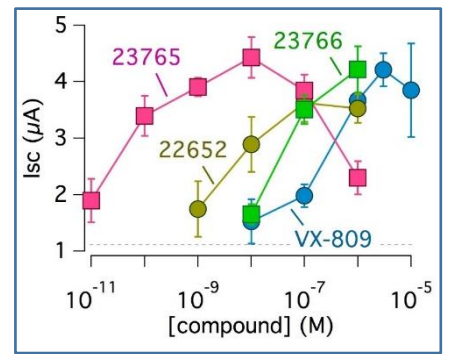


ca. 400 compounds synthesized and tested

E_{max} vs. EC_{50} on F508del-CFTR FRT cells (HS-YFP assay)



F508del/F508del HBE cells

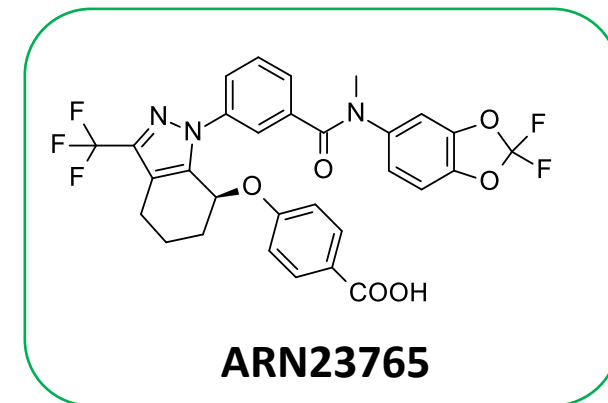
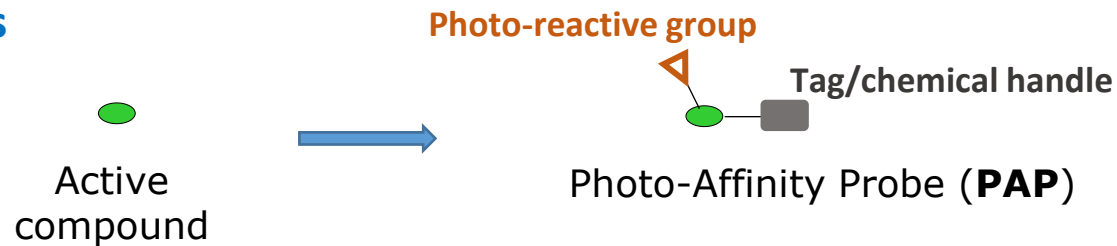


ARN23765 shows sub-nanomolar activity in rescuing F508del-CFTR in primary HBE cells from a F508del/F508del CF patient

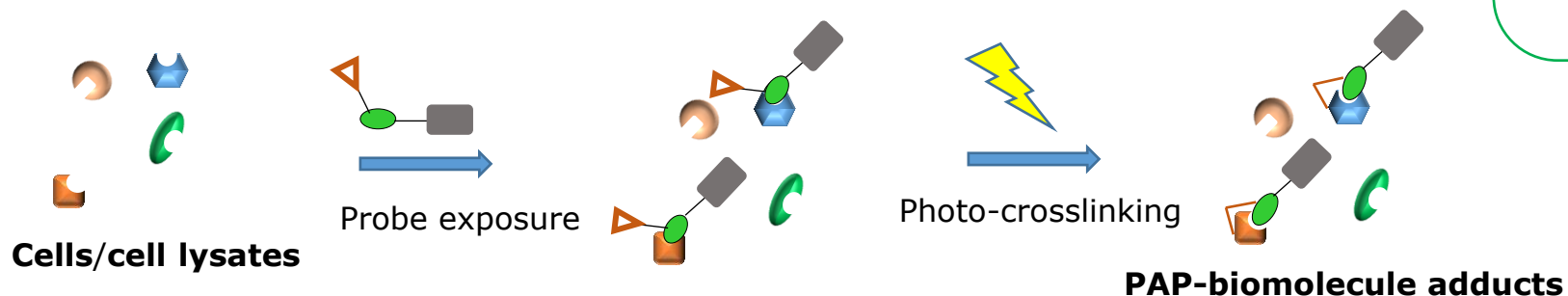
ARN23765 EC_{50} : **0.038 nM**
VX-809 EC_{50} : **~200 nM**

