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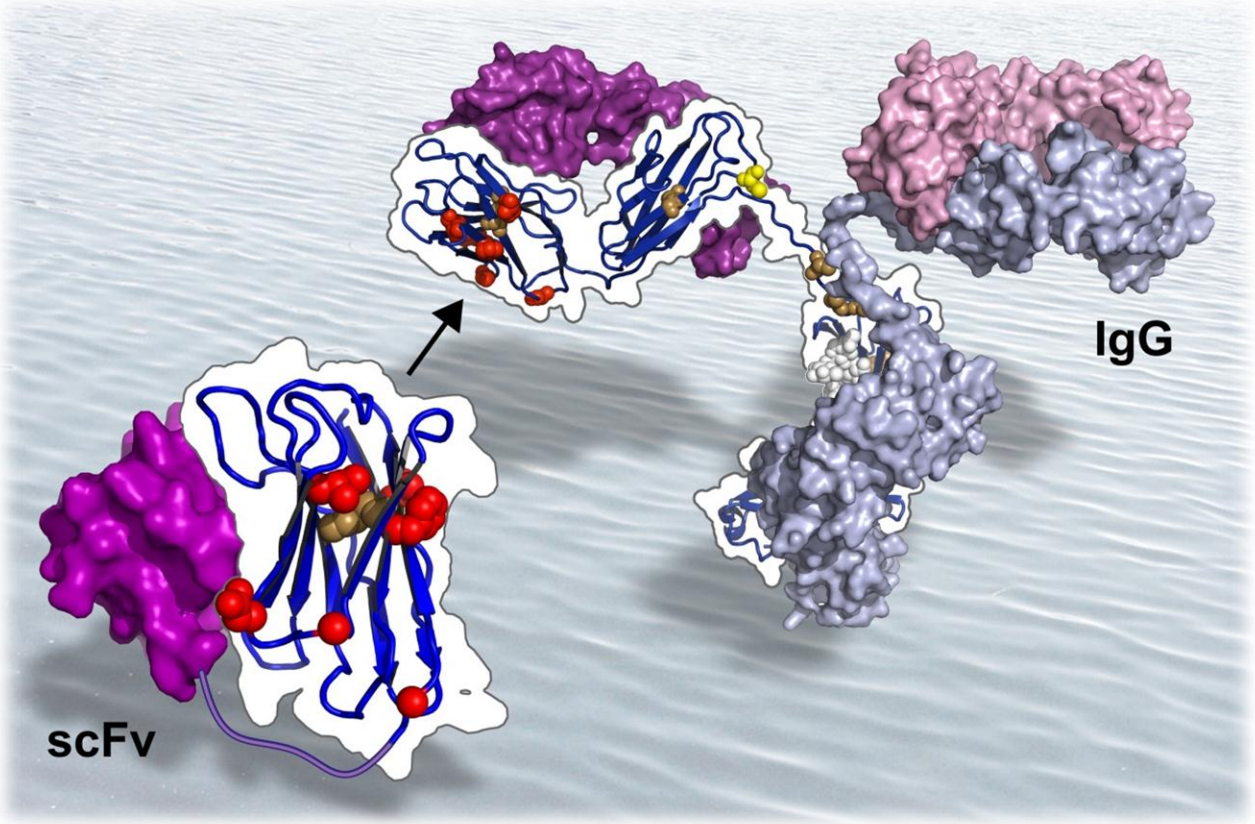
Optimize 2013
ANTIBODY ENGINEERING & DISCOVERY

February 26th, 2013

Transferring Engineered Properties between Antibody Formats and Expression Systems



Jonas V. Schaefer, PhD Department of Biochemistry , University of Zurich

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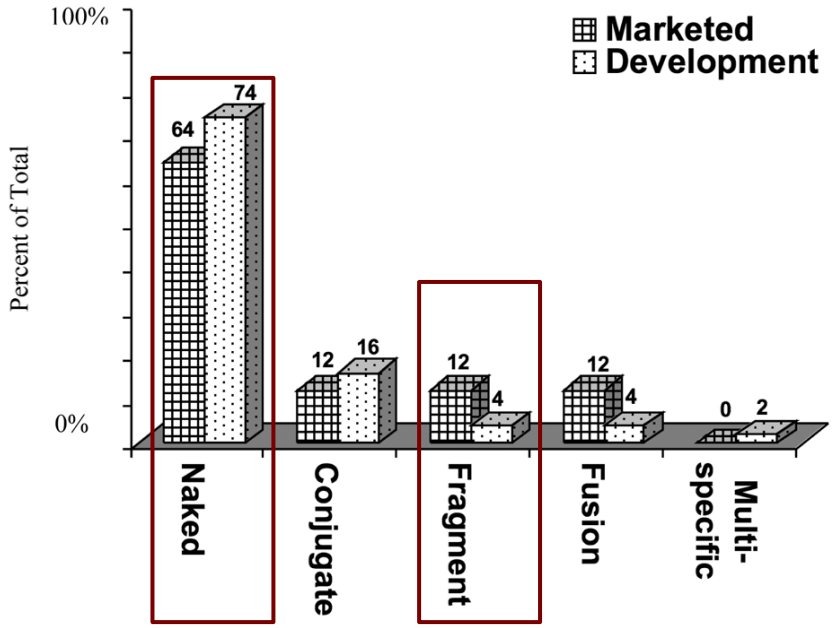
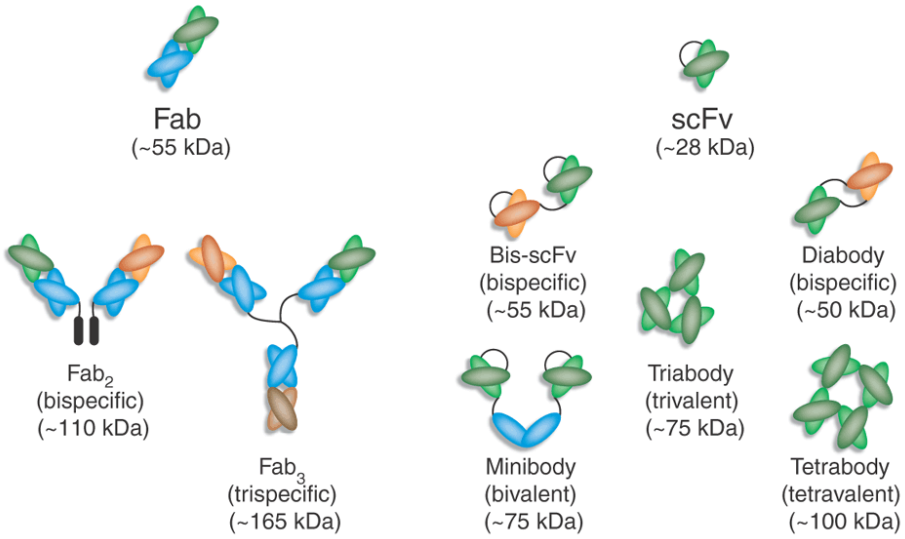
Antibody therapeutics vs. engineering





