

**A fully human antibody binds Tn and sTn carbohydrate antigens specifically on serine residues, without need for polypeptide interaction**

## Abstract LB-002

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## Abstract

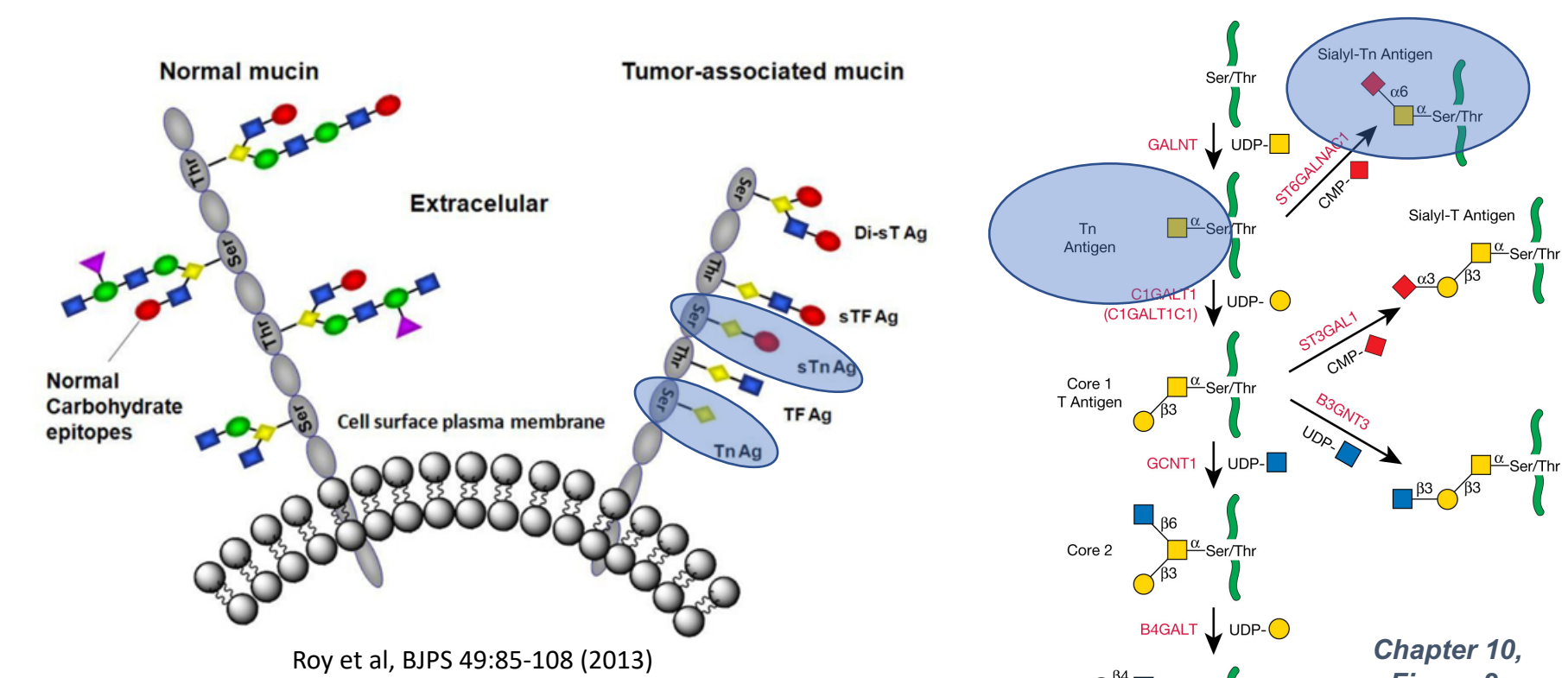
**Background:** Thomsen-nouvelle (Tn) antigen is an O-linked glycan that is associated with a broad array of tumors. Tn is a single alpha-linked GalNAc added to Ser or Thr as the first step of a major O-linked glycosylation pathway. In cancer, the glycosylation machinery can generate mucins with glycans aberrantly truncated at the initial GalNAc (Tn), or singly modified by addition of a sialic acid (sTn) on variable number 20 amino acid tandem repeat (VNTR) sequences. A mounting body of evidence supports that the modified residue (Ser or Thr) has a major impact on attached Tn structure. Accordingly, reports have described monoclonal antibodies and lectins that differentiate among contexts. Here we describe a fully human antibody that interacts preferentially with Ser-Tn/sTn, and shows broad tumor reactivity.