Photo-Affinity Labeling (PAL) strategy

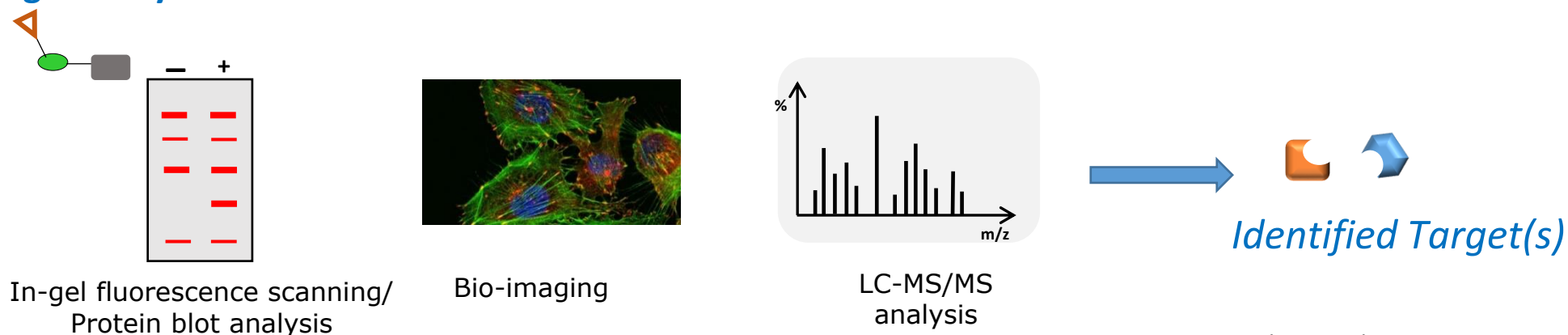
1) PAP design and synthesis



2) Target capturing



3) Target analysis and identification



Ge et al., *RCS Adv.* **2018**, 8, 29428

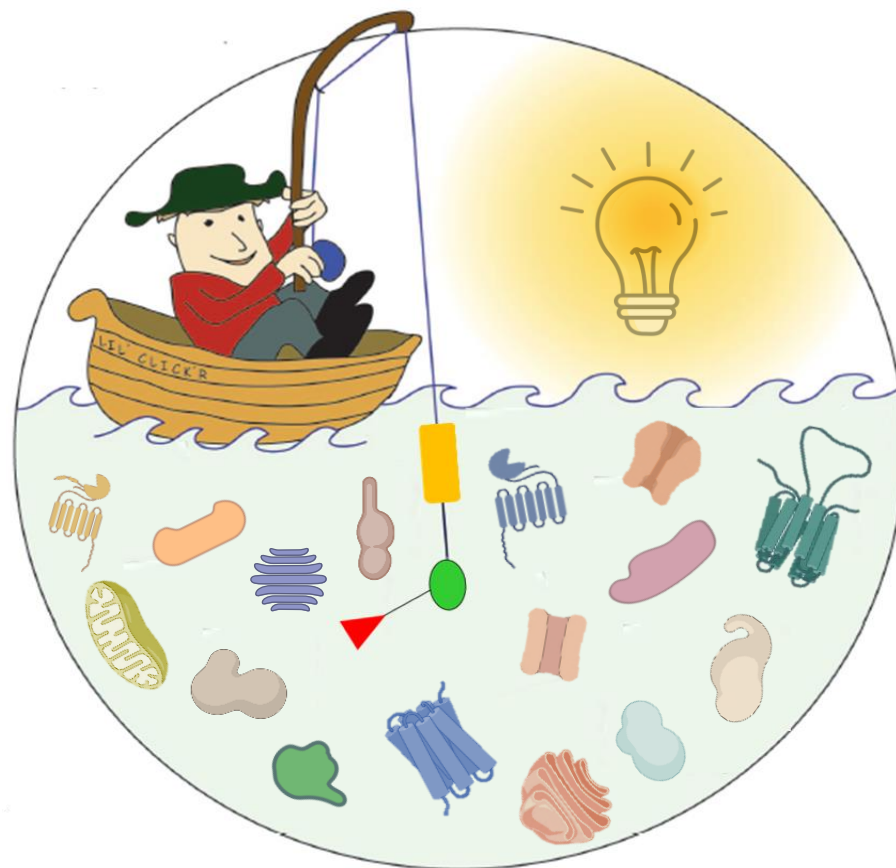
Hill et al., *J. Med. Chem.* **2018**, 61, 6945-6963

Dubinsky et al., *Bioorg. Med. Chem.* **2012**, 20, 554-570

Target ID: biased vs unbiased approach

BIASED APPROACH

We already have information on the compound's possible target and want to confirm it. This allows proceeding to investigate the MoA further