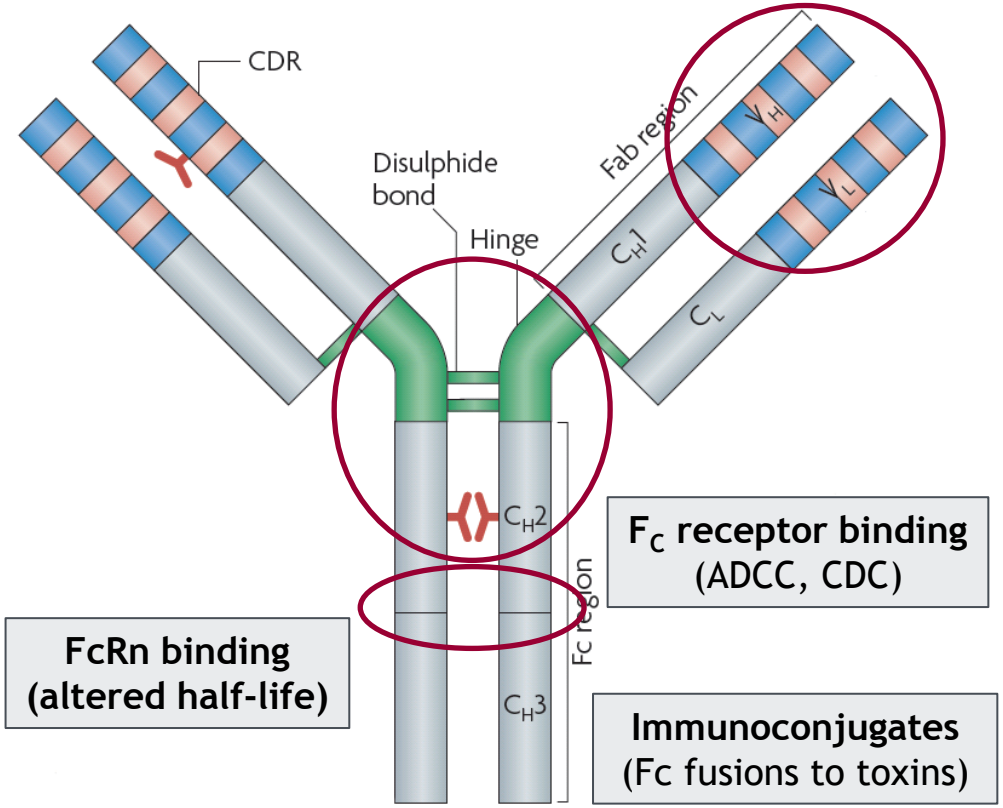
➡ while most antibodies on the market / in R&D are **full-length IgGs**, most of the antibody engineering is performed using **small fragments**

Zider and Drakeman, *Mabs* (2010)

Holliger and Hudson, *Nat. Biotechnol.* (2005)

Full-length IgG engineering

Optimization antigen binding
(affinity, humanization, decreased elimination)



F_C receptor binding
(ADCC, CDC)

FcRn binding
(altered half-life)

Immunoconjugates
(Fc fusions to toxins)

➡ variable domains mainly used for **individual optimization**

➡ most "transferable" engineering is focused on **F_C region**

adapted from Beck *et al.*, *Nat. Rev. Immunol.* (2010)

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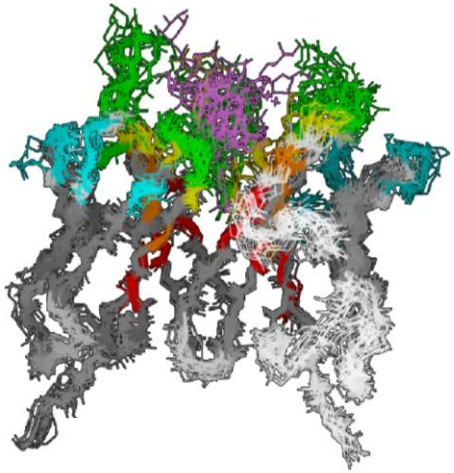
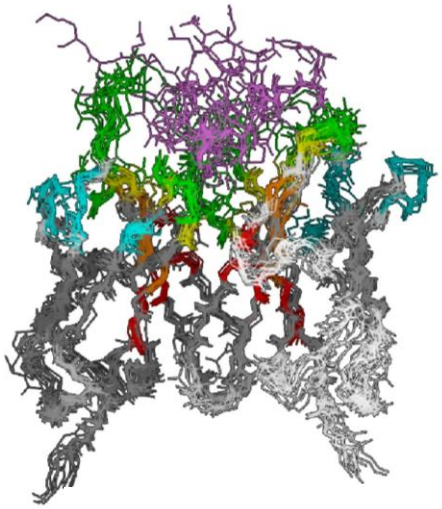
Why not just one "perfect" framework?

seven V_H germline families with different biophysical properties

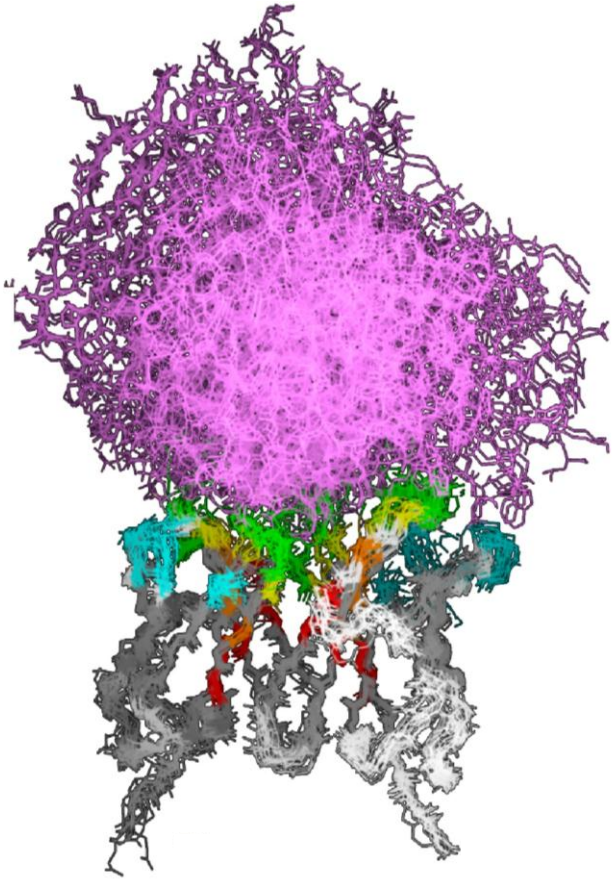
➡ variability in subfamilies increases **binding diversity**



Hapten



Peptides



Proteins

Ewert *et al.*, *Methods* (2004)