**Methods:** 2F3 anti-Tn/sTn was generated from a patient who received a vaccine that incorporated a MUC1 VNTR with Tn on both Ser and Thr. B cells were isolated, hybridomas generated, and supernatants screened by ELISA for reactivity with polymeric Tn on a synthetic backbone. Hits were screened by ELISA for reactivity to related glycans and binding to Tn+ cells was confirmed by FACS. A lead antibody, 2F3, was selected and used to determine target prevalence on tumor microarrays. 2F3 was affinity matured by error prone PCR. Hits were screened for specificity by glycan array and a panel of Tn glycopeptides through collaboration with the Consortium for Functional Glycomics to determine the preferred Tn context recognized by 2F3.

**Results:** Five hybridomas secreted antibodies that bound Tn/sTn by ELISA. 2F3 bound Tn<sup>+</sup> cells by FACS. 2F3 demonstrated strongest binding to multimeric Tn/sTn, and weaker binding to monomeric Tn. Tumor microarray studies demonstrated binding to 47% of ovarian and 75% of breast (including 89% of triple negative) cancer tissues. While MUC1 VNTR peptides with no Tn or with Tn on two different Thr residues showed no binding by ELISA, strong binding was observed to a MUC1 VNTR peptide modified on all 5 Ser/Thr. In order to determine whether spacing, number of Tn modifications, specific residues modified, or sequence context contributed to the specificity of binding by 2F3, we examined a panel of ~50 Tn glycopeptides. We observed binding only to peptides that contained Ser-Tn, and only those that were at least 5 amino acids from the attachment point to the solid matrix. Ser-Tn near free termini and multiple Ser-Tn in a single peptide increased binding, in this preliminary study. Finally, glycan array analysis demonstrated high specificity for Tn/sTn with no significant binding observed to almost 600 glycan structures found in mammals.

**Conclusions:** 2F3 anti-Tn/sTn antibody recognizes Tn preferentially on Ser residues that are in accessible contexts. 2F3 exhibits a pharmaceutically useful pattern of binding because high percentages of primary ovarian and breast cancer samples are recognized. We are now in preclinical exploration of the optimal MoA to exploit the specificity and selectivity of this candidate drug.

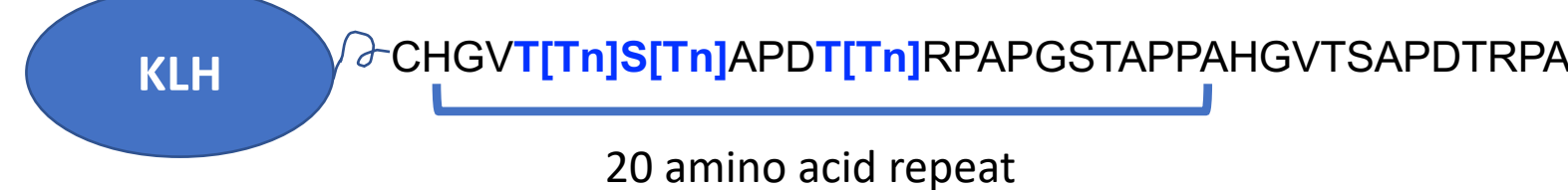
## Background



Early termination of O-glycan maturation pathway results in aberrant glycoforms on cell surface and secreted glycoproteins, including mucins. Mucins are a family of membrane associated and secreted glycoproteins that form a protective gel layer and participate in multiple cellular processes involving cell growth and survival. They contain variable numbers of 20 amino acid tandem repeat sequences, each with 5 Ser/Thr residues that are subject to O-linked glycosylation. The nature and extent of glycosylation varies significantly from health to disease.