UNBIASED APPROACH

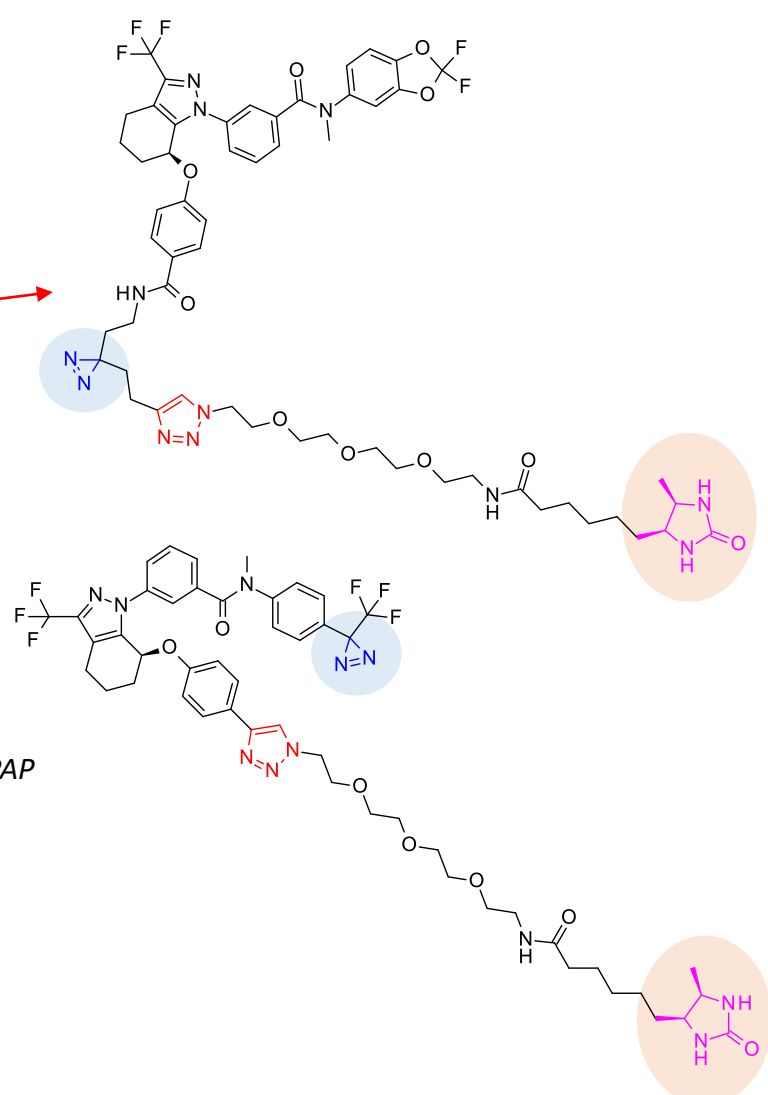
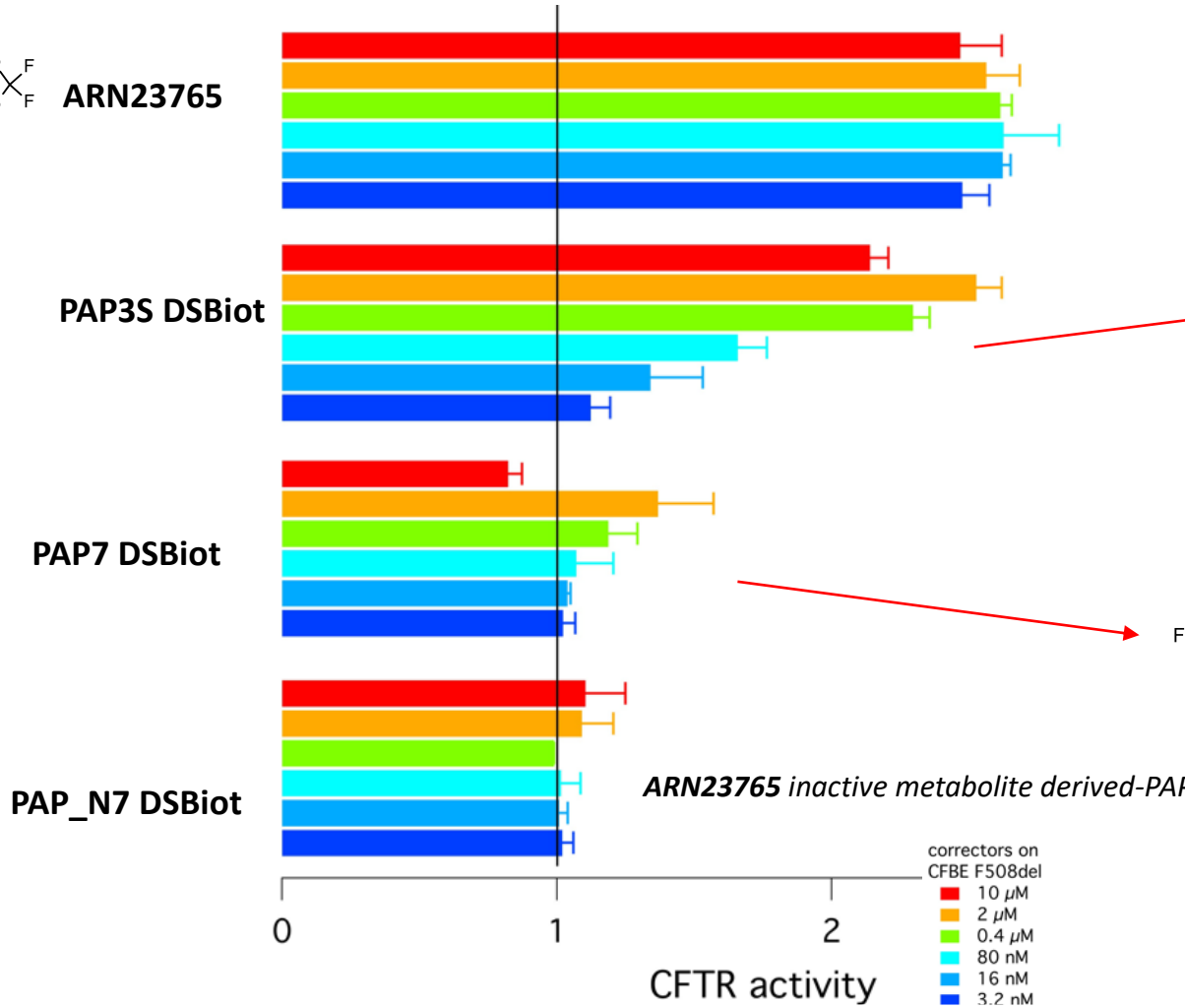
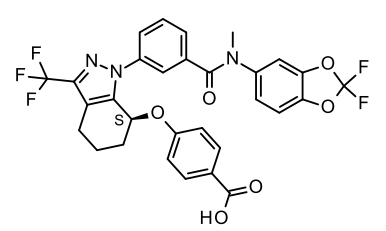
We do not have any information on the putative target(s)

OR

We want to explore other possible (off)-target(s) for a better characterization of compound's MoA