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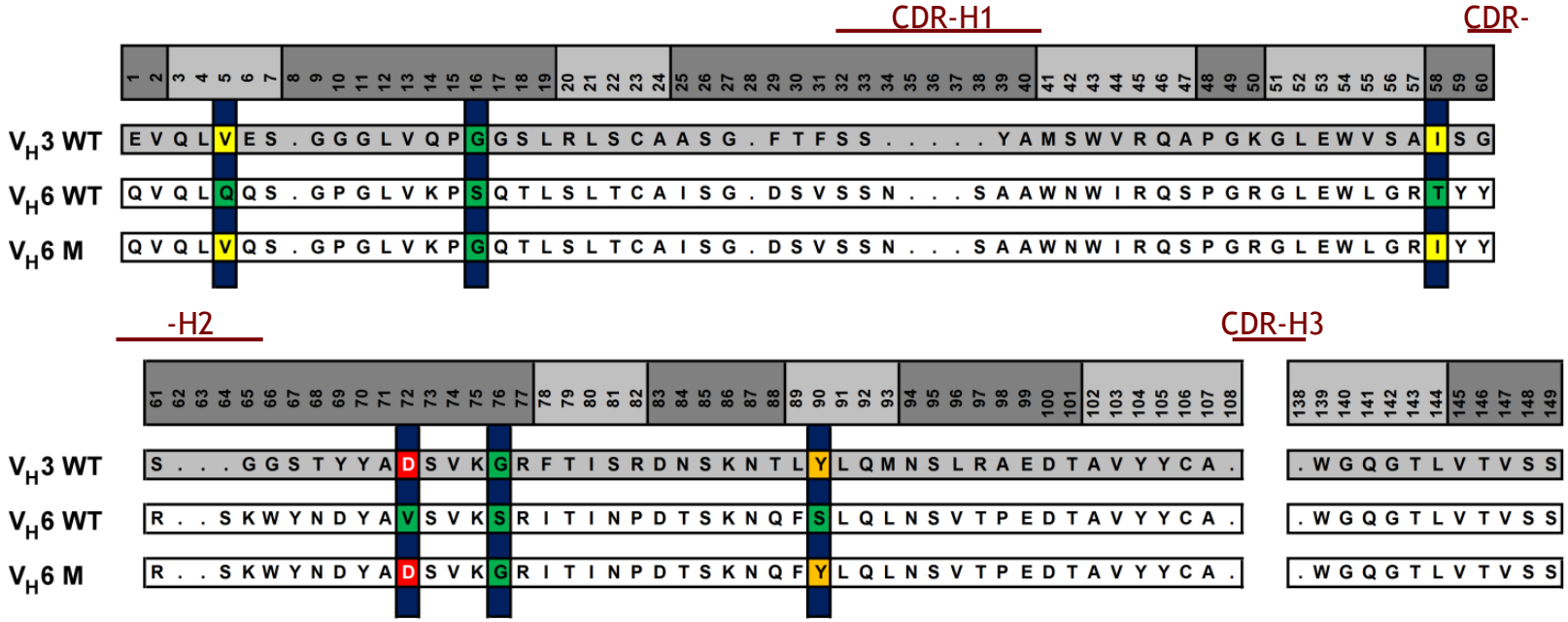
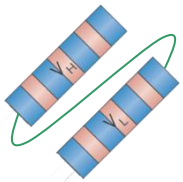
Engineering of unstable V_H6 domain

comparison of the human consensus V_H domains (germinal)



improved biophysical properties of scFv fragments expressed in *E. coli*:

- **increased stability: $\Delta\Delta G_{N-U}$ = 20.9 kJ/mol**
- **4-fold increase in expression levels**



Ewert *et al.*, *Biochemistry* (2003)

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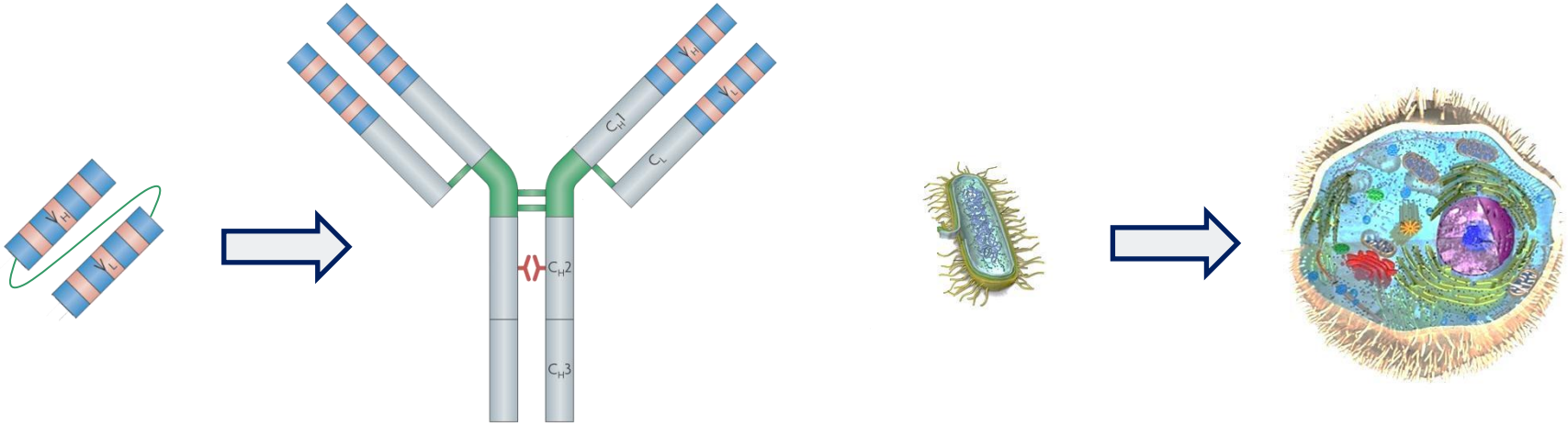
Are previous findings transferable?



scFv IgG prokaryote eukaryote

- Are the effects of the mutations "dampened" in a **larger assembly**?
- Does the eukaryotic **secretory quality control** overcome folding issues?

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Model antibodies

	IgG 6B3	IgG 2C2
heavy chain (HC)	V_H6	V_H6
antigen	protein	peptide
light chain (LC)	**$V_\lambda3$** (lambda)	**$V_\kappa3$** (kappa)

➡ chosen model IgGs differ in

- Fab stability: rather unstable (6B3) vs. extremely stable (2C2)
- pI: 6.9 (6B3) vs. 8.7 (2C2)
- antigen: protein vs. peptide

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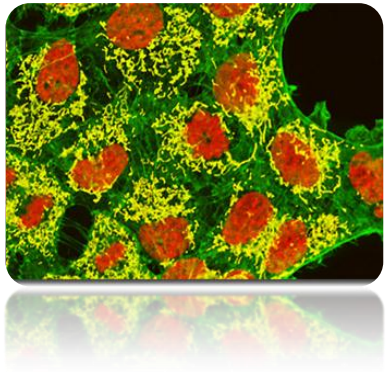
Eukaryotic expression systems