## Lead Generation

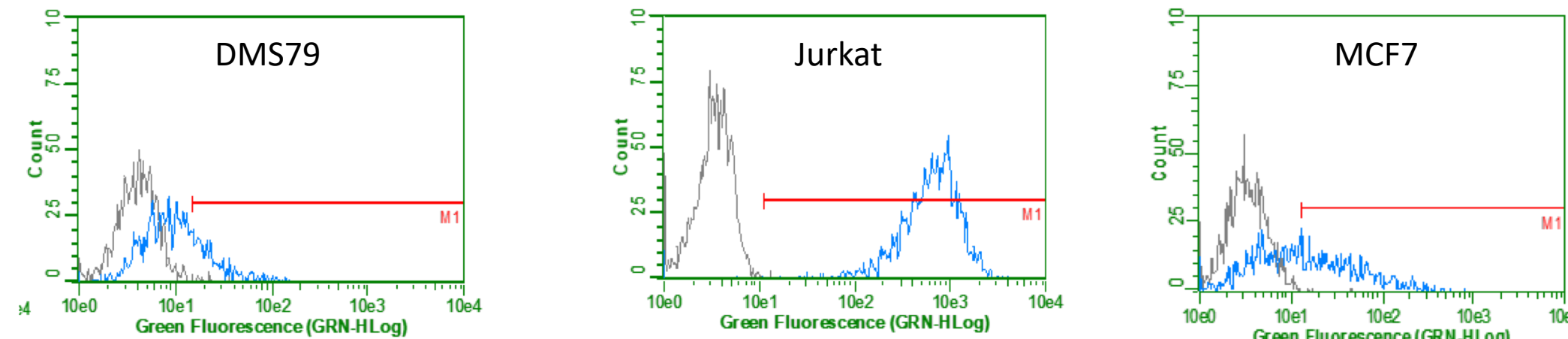
MUC-1-derived peptide used in vaccine that produced the lead candidate (Study 4)



### 5 Tn/sTn leads characterized by ELISA screen of related carbohydrate cancer antigens

| Clone ID | Specificity by ELISA        | PBS  | Gm2-PAA | Gd2-PAA | Gd3-PAA | Globo-H | Tn-PAA | sTn-PAA | TF-PAA | sLeA-PAA | EtOH | Gm2-cer | Gd2-cer | Gd3-cer | F-GM1-cer | Gm3-cer |
|----------|-----------------------------|------|---------|---------|---------|---------|--------|---------|--------|----------|------|---------|---------|---------|-----------|---------|
| 12E2b3   | Tn-PAA (Gm2/Gd2/Gd3-PAA)    | 0.21 | 0.41    | 0.38    | 0.39    | 0.23    | 0.69   | 0.20    | 0.29   | 0.19     | 0.13 | 0.12    | 0.11    | 0.12    | 0.12      | 0.12    |
| 3A7G3    | Tn-PAA/(sTn-PAA)            | 0.25 | 0.18    | 0.16    | 0.14    | 0.14    | 2.53   | 0.35    | 0.17   | 0.15     | 0.11 | 0.09    | 0.09    | 0.11    | 0.09      | 0.10    |
| 1A4G3    | Gm2-PAA/(sTn-PAA)           | 0.42 | 0.40    | 0.40    | 0.31    | 0.38    | 3.73   | 0.78    | 0.43   | 0.18     | 0.17 | 0.22    | 0.22    | 0.19    | 0.16      | 0.22    |
| 2F3      | Tn/sTn-PAA                  | 0.09 | 0.09    | 0.09    | 0.09    | 0.09    | 3.68   | 1.99    | 0.09   | 0.09     | 0.09 | 0.09    | 0.09    | 0.08    | 0.09      | 0.09    |
| 2A8      | (sTn-PAA, Gm3-cer, GD3-cer) | 0.17 | 0.18    | 0.20    | 0.21    | 0.18    | 0.19   | 0.24    | 0.13   | 0.22     | 0.09 | 0.11    | 0.11    | 0.19    | 0.10      | 0.28    |

mAb2F3 selected because it was the only candidate that binds Tn-positive cell lines