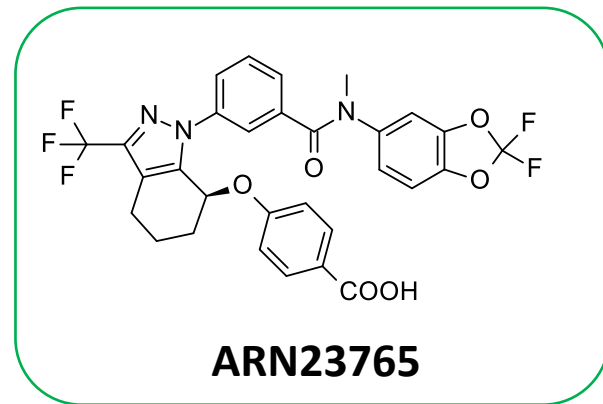
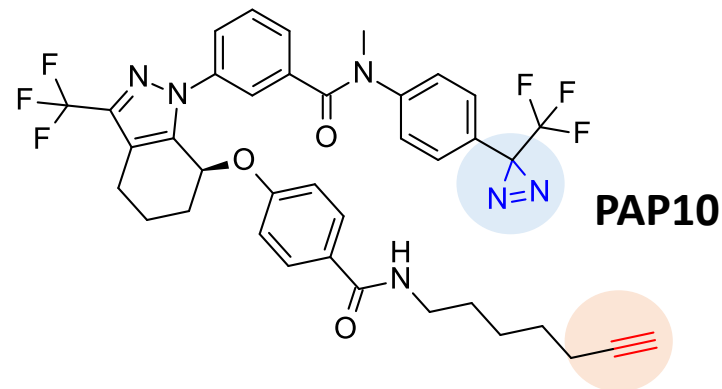
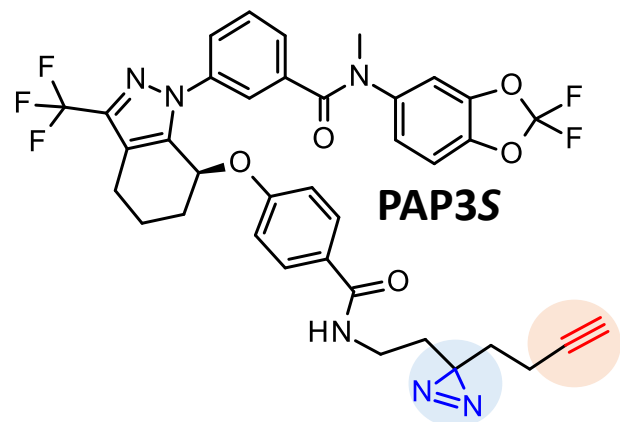
Adapted from: Sletten, E. M. et al., *Angew. Chem. Int. Ed. Engl.* **2009**, *48*, 6974-6798

Part I: CFTR identification with biotinylated PAPs

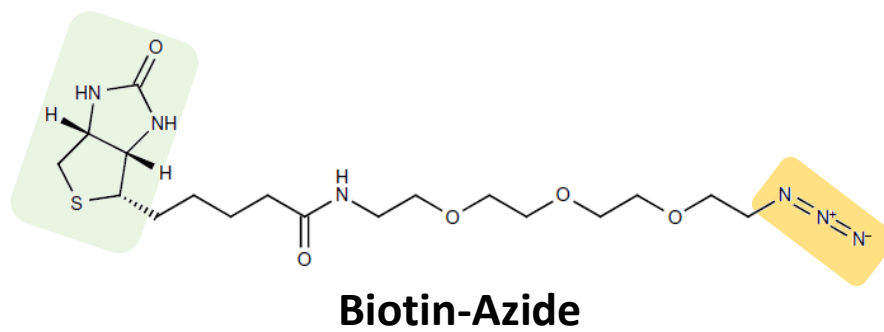
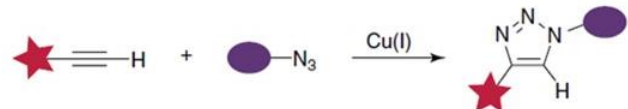


HS-YFP assay in F508del-CFTR CFBE410- cells

Part II: unbiased target ID (alkyne PAPs)