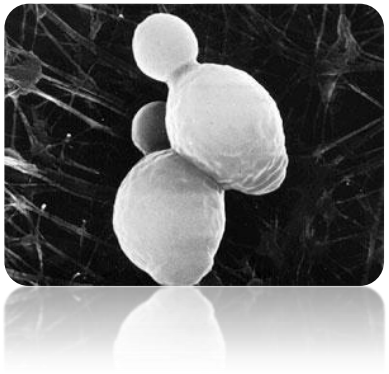
Mammalian cell culture (HEK)



stable HEK293 (Flp-In)

CMV promoters (**constitutive**)

Yeast *Pichia pastoris* (PP)



stable SMD1163

GAP promoters (**constitutive**)

- stable clones differ only in **point mutations** (same genetic locus)
- **constitutive expression** eliminates complications of induction strategy
- **expression level directly attributable to protein variant**

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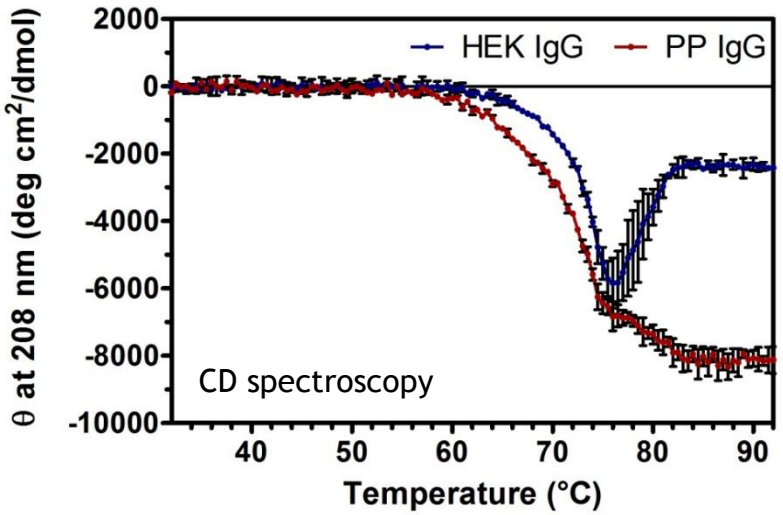
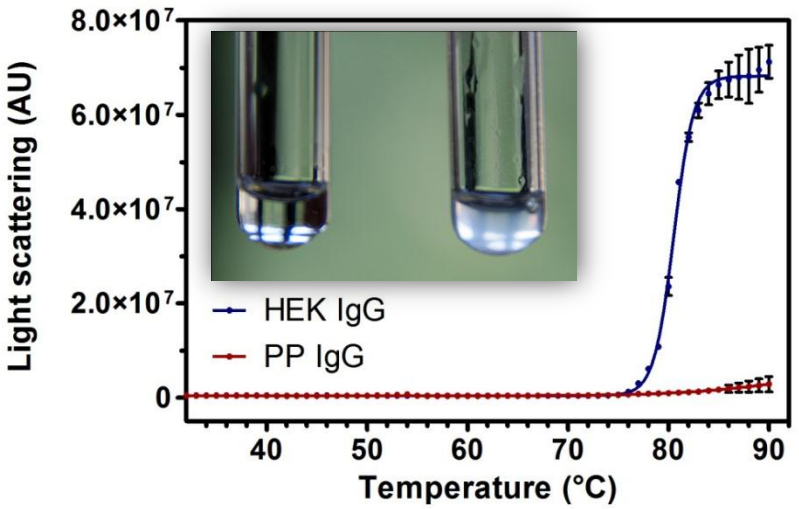
Difference in aggregation susceptibility





- ***Pichia*-derived glycans** reduce aggregation tendency
- **EAEA-peptide** (originating from yeast signal sequence) decreases aggregation susceptibility of HEK-IgG upon N-terminal addition

Schaefer and Plückthun, *J. Mol. Biol.* (2012)

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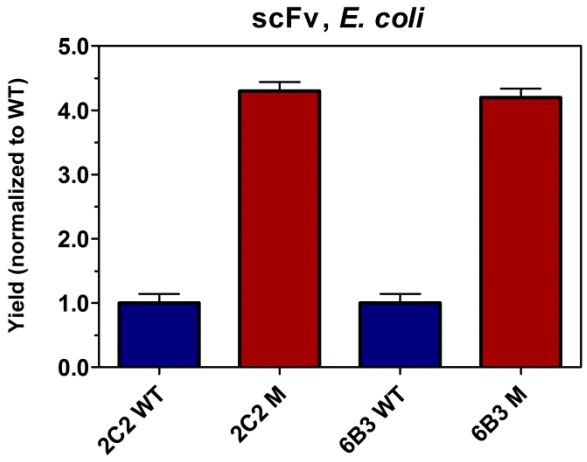
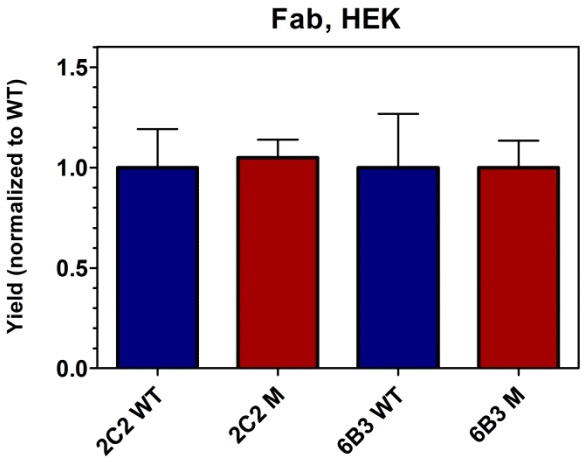
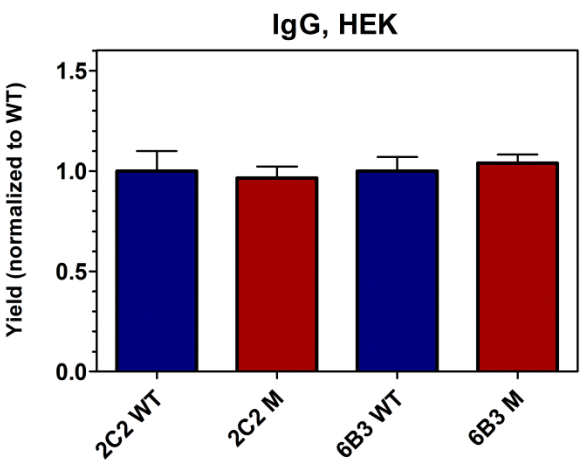
Comparison of expression levels

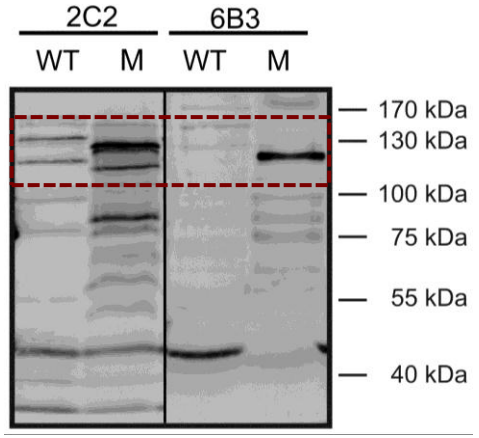






- eukaryotic **chaperons** and **quality control** systems equalize the expression yield between WT and stabilized V_H6

