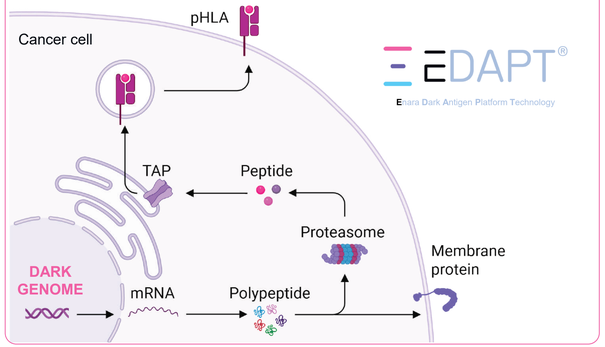
Discovery and validation of DARKFOX, a novel alternative open reading frame of FOXM1 that is an attractive cancer antigen for peptide-HLA targeting immunotherapy

Tatiana Lobry[1], Joana Senra[1], Samit Kundu[1], Magdalena von Essen[1], Masood Zaka[1], Richard Little[1], Veronica Freire-Beneitez[1], Vicki Jefferson[1], Carolin Christ[1], Luis Gonzalez[1], Lucy Gallacher[1], Emily Lam[1], Emily Tye[1], Eleanor Denham[1], Ilaria Michelozzi[1], Joseph Dukes[1*]

[1]Enara Bio Ltd., Oxford, U.K.
* Corresponding author

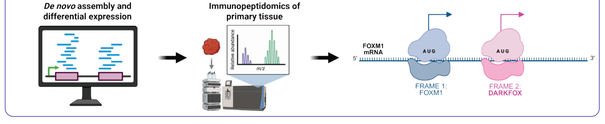
THE EDAPT PLATFORM FOR DISCOVERY AND VALIDATION OF NOVEL DARK ANTIGENS

- Enara built the EDAPT® platform to discover and validate novel tumor-specific antigens derived from the dark genome, which we have called Dark Antigens®
- Dark Antigen-encoding open reading frames (ORFs) are translated into polypeptides that are subject to cellular processing and presentation, resulting in Dark Antigen peptide-HLA molecules presented on tumor cells
- A subset of these novel ORFs encode for proteins possessing sequences that result in transmembrane expression and trafficking to the cell surface
- EDAPT was built to discover and validate targets that are highly cancer-specific, homogeneously expressed within tumors, and shared across broad patient populations
- These properties of Dark Antigens make them highly attractive as targets for bispecific T-cell engagers (TCEs), antibody-drug conjugates (ADCs), cancer vaccines and cell therapies

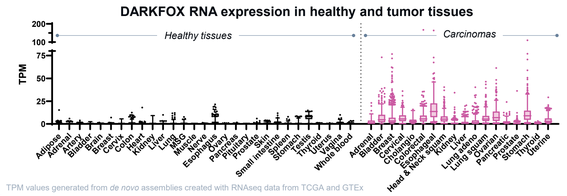


DARKFOX™ IS A NOVEL ALTERNATIVE OPEN READING FRAME IN THE FOXM1 GENE

- *De novo* assemblies were generated from high quality esophageal adenocarcinoma RNAseq datasets (TCGA) and subject to differential expression analysis with normal tissues (GTEx)
- Putative ORFs from transcripts with evidence of cancer-specific expression were predicted and used as a reference proteome to query immunopeptidomics data from 100's of primary tumors
- 2515 peptides with MS observations were mapped to 6606 novel ORFs where 1 of these ORFs was an alternative ORF found in a non-canonical reading frame of the FOXM1 gene



DARKFOX EXHIBITS PAN-TUMOR AND MINIMAL HEALTHY TISSUE EXPRESSION

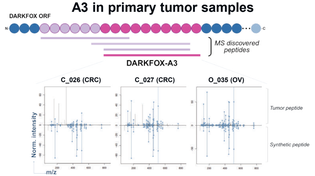


TPM values generated from *de novo* assemblies created with RNAseq data from TCGA and GTEx

