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**Structural and functional insights on the *Drosophila*
TNFR Grindelwald, coupling loss of cell polarity and
neoplastic growth**

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GLOSSARY

ADAM	A disintegrin And Metalloproteinase domain
AP-1	Activator Protein-1
aPKC	Atypical Protein Kinase C
AJ	Adherence Junction
Apaf-1	Apoptotic protease-activating factor-1
Avl	Avalanche
BAFF	B-cell Activating Factor
BAFFR	B-cell Activating Factor Receptor
Bsk	Basket
Baz	Bazooka
BCMA	B Cell Maturation Antigen
Ben	Bendless
Cask	Calmodulin-associated Ser/Thr kinase
CD	Cluster of differentiation
Crb	Crumbs
CRD	Cysteine-Rich Domain
CYLD	Cylindromatosis
DcR	Decoy Receptor
DD	Death Domain
DED	Death Effector Domain
Dlg1	Disc large 1
DIAP1	<i>Drosophila</i> Inhibitors of Apoptosis Protein-1
DISC	Death-Inducing Signalling Complex
DR	Death Receptor
dUev1a	<i>Drosophila</i> Ubiquitin-conjugating enzyme variant 1a
ECD	Extracellular Domain

EDA	Ectodysplasin-A
EDAR	Ectodysplasin-A Receptor
Egr	Eiger
EMT	Epithelial-to-Mesenchymal Transition
ERK	Extracellular signal- Regulated Kinase
FA	FERM-Associated
FADD	Fas-Associated Death Domain protein
FasL	Fas Ligand
FERM	4.1/Ezrin/Radizin/Moesin
FL	Full-length
GBM	Glycosphingolipid-Binding Motif
Grnd	Grindelwald
Hep	Hemipterous
IP	Immunoprecipitation
ICD	Intracellular Domain
JNK	Jun N-terminal Kinase
K_d	Dissociation constant
KD	Knockdown
KO	Knockout
ITC	Isothermal Titration Calorimetry
L27	Lin2-Lin7
Lgl	Lethal giant larvae
LRR	Lysine-Rich Region
LTβ	Lymphotoxin-β
LTβR	Lymphotoxin-β Receptor
MAGUK	Membrane-Associated Gualylate Kinase
MAPK	Mitogen-Activated Protein Kinase
MATH	Meprin And Traf Homology

MMP1	Matrix Metalloproteinase 1
MW	Molecular Weight
NGFR	Nerve Growth Factor Receptor
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-Aspartate receptor
NOPO	No Poles
OPG	Osteoprotegerin
Pals1	Protein-associated with Lin7, Stardust and MPP5
PatJ	Pals1-associated tight junction protein
Par	Partition
PB1	Phox/Bem1
PLAD	Pre-Ligand Assembly Domain
PDZ	PSD95, Discs large, ZO-1
Puc	Puckered
RANK	Receptor Activator of NF- κ B
RELT	Receptor Expressed in Lymphoid Tissues
RING	Really Interesting New Gene
RNAi	RNA interference
S2 cells	<i>Drosophila</i> Schneider-2 embryonic cells
Scrib	Scribble
Sdt	Stardust
SH3	Src-Homology 3
SLS	Static Light Scattering
SMAC	Second-Mitochondria derived Activator of Caspase
SJ	Septate Junction
Tab2	Tak1-binding protein 2
TACE	TNF- α Converting Enzyme
TACI	Transmembrane Activator and CAML Interactor

Tak1	TGFβ-activated kinase 1
THD	TNF Homology Domain
TIM	Traf-Interacting Motif
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRAF	TNF Receptor-Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL-R	TNF-Related Apoptosis-Inducing Ligand Receptor
TJ	Tight Junction
Veli	Vertebrate Lin-7 homolog
Wg	Wingless
Wng	Wengen
WT	Wild-type
XEDAR	X-linked Ectodysplasin-A2 Receptor
ZA	Zonula Adherens