- **prokaryotic expression** of IgGs indicates increased periplasmatic levels of the M variants



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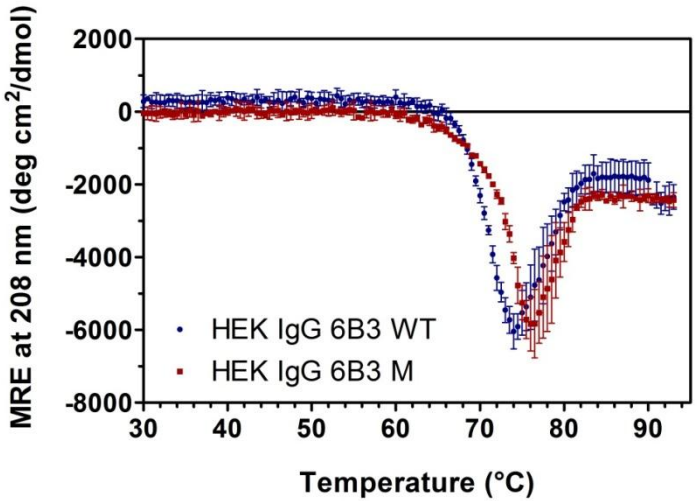
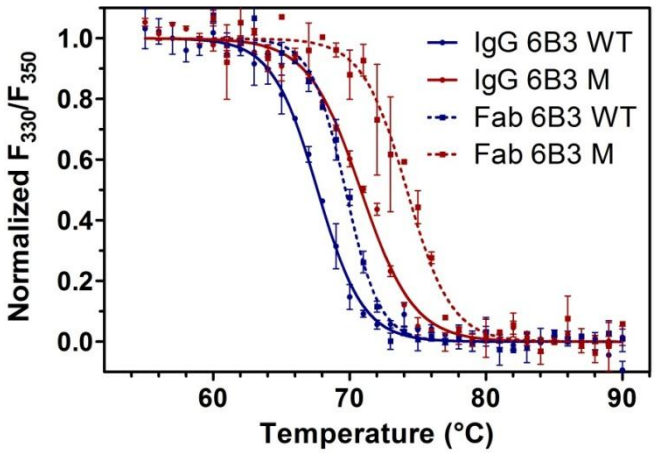
Stabilizing effects of V_H6 mutations

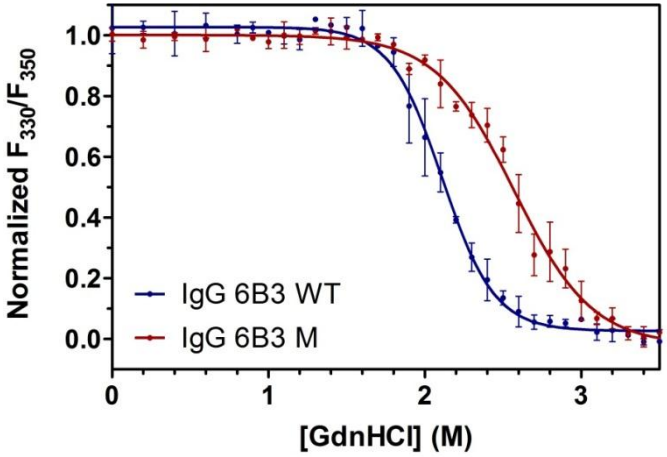
CD208nm

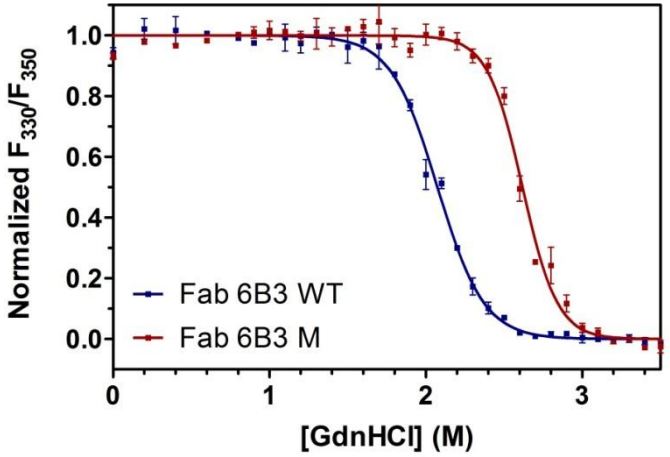


ITF



GdnHCl-unfolding





Methods described in Garidel *et al.*, *Biotechnol. J.*(2008)

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Stability overview

			ITF	GdnHCl	DSF	DSC
IgG 2C2	WT		70.4°C*	2.5 M	n.d.	86.0°C
	M		71.8°C*	3.8 M	n.d.	87.8°C
		Δ =	**1.4°C**	**1.3 M**	**-**	**1.8°C**
IgG 6B3	WT		67.6°C	2.0 M	74.5°C	72.1°C
	M		70.8°C	2.6 M	77.0°C	74.3°C
		Δ =	**3.2°C**	**0.6 M**	**2.5°C**	**2.2°C**
Fab 6B3	WT		69.7°C	2.0 M	76.5°C	72.6°C
	M		74.2°C	2.6 M	80.0°C	76.6°C
		Δ =	**4.5°C**	**0.6 M**	**3.5°C**	**4.0°C**

* – determined in the presence of 1 M GdnHCl

n.d. – not determined

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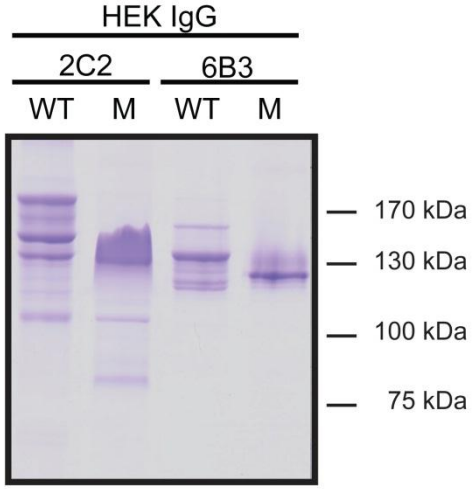
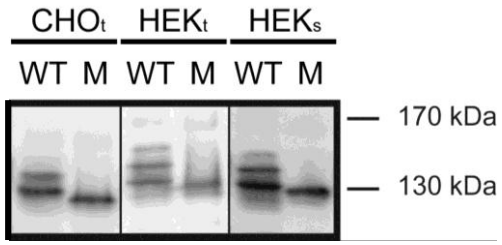
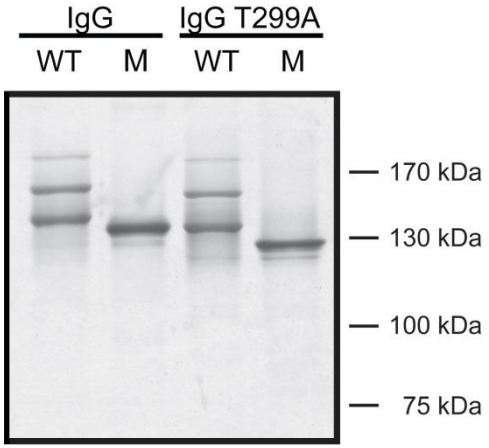
Electrophoretic analyses of IgGs