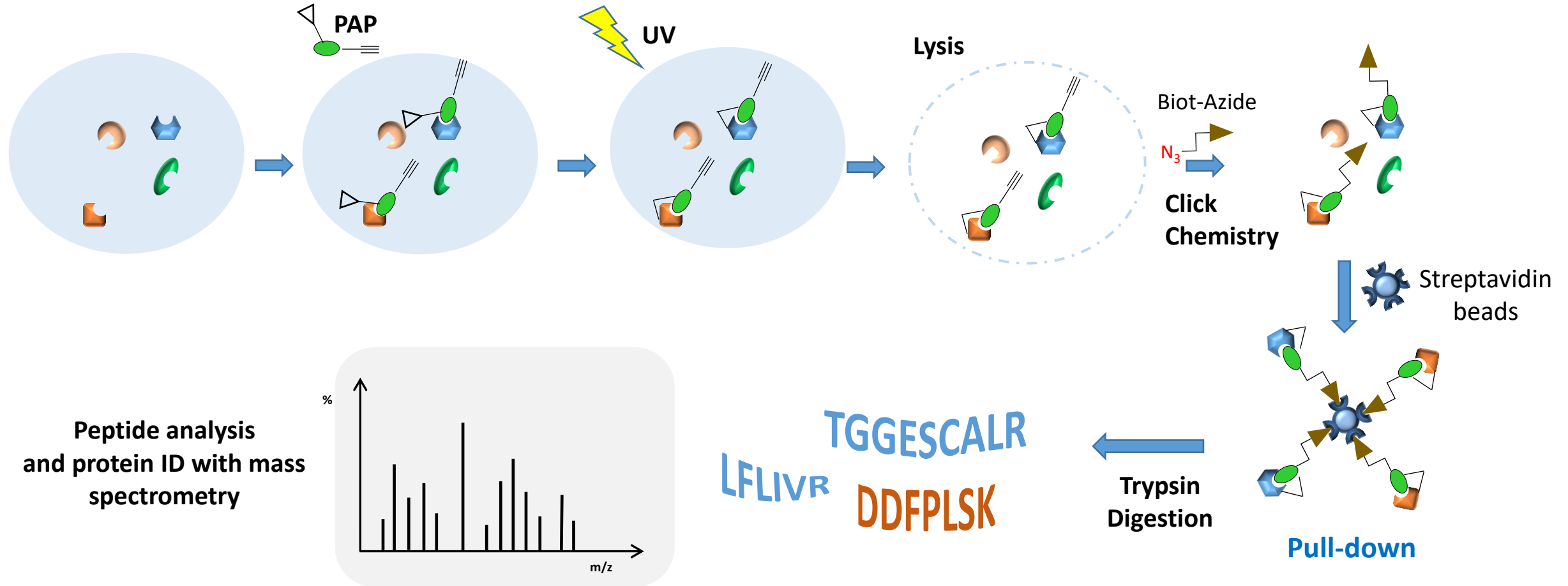


**Biotin-Tag addition
by Click Chemistry**



Sletten, E. M. et al., *Angew. Chem. Int. Ed. Engl.* **2009**, 48, 6974-6798

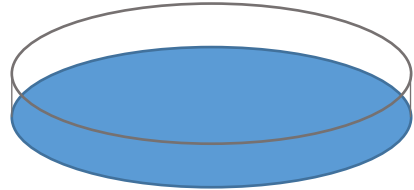
PAL with alkyne PAPs and Click Chemistry



- Stable isotope labeling using amino acids in cell culture (SILAC) method uses a non-radioactive metabolic labeling strategy to incorporate "heavy" ^{13}C - and/or ^{15}N -labeled amino acids in vivo into proteins during translation
- It allows comparing quantitatively two cell populations receiving different treatments and fed with either labeled or not labeled medium
- Proteins from both cell populations are combined and analyzed together by mass spectrometry as pairs of chemically identical peptides that can be differentiated owing to their different isotope composition (i.e., mass difference)
- The ratio of peak intensities in the mass spectrum for such peptide pairs reflects the abundance ratio for the two proteins

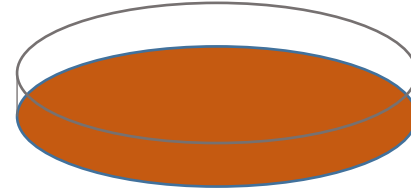
Live-cell PAL and quantitative proteomics (SILAC approach)

“Light” medium



Competitive PAL
(parental cmpd + probe)

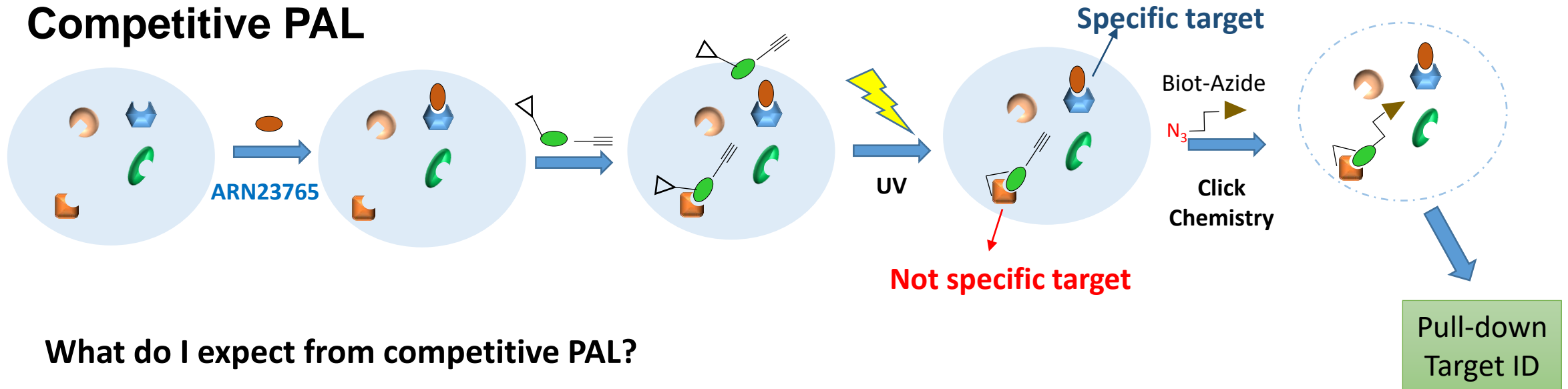
“Heavy” medium



Classical PAL
(probe only)

L-Arg ($^{13}\text{C}_6$ $^{15}\text{N}_4$)
L-Lys ($^{13}\text{C}_6$ $^{15}\text{N}_2$)