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ABSTRACT

Disruption of polarity and impairment of programmed cell death are signalling pathways of advanced epithelial tumors, whose progression often involves the activation of signalling controlled by TNF receptors, such as the JNK pathway. However, the link between loss of polarity and JNK pathway still remains elusive. In a disc-specific *rn>avl* RNAi screen aimed at finding new genes involved in neoplastic onset, a novel *Drosophila* TNFR was identified, named Grindelwald (Grnd). Grnd and the unique fly TNF Eiger activate the JNK pathway, and induce apoptosis or malignancy in a cell context-dependent manner. My PhD project focused on the characterization of the molecular mechanisms underlying Grnd activation and transduction of the JNK pathway.

Upon binding to Eiger, Grnd recruits to the intracellular membrane Traf2, the most upstream component of the JNK pathway, and activates the JNK cascade. To shed light on the mechanism of Grnd activation by Eiger, I determined the crystal structure of the extracellular domain (ECD) of Grnd, in isolation and in complex with Eiger. Grnd-ECD comprises a single cysteine-rich domain, organized in two modules, named X2 and C2, and stabilized by disulphide bridges. The crystal structure of the Eiger:Grnd complex showed that the Eiger-TNF domain folds in a typical “jelly-roll” antiparallel β -sheet, and forms hetero-hexamers with three Grnd molecules. To map the interacting surface between Grnd and Eiger, we engineered point mutations and performed binding assays revealing that Thr51, His66-Asn67 of Grnd and Asp337, His388-Arg391, and Arg396-Arg401 of Eiger are essential for the nanomolar affinity between the ligand and the receptor. *In vitro* studies suggest that glycosylation on Asn332 of Eiger lowers the affinity between Eiger and Grnd. We are currently generating collaboratively Grnd mutant flies to assess the relevance of the Eiger-Grnd interaction in activating the JNK pathway and inducing the small-eye phenotype, or in promoting malignancy in *scrib*^{-/-}; *ras*^{V12} clones.

Previous results showed that the JNK pathway was activated in polarity-deficient cells to drive neoplastic growth. In the *rn>avl* RNAi screen, Grnd, but not Eiger, was necessary for the JNK pathway to be induced. I showed that Grnd directly interacts with the polarity protein Veli, to promote hyper-proliferation and invasiveness. Collectively, these findings depict Grnd as the first TNFR able to couple cell polarity with tumor overgrowth.

One major goal of my project was also the identification of a Grnd orthologue in human, able to couple polarity and signal transduction. Biochemical analyses revealed that Fas is the only TNFR interacting with Veli and with the basolateral polarity protein Dlg1, and provided evidence that Dlg1 compete with Veli for binding to Fas. The relevance of these interactions for the Fas biology is currently under investigation.

1 INTRODUCTION

During my PhD, I studied the regulation of specific Tumour Necrosis Factor Receptors (TNFRs), comparing their working principles in *Drosophila melanogaster* and in humans. For this reason, the Introduction below will contextualize TNFRs in the known literature mainly in flies and human.

1.1 Tumour Necrosis Factors and Tumour Necrosis Factor Receptors

1.1.1 The Tumour Necrosis Factor family

The discovery of Tumour Necrosis Factor (TNF) was a step-by-step finding. In late XIX century, the physician Bruns noticed tumour regression after bacterial infection. In 1943, lipopolysaccharide (LPS) was identified as the responsible for the tumour regression, but only after about 20 years it was found that the LPS effect was not direct, but mediated by a factor released in the medium. This was named “tumour necrotizing factor”, then renamed “Tumour Necrosis Factor” by Old in 1975 (1).