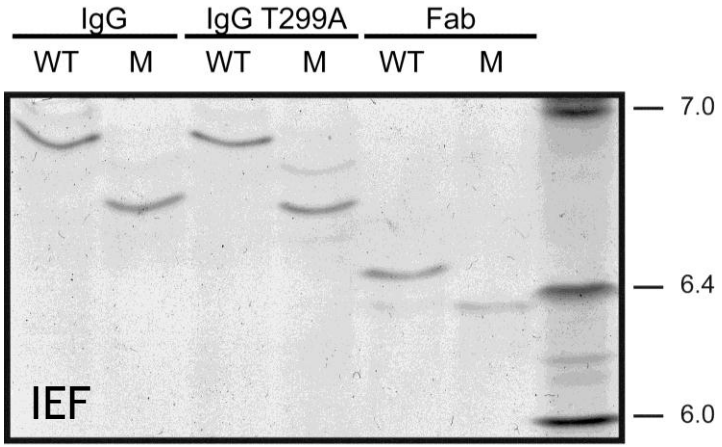




➡ non-reducing SDS-PAGE reveals **inhomogeneity of WT, but not of M variants**

➡ banding pattern is not caused by:

- **glycosylation**
- **proteolysis**
- **charge heterogeneity**



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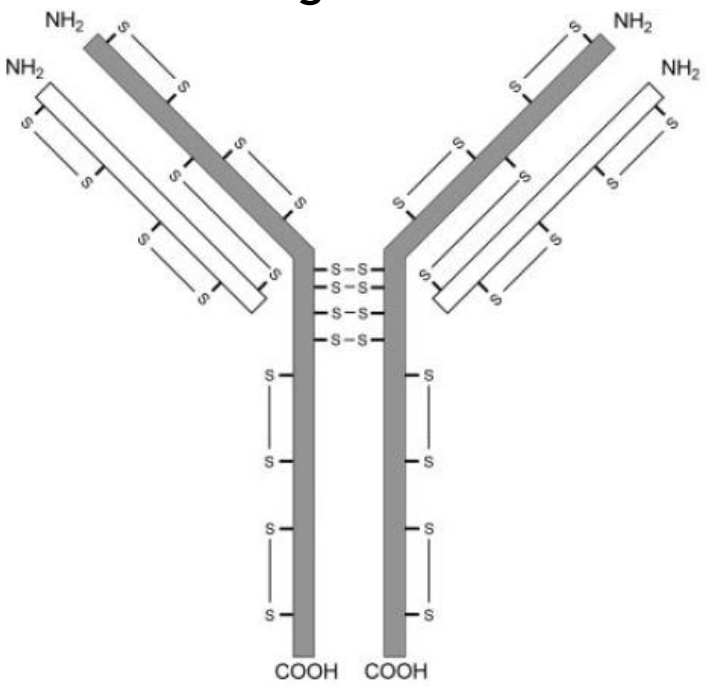
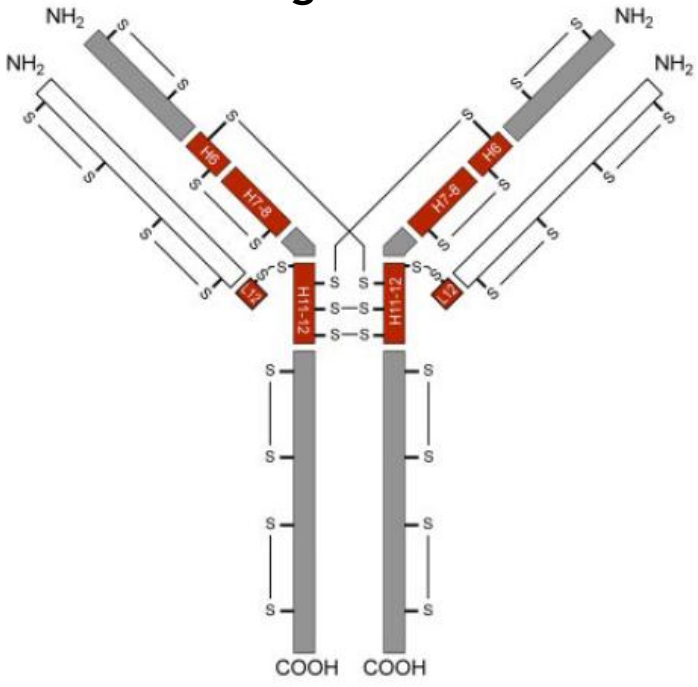
Disulfide bond scrambling in IgG2

IgG2-A



IgG2-B



➡ **Disulfide shuffling** as source of multiple bands?

Wypych *et al.*, *J. Biol. Chem.* (2008)

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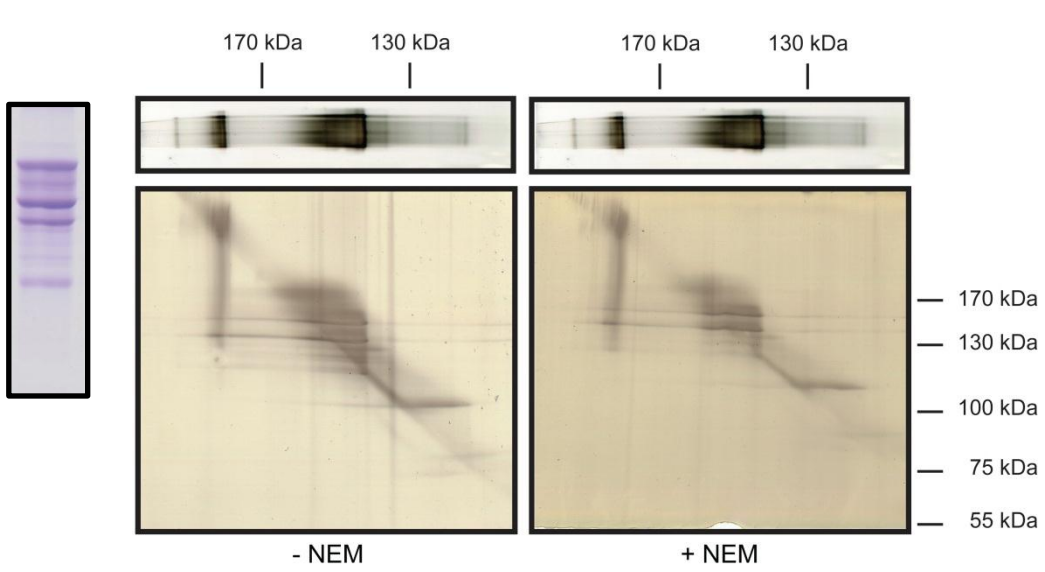
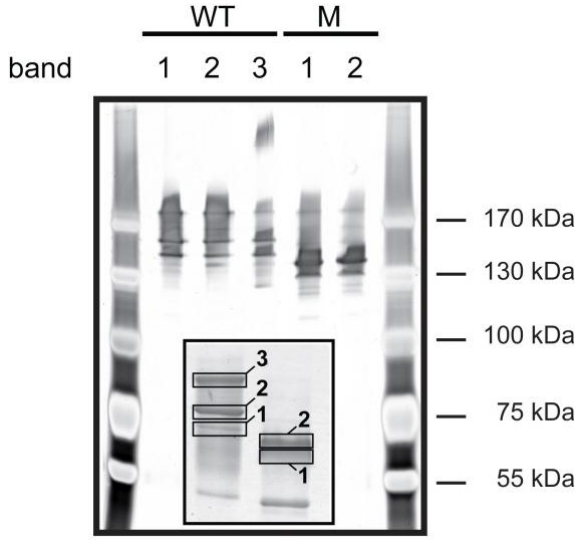
2D-electrophoresis