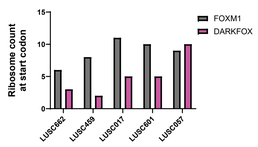
DARKFOX TRANSLATION CONFIRMED BY IMMUNOPEPTIDOMIC AND RIBO-SEQ ANALYSES OF MULTIPLE PRIMARY TUMOR SAMPLES

- MS-based immunopeptidomics of primary tumor tissues identified 4 peptides that mapped exclusively to the DARKFOX ORF with no observations in benign or NAT tissues
- The most abundant peptide, DARKFOX-A3, was observed in HLA-A3+ tumor samples
- DARKFOX-A3 was validated in primary tumors with fragmentation analysis of synthetic peptide

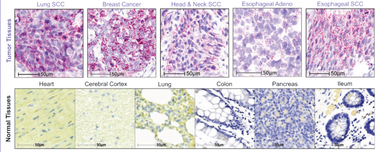
Immunopeptidomic validation of DARKFOX-A3 in primary tumor samples



Ribo-seq analysis of lung squamous tumors for FOXM1 and DARKFOX



EXPRESSION OF DARKFOX IS TUMOR-SPECIFIC, ABUNDANT AND PAN-CANCER



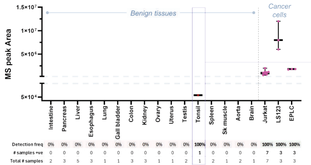
Cancer Indication	n	DARKFOX expression-prevalence (Score ≥ 2 by RNAscope) % samples positive
Lung Squamous Cell Carcinoma	43	> 70%
Breast Cancer	52	
H&N Squamous Cell Carcinoma	71	
Esophageal Squamous Cell Carcinoma	53	50 – 70%
Ovarian Serous Adenocarcinoma	28	
Esophagus Adenocarcinoma	26	
Lung Adenocarcinoma	79	25 – 50%
Melanoma	11	
Gastric Adenocarcinoma	41	
Bladder Cancer	5	
Colorectal Adenocarcinoma	135	

- RNA *in situ* hybridization (RNA-ISH) was performed on >600 primary tumor and benign tissue samples from a variety of tissue types
- DARKFOX demonstrated broad, pan-cancer expression (score ≥2), with high prevalence observed in several major solid tumor types
- No DARKFOX expression was observed in critical, healthy tissues

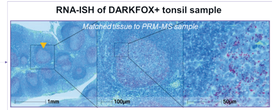
DARKFOX-A3 PEPTIDE IS NOT FOUND IN BENIGN TISSUES EXCEPT FOR A TONSIL SAMPLE AT LEVELS MUCH LOWER THAN IN TUMOR CELLS

- PRM-MS was performed on 32 x HLA-A3+ benign tissue samples (16 anatomical sites) and 3 control tumor cell lines (varying in DARKFOX expression) for detection of DARKFOX-A3 peptide
- No DARKFOX-A3 peptide was detected in 31 benign samples, and a positive signal was observed in a single tonsil sample (sample acquired from an adult with chronic tonsilitis)
- The MS peak area signal for the positive detection in the tonsil sample was orders of magnitude lower than the cancer cell line controls, including the lowest expressing lines
- RNA-ISH on the matched tonsil sample confirms DARKFOX expression restricted to germinal B cell centers

PRM-MS analysis of DARKFOX on HLA-A3⁺ tissues and cancer cell lines



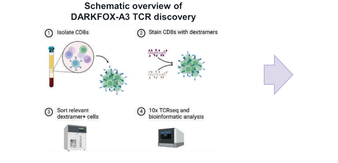
RNA-ISH of DARKFOX+ tonsil sample



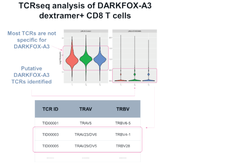
DARKFOX-A3 IS IMMUNOGENIC AND WILD-TYPE TCRS CAN RECOGNIZE ENDOGENOUS LEVELS OF THE ANTIGEN ON TUMOR CELLS