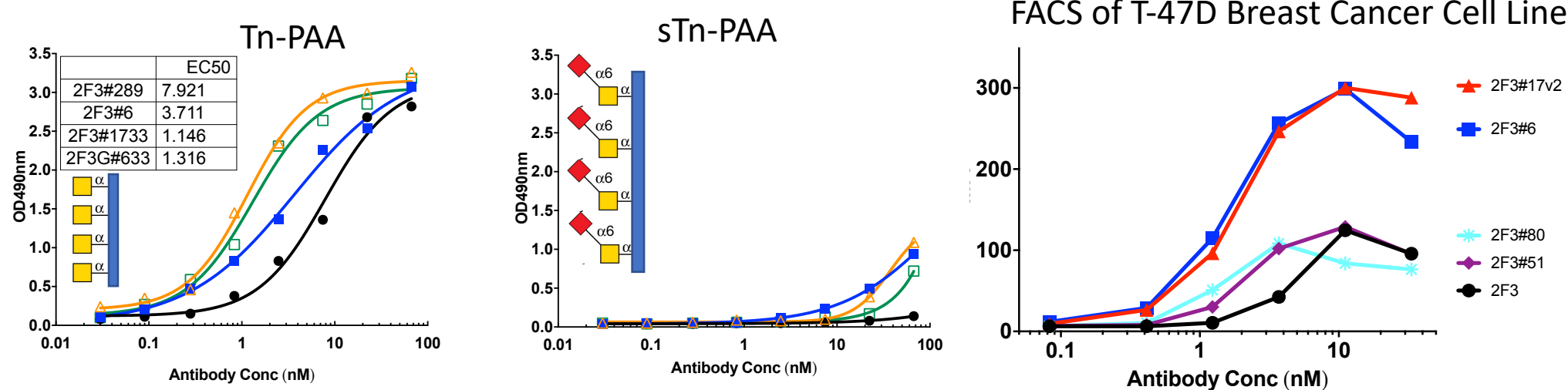
- Thousands of hybridomas from two vaccine trials. 5 Tn and/or sTn-reactive leads identified
- 2F3 has favorable specificity and binds Tn-positive cell lines by FACS

## Target and Lead Validation

### Lung, ovarian, breast, and colon tumor microarrays stained with 2F3G3

| Tumor              | Positive/ total cores | % 2F3G3 positive |
|--------------------|-----------------------|------------------|
| Lung (SCLC)        | 3/80                  | 4%               |
| Ovarian            | 35/75                 | 47%              |
| Breast             | 107/142               | 75%              |
| TNBC               | 24/27                 | 89%              |
| Colon (malignant)  | 6/20                  | 30%              |
| Colon (metastatic) | 10/20                 | 50%              |

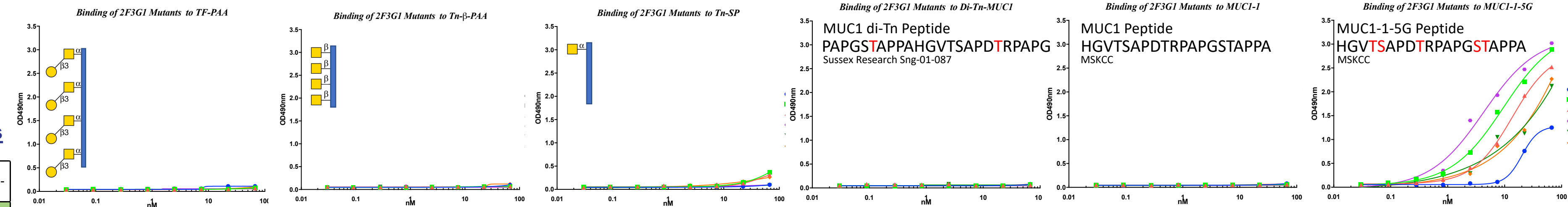
### Affinity matured mutants improve affinity for synthetic antigens and Tn/sTn on cells