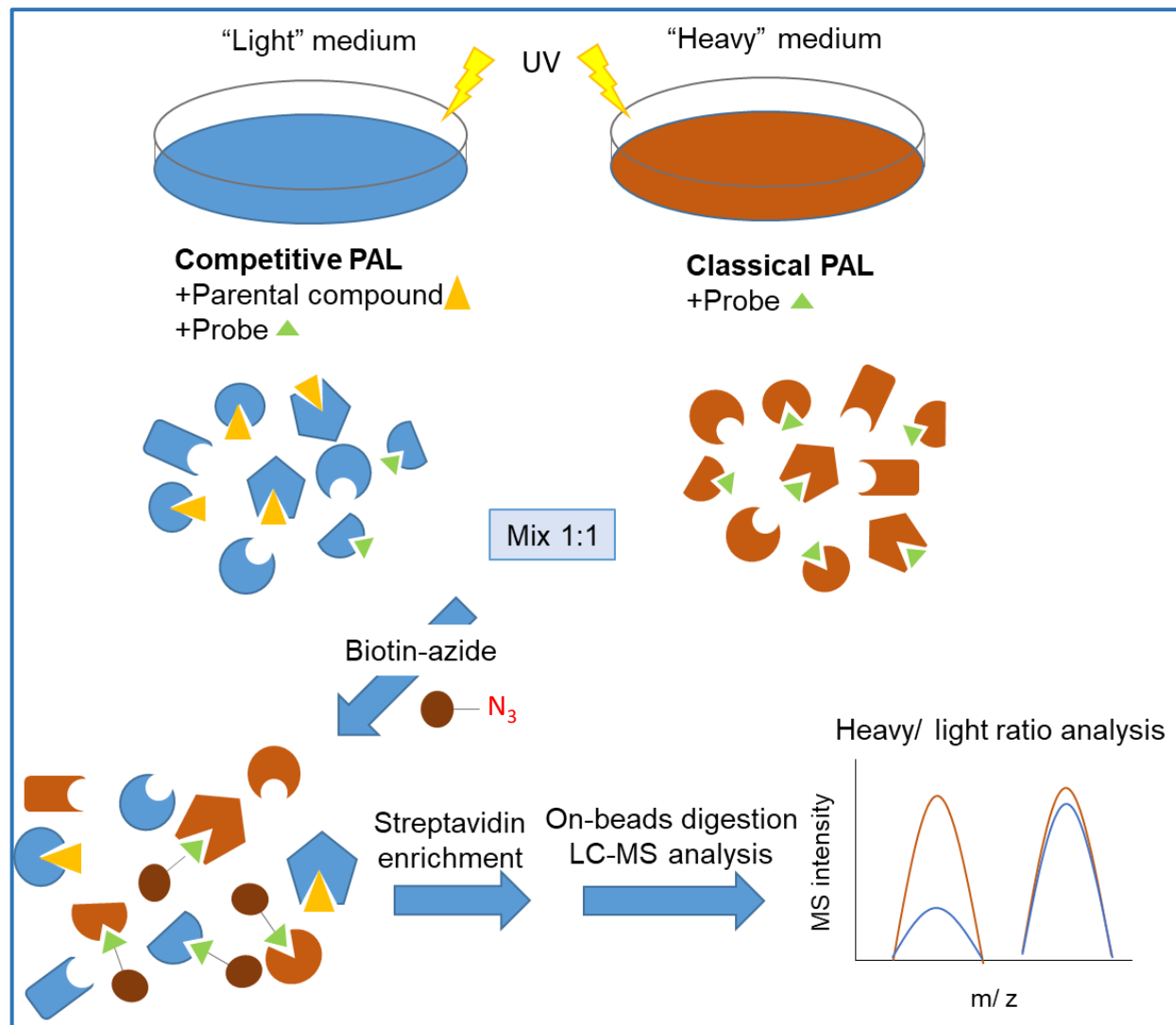
Competitive PAL



What do I expect from competitive PAL?

- lower recovery of ARN23765-bound proteins (i.e. specific targets)

Live-cell PAL and quantitative proteomics (SILAC approach)



What do I expect from competitive PAL + SILAC quantitative proteomics?

When “true” target: $\frac{\text{Classic PAL}}{\text{Competitive PAL}} > 1$

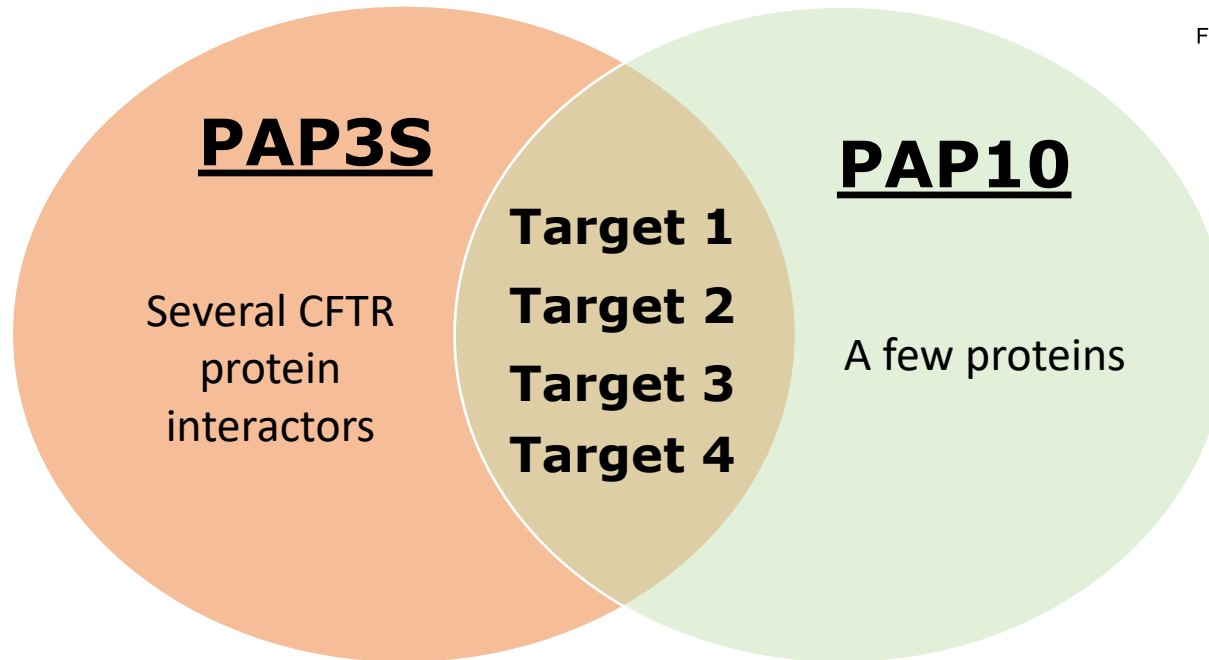
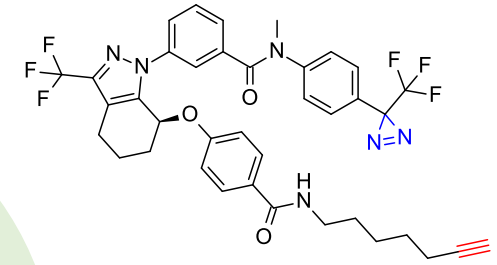
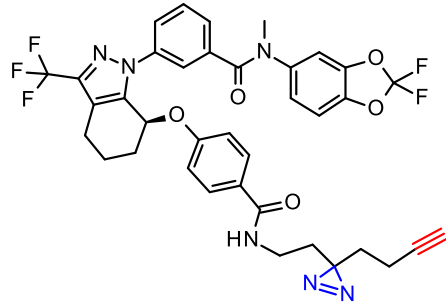
In our experiments:

$\frac{\text{Heavy peptide}}{\text{Light peptide}} > 1$ **Cut-off ≥ 1.5**

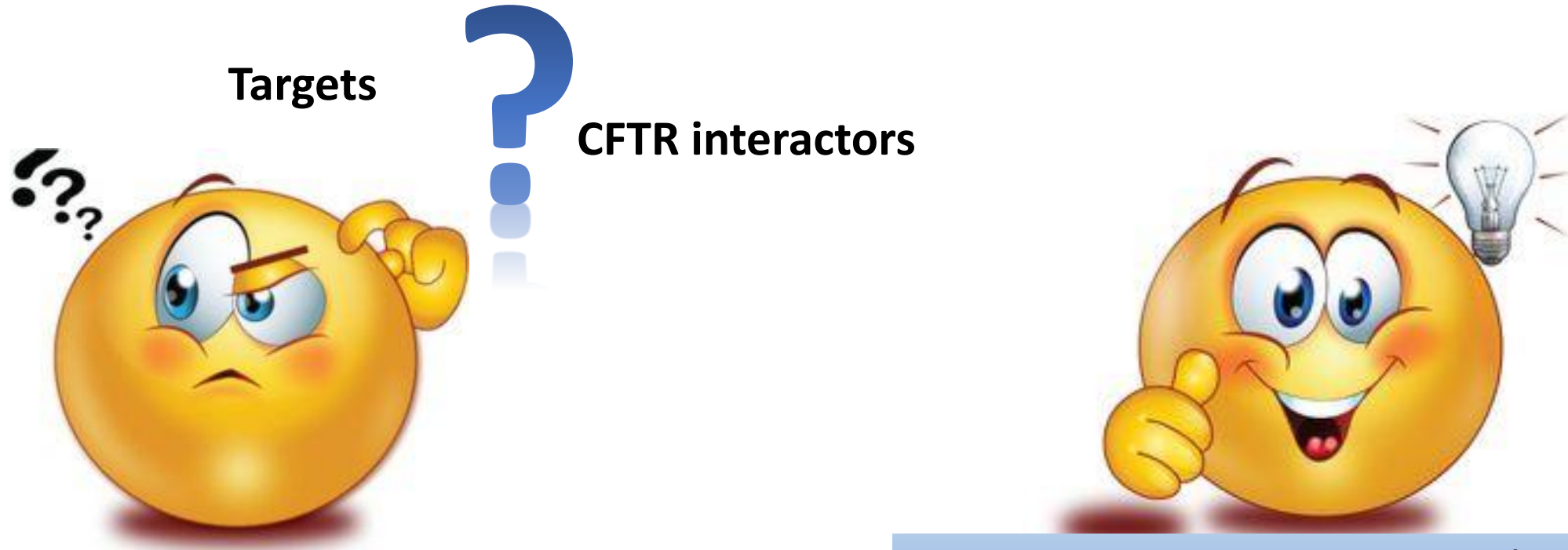
Selection criteria

Protein identified by ≥ 2 peptides
Protein identified in all samples
Heavy/ light ≥ 1.5 with % CV $< 25\%$

Proteins common to PAP3S and PAP10



Target validation (I)



Target confirmation in CFTR ^{-/-} cell lines

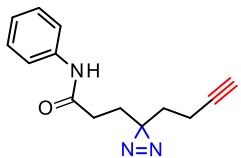
Target ID in CFTR^{-/-} cells

Target ID in CFTR^{-/-} CFBE41o- cells

<i>Identified Proteins with at least 2 peptides</i>	
PAP3S_Rep1	112
PAP3S_Rep2	113
PAP3S_Rep3	91
PAP10_Rep1	69
PAP10_Rep2	24
PAP10_Rep3	2
NP_Rep1	37
NP_Rep2	21
NP_Rep3	203

Target ID in HEK-293 cells

<i>Identified Proteins with at least 2 peptides</i>		
NP	NP_1	371
	NP_2	508
	NP_3	437
	NP_4	399
PAP 3S	PAP3S_1	578
	PAP3S_2	540
	PAP3S_3	653
	PAP3S_4	567
PAP 10	PAP10_1	502
	PAP10_2	493
	PAP10_3	430
	PAP10_4	481