At present, the human TNF superfamily includes 19 members, while a unique *Drosophila* protein has been identified, Eiger (reviewed in (2, 3)). TNFs are mostly classified as type II transmembrane proteins, *i.e.* organized in a signal peptide, an intracellular N-terminus and an extracellular C-terminus. Exceptions are represented by human VEGI and LT α , which are not equipped with the intracellular domain (Fig. 1). TNFs are usually produced as transmembrane proteins, and only a few of them can generate soluble forms by proteolysis mediated by the protease TACE/ADAM17 (TNF- α -Converting Enzyme/A Disintegrin And Metalloproteinases domain) (4), matrilysin (5), or members of the subtilysin-like furin family (6). Extracellular cleaved TNFs are able to act at a long-range distance, spreading signal activation of their cognate receptors in a non-cell autonomous manner. Nevertheless,

certain soluble ligands have been reported to induce opposite outcomes when compared with the membrane-bound form, as in the case of FasL (7, 8).

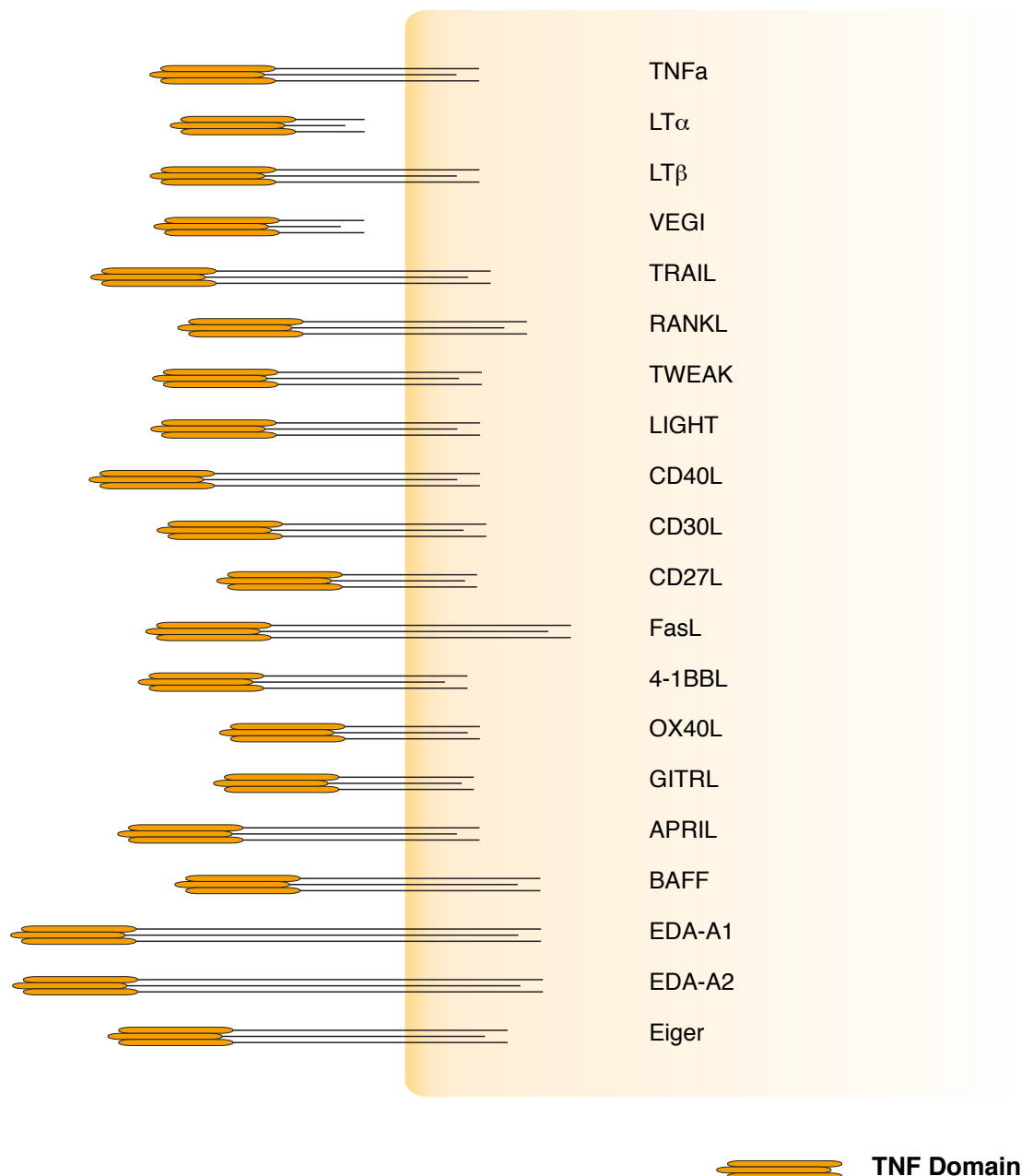


Fig. 1. The TNF family. So far, 19 human TNFs and a single *Drosophila* TNF have been identified. TNFs are represented as transmembrane proteins, with the exception of VEGI and LT α that do not possess a transmembrane region. The TNF homology domain is indicated as a trimeric orange structure.