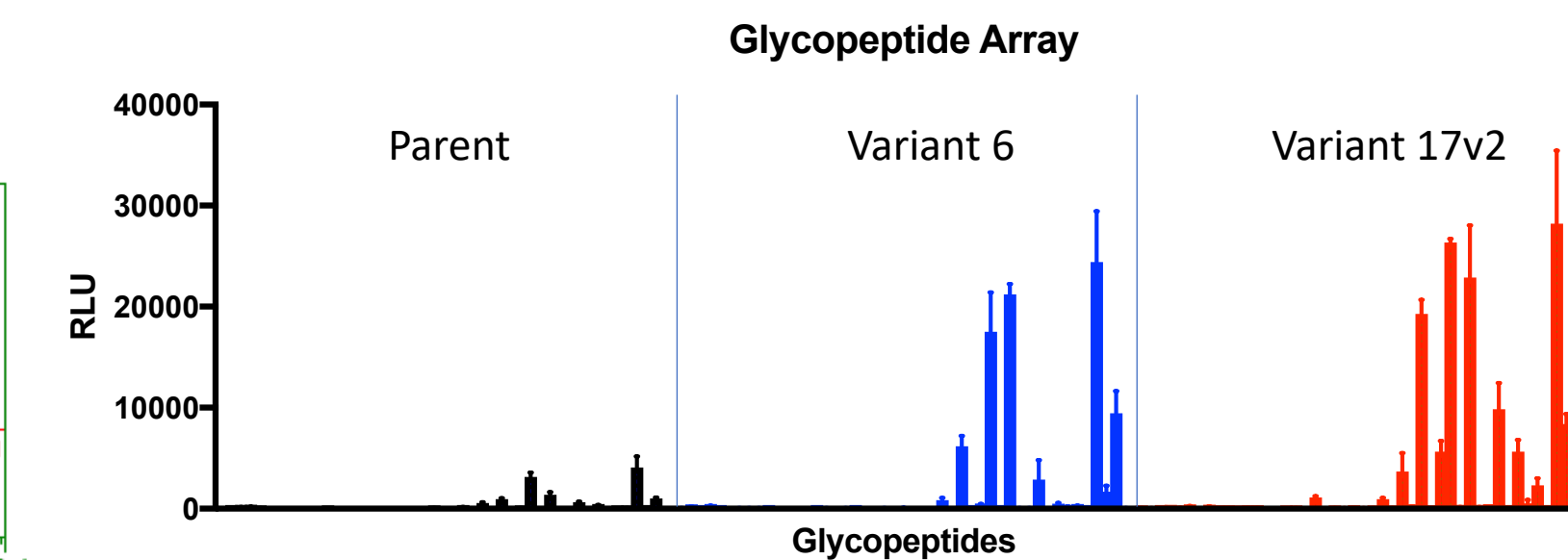
ELISA and FACS-based binding experiments demonstrate clear differentiation among mutants with marked improvement for multiple lead variants.

## Glycan Epitope Determination

### Mutants retain specificity of parents by ELISA and only bind MUC1 peptides if they are modified by Tn on a Ser residue