- DARKFOX-A3-specific TCRs were identified from peripheral blood of healthy donors using a dextramer-based isolation method and the 10x platform for single-cell TCR sequencing
- Identified TCRs (TCR#3 and #5) were validated by expression in Jurkat cells and 2 of the validated TCRs also recognized cell lines endogenously expressing DARKFOX and HLA-A*03:01

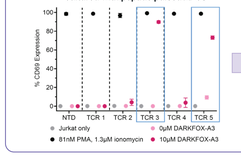
Schematic overview of DARKFOX-A3 TCR discovery



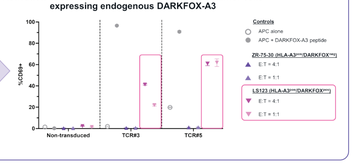
TCRseq analysis of DARKFOX-A3 dextramer+ CD8 T cells



Activation of Jurkat cells expressing TCRs co-cultured with peptide pulsed APCs



Activation of Jurkat-TCR cells in the presence of tumor cells expressing endogenous DARKFOX-A3



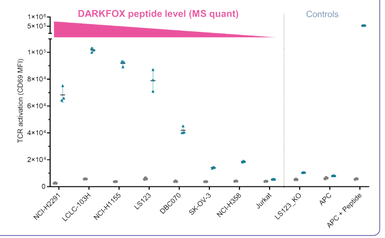
DARKFOX-A3-SPECIFIC TCRS ACTIVATE T CELLS IN LINE WITH PRESENTED PEPTIDE

- PRM-MS was performed on eluted pHLAs from HLA-A*03:01+/DARKFOX+ and control cell lines with samples referenced to the DARKFOX-low expressing T cell leukemia cell line, Jurkat
- A DARKFOX-A3-specific wild type TCR (TCR#3) expressed in Jurkats (β2M knock-out) activated the T cell line in a DARKFOX-A3 peptide-dependent manner

DARKFOX-A3 PRM-MS analysis

Reference Sample	Peptide quant (amole)
Jurkat (T cell leukemia)	15.75 – 21.45
Test Sample	**Relative peptide** (normalized to Jurkat)
NCI-H2291 (NSCLC)	~60-fold higher
LCLC-103H (NSCLC)	~30-fold higher
LS123 (CRC)	~20-fold higher
NCI-H1155 (NSCLC)	~20-fold higher
DBC070 (BC)	~3-fold higher
SK-OV-3 (Ovarian)	~3-fold higher
NCI-H358 (NSCLC)	~2.5-fold higher
LS123_KO	Not detected

DARKFOX-A3 TCR#3 activation with cancer cell lines



CONCLUSIONS

- Enara Bio's EDAPT® pipeline discovered and exhaustively validated DARKFOX™, a novel alternative open reading frame found in the FOXM1 gene
- DARKFOX represents a compelling target for cancer immunotherapy that is highly prevalent in multiple solid tumors with homogenous intra-tumoral expression, and is characterised by minimal presence in normal tissues
- The DARKFOX-A3 peptide is robustly presented on HLA-A*03/DARKFOX-positive tumor cells with no peptide detected in benign tissues except at very low levels in a single tonsil sample where expression analyses suggest it originates from activated B cells, consistent with prior data on FOXM1 expression
- DARKFOX-A3 is immunogenic, and specific TCRs can be identified from healthy donors that have sufficient affinity to recognize endogenous expression of DARKFOX-A3 pHLA
- ENA101, an EnTiCE® bispecific T-cell engager targeting DARKFOX-A3, is currently in IND-enabling studies

Acknowledgements

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Ethics Approval

All work involving the use of human tissues was approved by the NHS Health Research Authority Northwest Haydock Research Ethics Committee (reference number 19/NW/0216).

Ethical approval for work using human blood derived cells was received from NHS London-Bromley Research Ethics Committee REC reference 22/PR/1176. Commercial blood sources were supplied with informed consent and with certification from local research ethics committees.

Scan for more info!

Presented at The Society for Immunotherapy of Cancer's 40th Annual Meeting (SITC 2025)