Analysis of IgG variants on "non-conventional" 2D-SDS-PAGE

HEK IgG 2C2 WT





- distinct bands of 1st dimension resolve again in the 2nd dimension
- multiple bands are **not caused by disulfide heterogeneity / shuffling** (confirmed by MS analyses and determination of unpaired cysteines)

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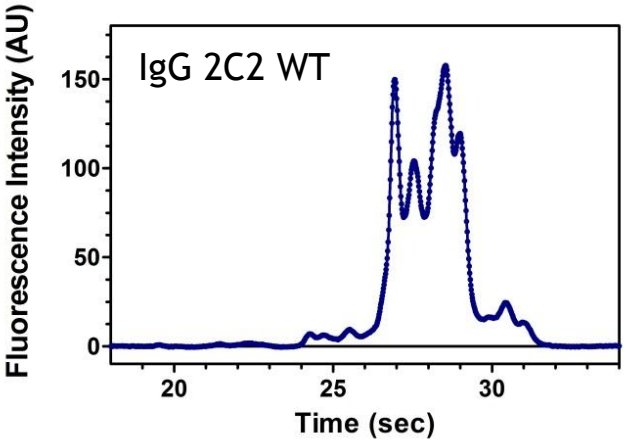
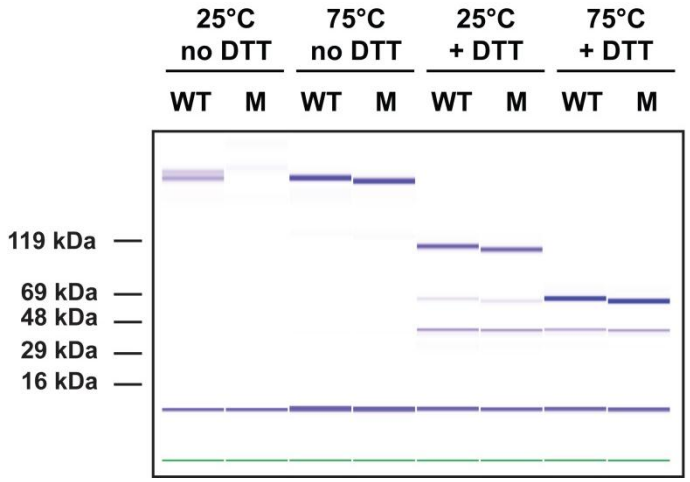
Stability probed by dye binding

Analysis by capillary electrophoresis (performed in microfluid chip)

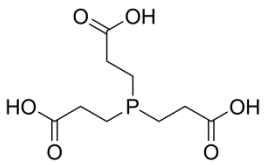


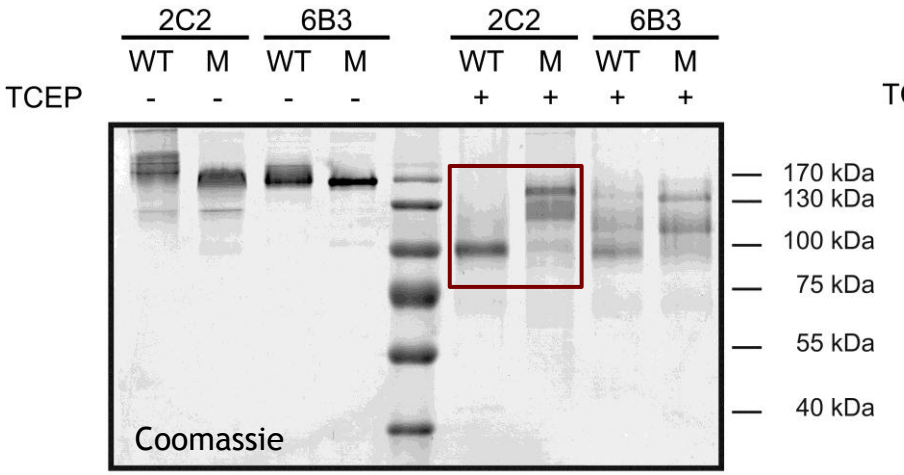


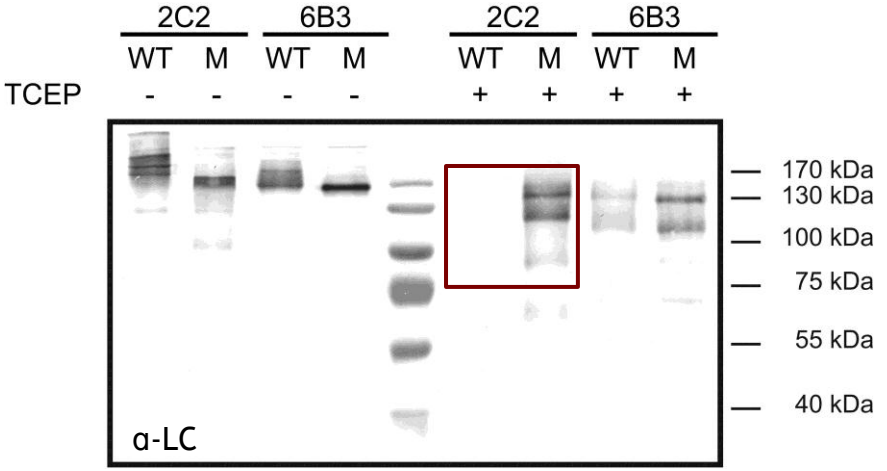
➡ M variant seems **more densely packed** (less SDS-micelles can bind)

Stability probed by partial reduction

Partial reduction of IgG by hydrophilic TCEP

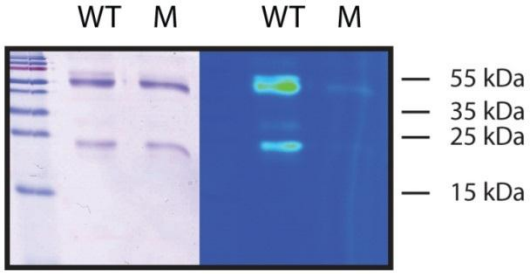






➡ TCEP treatment reduces **inter-molecular disulfide bond** only in WT IgGs

➡ labeling of free Cys with fluorescent 5-IAF confirms improved structural integrity / compactness