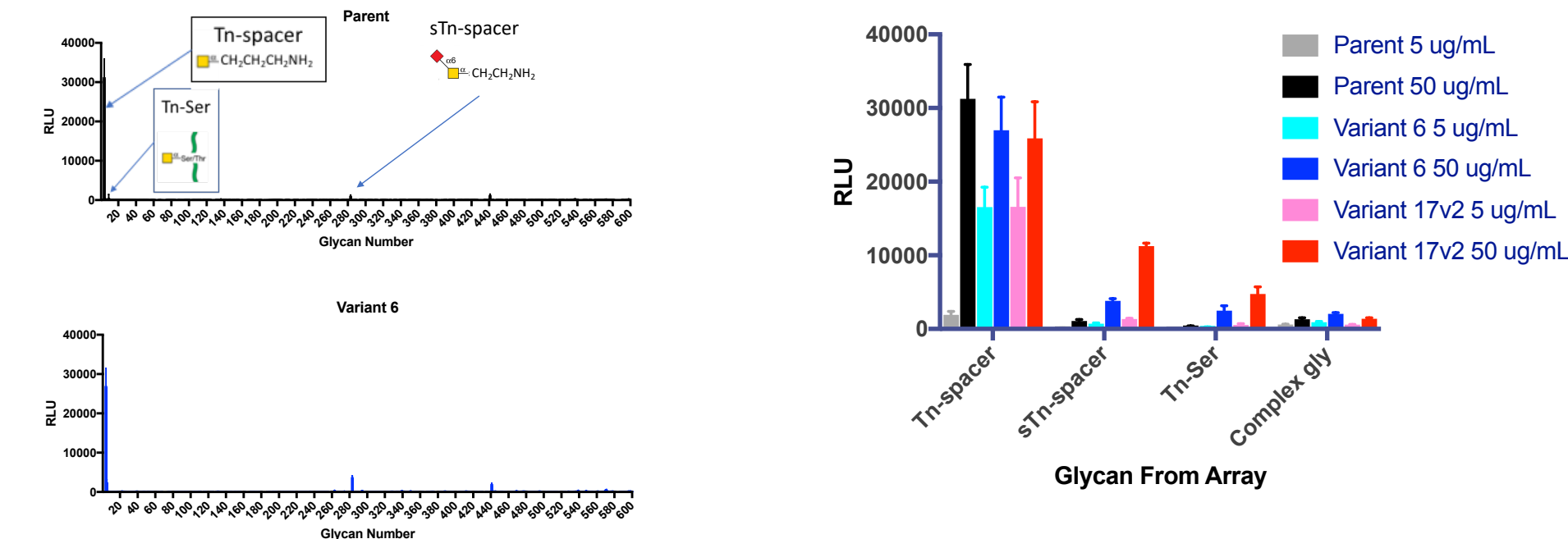
### 2F3 and Variants only bind to peptides containing Ser-Tn