1.1.2 Structural features of TNFs

TNFs are active as trimeric ligands, characterized by the presence of a TNF homology domain (THD) on the extracellular domain (hereon referred to as ECD), responsible for binding to the receptor (the TNFR). The THD domain folds as a compact “jelly-roll” β -sandwich, with an inner and an outer β -sheets composed by five antiparallel β -strands each, named A’AHCF and B’BGDE, respectively. The outer β -sheet creates the external surface and comprises five β -strands, with the connecting loops that are responsible for the binding to receptors. The inner β -sheet shows 25-30 % sequence homology among ligands, and contains hydrophobic and aromatic residues involved in trimer formation (Fig. 2).

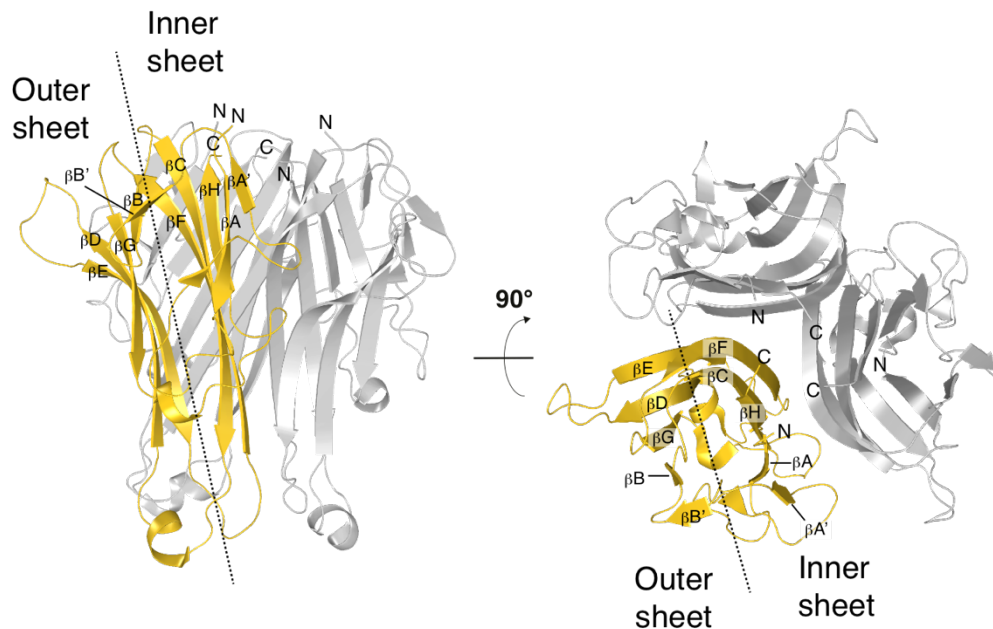


Fig. 2. The TNF homology domain (THD). Cartoon representation of the LT α THD (PDB: 1TNR). The THD consists of two β -sheets organized in a jelly-roll fold. The inner sheet is composed by the AA’HCF β -strands and is packed against the other two THDs of the trimeric TNF arrangement. The outer β -sheet consists of the β -strands B’BGDE, and is involved in binding the TNFRs.