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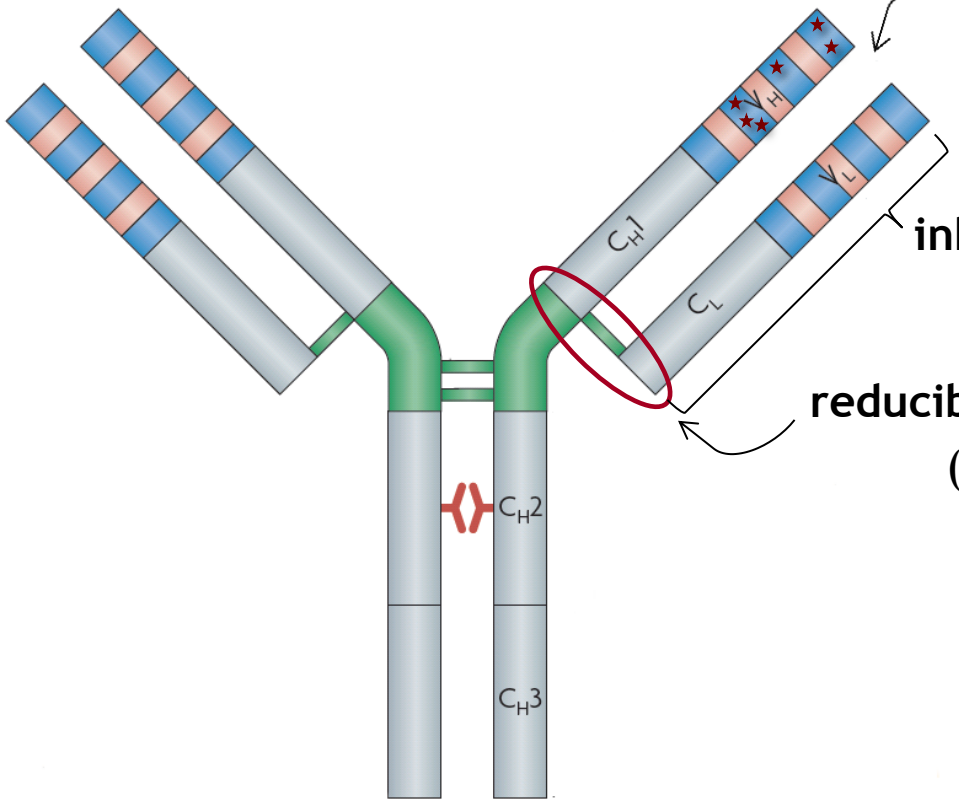
Conclusions

mutations affect **structural integrity and homogeneity**



packing of V_H-V_L interface
(dye accessibility)

inhomogeneous banding pattern
(in equilibrium)

reducibility of disulfide bond
(TCEP reduction)

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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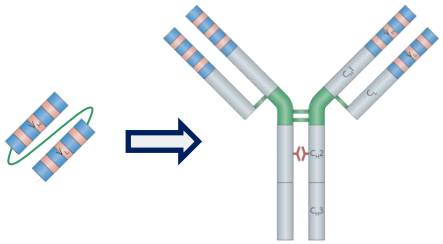
Conclusions

- variable domain mutations: effects on **expression level**
  - strong influence in *E. coli*
  - moderate influence in *Pichia pastoris*
  - no influence in HEK293

- mutations influence the biophysical properties of the IgG: **thermal and denaturant-induced unfolding**

- increased stability independent of the expression system used

- **transferability of improvements implemented in smaller fragments onto full-length IgG**



Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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Thomas Müller-Späth (ChromaCon)

Stefan Duhr (NanoTemper)

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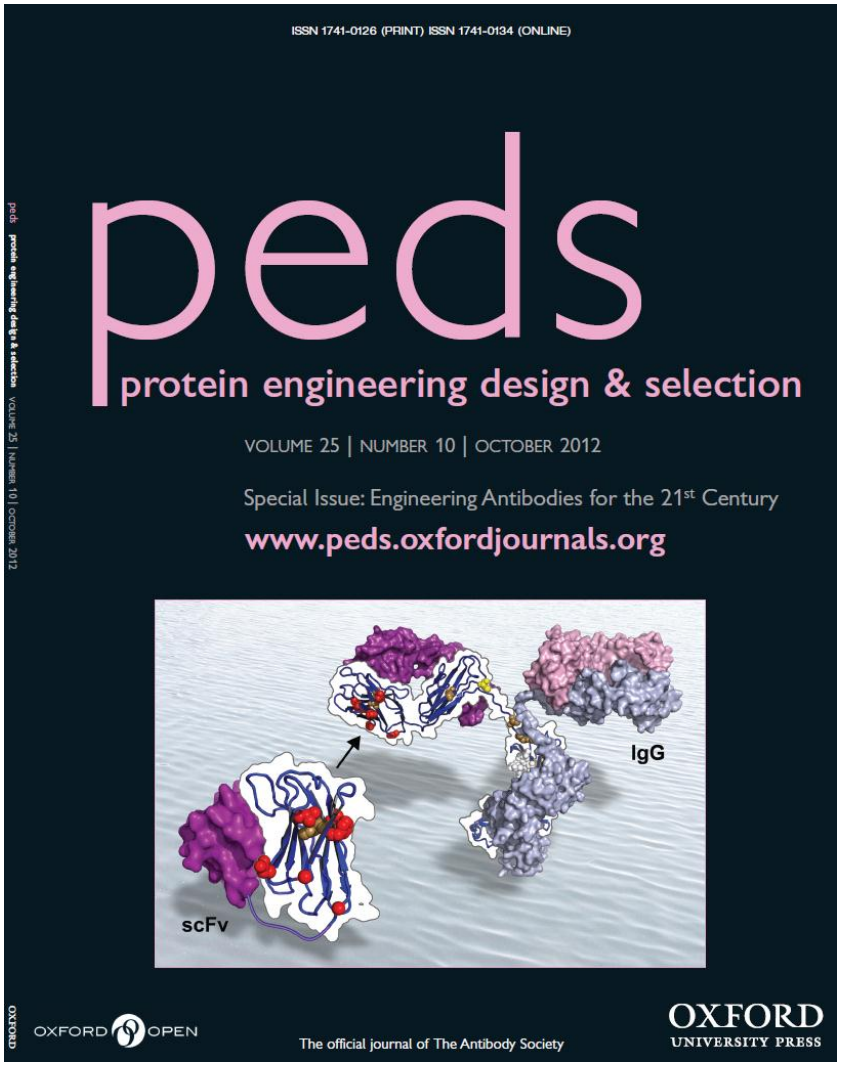

Schaefer and Plückthun (2012) *Protein Eng. Sel. Des.* 25, 485-506