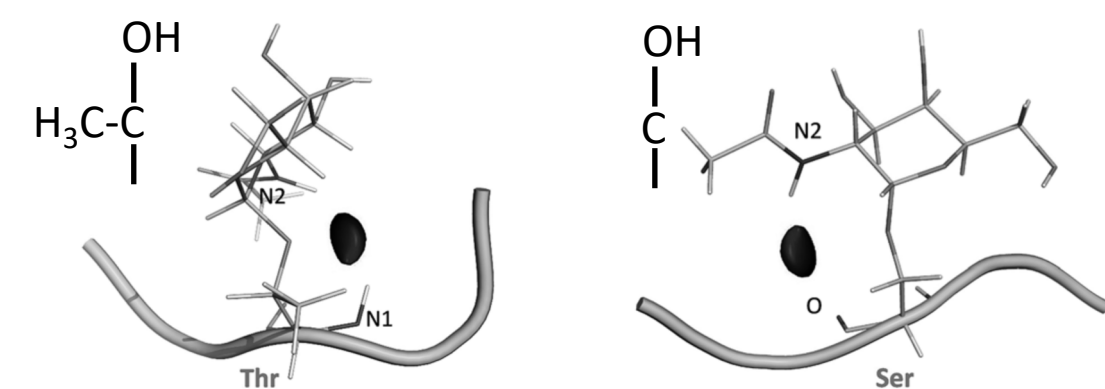
| Description             | Sequence                                              | Parent | Variant 6 | Variant 17 | 1+ Ser | Only Thr | No To |
|-------------------------|-------------------------------------------------------|--------|-----------|------------|--------|----------|-------|
| Phosphate Buffer        | buffer control                                        | 3      | 5         | 47         |        |          |       |
| Mannose-6s (Man6s)      | glycan control                                        | 140    | 185       | 23         |        |          | x     |
| Blood group A tetraose  | <b>GaiNAca</b> 1-3(Fuca)1-2(Galb1-3GlcNAc-AEAB        | 147    | 64        | 109        |        |          |       |
| Blood group A pentaoase | <b>GaiNAca</b> 1-3(Fuca)1-2(Galb1-3GlcNAcb1-3Gal-AEAB | 188    | 248       | 145        |        |          |       |
| A-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 45     | 55        | 81         |        |          |       |
| B-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 19     | 23        | 237        | x      |          |       |
| C-MUC2                  | <b>Ac-PTTT</b> PLK-NH2                                | 9      | 22        | 30         |        | x        |       |
| D-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 12     | 12        | 179        |        |          |       |
| E-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 8      | 27        | 87         |        |          |       |
| F-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 11     | 54        | 70         |        | x        |       |
| G-MUC2                  | <b>Ac-PTT</b> TLK-NH2                                 | 10     | -39       | 51         |        |          |       |
| a-Dystroglycan          | <b>Ac-PPTTT</b> TKP-NH2                               | 37     | -4        | 86         |        |          | x     |
| MUC5AC                  | <b>H2N-GTTSPVPVTT</b> TSITSAP-OH                      | 23     | -14       | 87         |        | x        |       |
| EAG                     | <b>Ac-PTDST</b> TAPTK-NH2                             | 11     | 18        | 16         |        |          |       |
| EAG-R                   | <b>Ac-PTDST</b> TAPTK-NH2                             | 15     | 45        | 29         |        |          |       |
| a-Dystroglycan          | <b>Ac-PTTT</b> TTKP-NH2                               | 24     | 28        | 51         |        |          |       |
| MUC1-1                  | <b>H2N-TSAPD</b> TRDAP-NH2                            | 11     | 14        | 61         | x      |          |       |
| MUC1-1R                 | H2N-TSAPDTRDAP-NH2                                    | 7      | 7         | 19         |        |          |       |
| MUC1-2                  | <b>H2N-APGS</b> TAPP-NH2                              | 33     | 59        | 1121       | x      |          |       |
| MUC1-2R                 | H2N-APGSTAPP-NH2                                      | 4      | 0         | 6          |        |          | x     |
| PADRE Tn3b              | <b>H2N-GaKaCAAA</b> TKLAAaTtTtTtG-CONH2               | 13     | -84       | 49         |        | x        |       |
| Tn3 linker              | <b>Ac-TT</b> THNH(C2)3NH2                             | 8      | 21        | 24         |        |          |       |
| Peptide-4               | <b>Ac-T</b> THNH(C2)3NH2                              | 34     | 2         | 70         |        |          |       |
| Peptide-4               | H2N-KTTT-CONH2                                        | 7      | 31        | 33         |        | x        |       |
| Peptide-5               | H2N-KTTTG-CONH2                                       | 10     | -6        | 41         |        |          |       |
| Ser-GaiNAc1             | <b>H2N-Seria</b> (a-D-GaiNAc)-NH2                     | 105    | -1        | 960        | x      |          |       |
| Ser-GaiNAc2             | <b>H2N-Seria</b> (a-D-GaiNAc)-OH                      | 10     | -3        | 42         | x      |          |       |
| Thr-GaiNAc1             | <b>H2N-Thra</b> (a-D-GaiNAc)-NH2                      | 520    | 810       | 3674       |        | x        |       |
| Thr-GaiNAc2             | <b>H2N-Thra</b> (a-D-GaiNAc)-OH                       | 4      | 6         | 235        | x      |          |       |
| IgA-Pep01               | <b>H2N-KPVPST</b> PTTPSC-OH                           | 990    | 6199      | 1975       | x      |          |       |
| IgA-Pep02               | H2N-KPVPSTPTTPSC-OH                                   | 26     | 35        | 75         |        | x        |       |
| IgA-Pep03               | <b>H2N-KPVP</b> STPTTPSC-OH                           | 58     | 353       | 5653       | x      |          |       |
| IgA-Pep04               | <b>H2N-KPST</b> PTPTPS-CO-H                           | 3151   | 17492     | 26366      | x      |          |       |
| IgA-Pep05               | H2N-KPSTPTPTSPSC-OH                                   | 28     | 60        | 149        |        |          | x     |
| IgA-Pep06               | <b>H2N-KT</b> PTPTPS-CO-TPC-OH                        | 1373   | 21199     | 22880      | x      |          |       |
| IgA-Pep07               | H2N-KPTPTPTSPSC-OH                                    | 20     | 30        | 141        |        |          | x     |
| IgA-Pep08               | H2N-KTPTPTPTTPSC-OH                                   | 22     | -6        | 292        |        | x        |       |
| IgA-Pep09               | <b>H2N-KPT</b> PSPTPTTPC-OH                           | 647    | 2844      | 5635       | x      |          |       |
| IgA-Pep10               | H2N-KPSPSTPTTPSC-OH                                   | 19     | -127      | 125        |        |          | x     |
| IgA-Pep11               | <b>H2N-KPS</b> PSPTPTTPSC-OH                          | 315    | 311       | 5649       | x      |          |       |
| IgA-Pep12               | H2N-KPSTPTPTSPSC-OH                                   | 20     | 83        | 457        |        |          | x     |
| IgA-Pep13               | <b>H2N-KPS</b> TPPTPTPS-CO-OH                         | 40     | 253       | 2319       | x      |          |       |
| IgA-Pep14               | H2N-KPSTPTPTSPSC-OH                                   | 43     | 38        | 112        |        |          | x     |
| IgA-Pep15               | <b>H2N-KPST</b> PTPTPS-CO-H                           | 4096   | 30379     | 28214      | x      |          |       |
| IgA-Pep16               | H2N-KPSTPTPTPS-CO-OH                                  | 115    | 1649      | 5354       | x      |          |       |
| IgA-Pep17               | <b>H2N-KPST</b> PTPTPS-CO-OH                          | 1010   | 9417      | 1956       | x      |          |       |
| IgA-Pep18               | <b>H2N-KPST</b> PTPTSPSC-OH                           | 16     | 22        | 174        |        |          |       |