1.1.3 The Tumour Necrosis Factor Receptors superfamily

TNFRs superfamily comprises 29 members in human, and a single one in *Drosophila melanogaster*, named Wengen (reviewed in (3, 9)). TNFRs are mostly type I transmembrane proteins, consisting of a signal peptide, an N-terminal extracellular region, a transmembrane helix (TM), and a C-terminal intracellular domain. Exceptions to this domain structure are

present among TNFRs. In particular, human BCMA (B Cell Maturation Antigen), TACI (Transmembrane Activator and CAML Interactor), BAFFR (B-cell Activating Factor Receptor) and XEDAR (X-linked Ectodysplasin-A2 Receptor) are classified as type III transmembrane proteins because they lack the signal peptide, whereas DcR3 (Decoy Receptor 3) and OPG (Osteoprotegerin) are produced and secreted as soluble receptors. In addition, TRAIL-R3 (TNF-related Apoptosis-Inducing Ligand Receptor 3) is anchored to the membrane by C-terminal glycolipids (10). Most TNF ligands bind only to one TNFR, but some of them including TNF α , FasL, April, and BAFF, associate with more than one. Notably, DR6 (Death Receptor 6), RELT (Receptor Expressed in Lymphoid tissues), and TNFR19 are so far orphan receptors as their ligand has been not yet identified, whereas NGFR (Nerve Growth Factor Receptor) binds ligands that do not belong to the TNF family. A TNFRs hallmark is the presence of cysteine-rich domains (CRD) in the extracellular region, and of death domains or Traf-interacting motifs (TIM) in the intracellular portion, which transduce extracellular signals to the cell (Fig. 3). These extracellular and intracellular features are described in details in the next paragraphs.