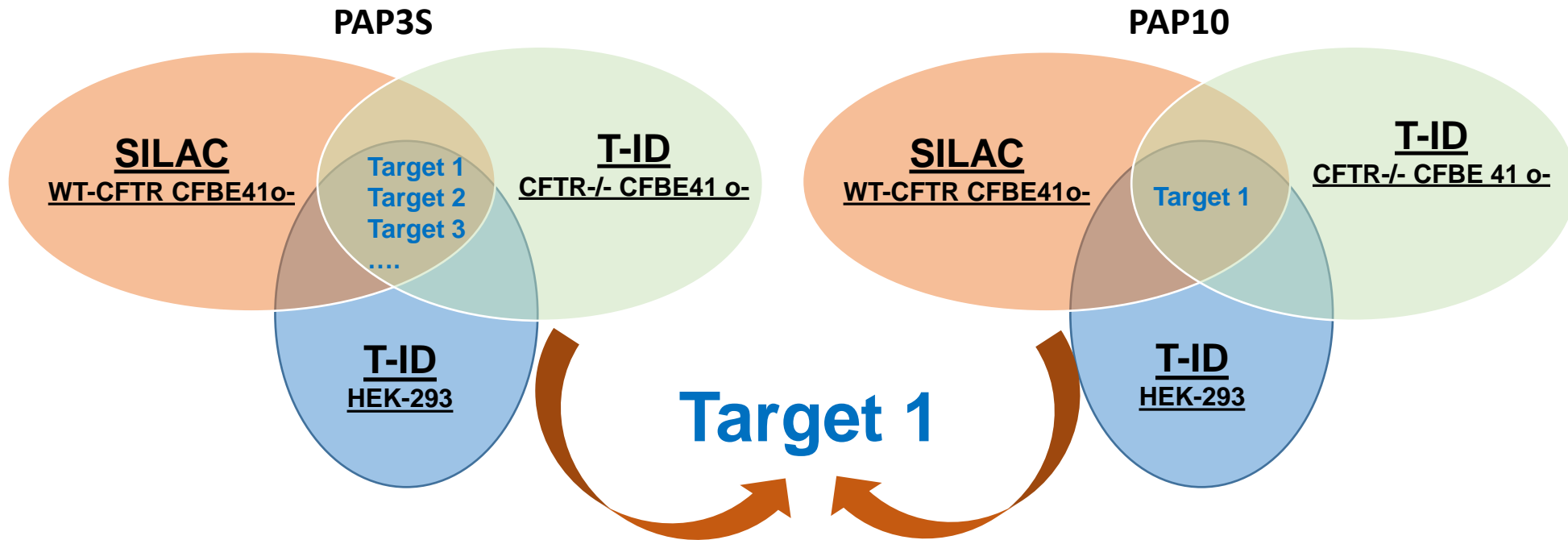


NP

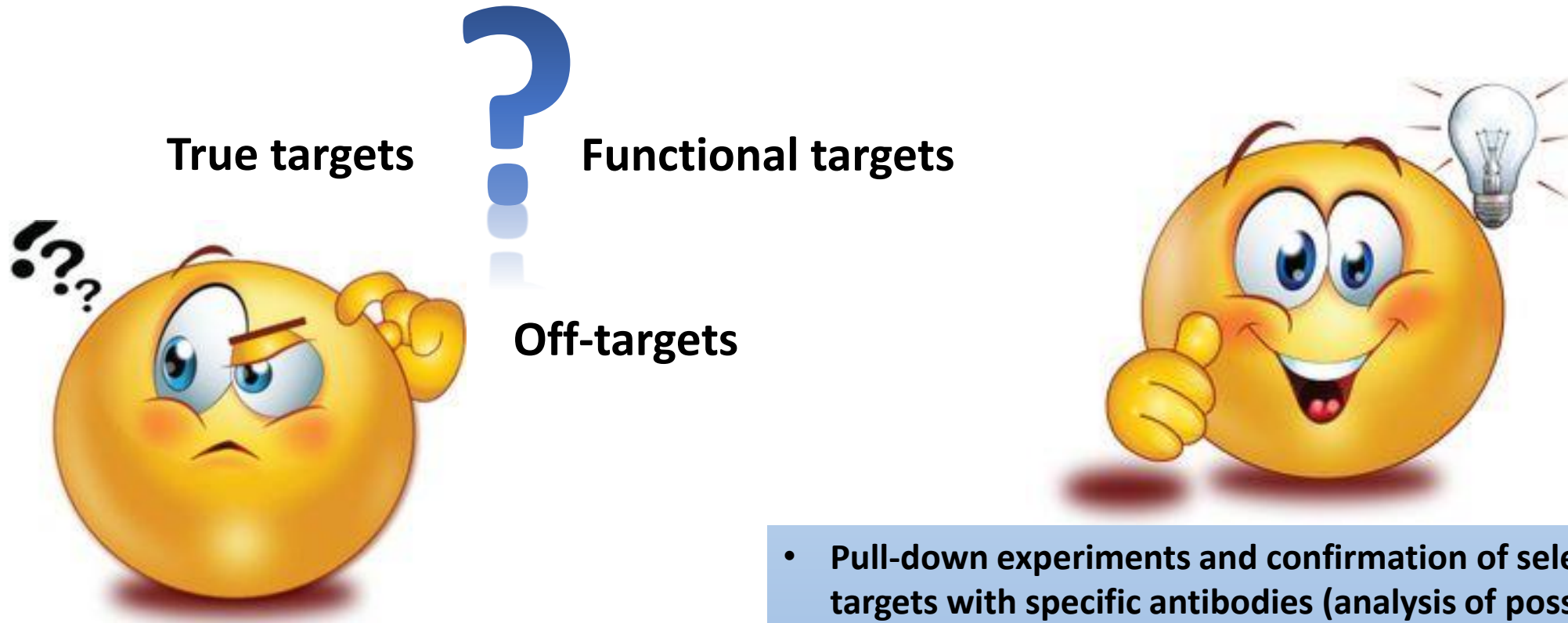
Overall analysis (II)

Proteins identified with both PAP3S and PAP10 in all experiments

Strategy summary



Target validation (II) - Future perspectives



- Pull-down experiments and confirmation of selected targets with specific antibodies (analysis of possible Target 1-isoforms)
- Competition assays with ARN23765 to confirm target specificity
- Functional assays

Target 1

Acknowledgments



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italian cystic fibrosis research foundation