Tn glycan array analysis performed by the Consortium for Functional Glycomics. Yellow>mean, orange>mean+1SD, red>mean+2SD.

### 2F3 and Variants have a very clean profile across mammalian glycans



### Structural variation between Tn-Ser and Tn-Thr



Madariaga et al, Chem. Eur. J. 20, 12616-12627 (2014)

## Summary

### Fully human lead anti-Tn antibody identified and optimized

- 2F3 binds Tn and sTn. Affinity improved >25X by ELISA and ~10X in cell-based binding
  - Lead variants do not bind closely-related antigens, retain high specificity of parent
- Lead antibody used to further validate Tn target**
- Binds to high percentage of colon, ovarian, and breast tumor tissue, including breast tumors that are negative for ER, PR, and HER2 (TNBC)
- Specificity for Tn-Ser**
- 2F3 and Variants appear to preferentially bind to Tn on Ser
  - Dramatic structural variation has been reported that can explain this phenomenon

## References

1. Roy et al, *BIPS* 49:85-108 (2013)
2. Essentials of Glycobiology, 3<sup>rd</sup> edition. Chapter 15. Varki, A. Executive editor.
3. Borgert et al, *ACS Chem. Biol.*, 7 (6), pp 1031–1039 (2012)
4. Madariaga et al, *Chem. Eur. J.* 20, 12616-12627 (2014)

Borgert et al, *ACS Chem. Biol.*, 7 (6), pp 1031–1039 (2012)