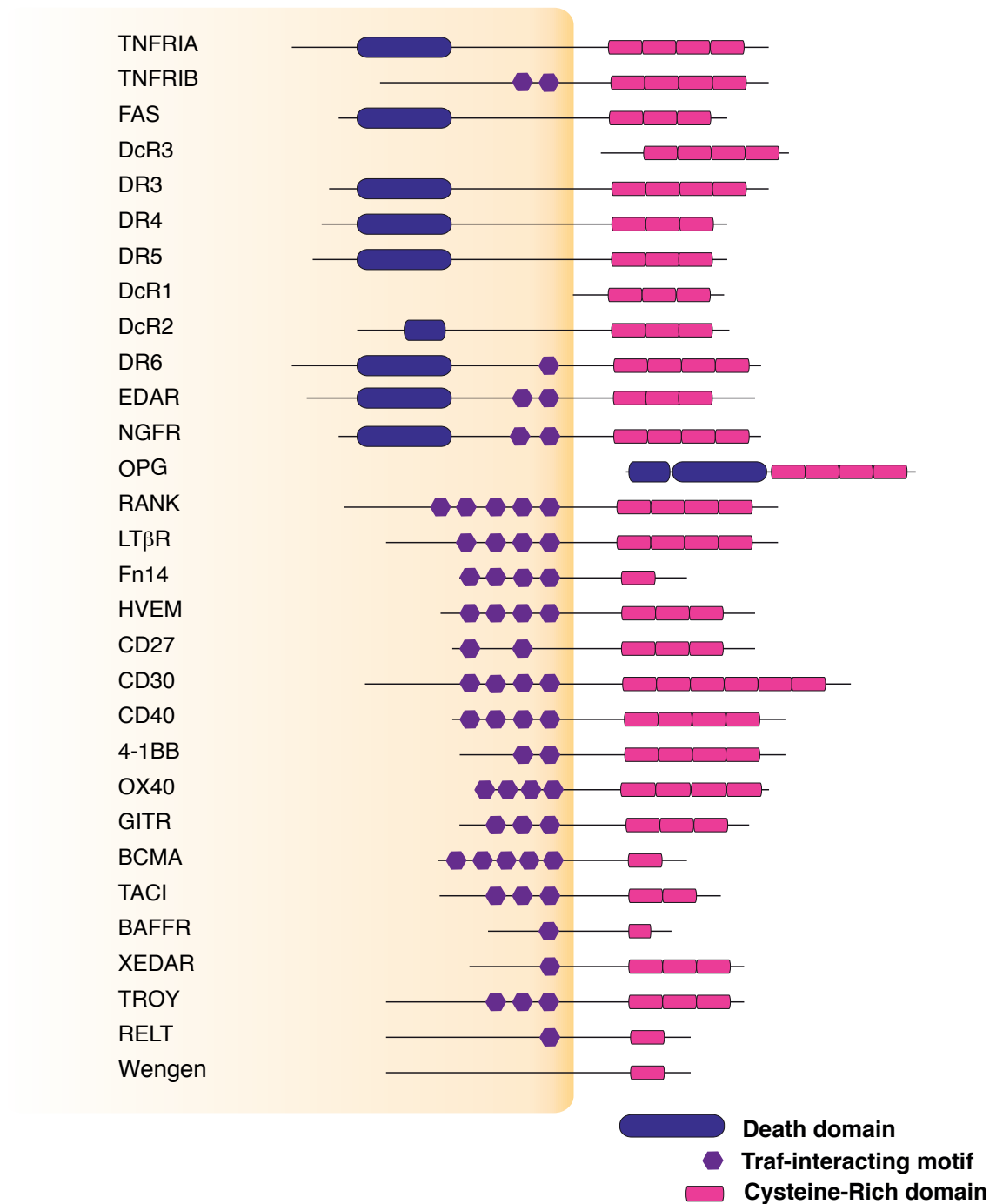


Fig. 3. The TNFR superfamily. The 29 human TNFRs and the *Drosophila* TNFR Wengen are shown as transmembrane proteins, with the decoy receptors DcR3 and OPG directly synthesized as secreted receptors. CRDs in the ECD are indicated as magenta boxes, whereas the intracellular death domain of TNFR1, Fas, DR3, DR4, DR5, DR6, EDAR, and NGFR is depicted in blue. Receptors equipped with an intracellular domain, but not comprising the DD, belong to the TIM-containing family. The TIM is shown as a purple hexagon.

1.1.4 Structural features of TNFRs and signal transduction mechanisms

The extracellular cysteine-rich domains (CRDs) present on all TNFRs are pseudo-repeat regions about 40 residues long, containing usually six cysteines engaged in three disulphide bonds that stabilize the fold. The CRD number varies among receptors from one to four, with CD30 showing six CRDs probably deriving from partial duplication of the first three (11), and BAFFR equipped with half CRD (12). A sequence-based classification of CRDs is made difficult by the different spacing among cysteines observed in the various TNFRs, thus a more structural approach is generally used (13). According to this approach, each CRD is classified on the basis of the structural organization of the two modules, and on the disulphide bonds pattern. The module types are indicated by a letter, indicating the helical or β -strand kind of arrangement, and a number, corresponding to the number of disulphide bridges. A1 and A2 modules are generally 12-17 residue long, and consist of two β -strands connected by turns generating a C-shape morphology. B1 and B2 modules are 21-24 residues long, with three β -strands organized in a S-shaped fold. A and B modules are the most common. However, rarer ones have been found. One example is the X2 module of BAFFR, characterized by the presence of three β -strands, with an intercalated pattern of cysteines. (14, 15). C2 and D2 modules are both formed by two α -helices connected by a loop, with a nested or an intercalated pattern of cysteines, respectively. C2 module has been described for Fn14 and TNFRI-CRD4 (16-18), whereas the D2 module is present in BCMA and TACI-CRD2 (18, 19).

The conventional model for TNF:TNFR association assumes that trimeric ligands bind three monomeric receptors, forming 3:3 hetero-hexamers. TNFRs with multiple CRDs usually bind their ligand via the second and the third CRD (20-22). The first CRD is conserved, but it is usually not involved in ligand recognition. Interestingly, Naismith *et al.* in 1995 crystallized the unligated form of TNFRI, which contains 4 CRDs and folds as a dimer in which the initial part of the first CRD is the region responsible for the self-association (23) (Fig. 4A). Later works illustrated that also CD27 and CD40 dimerize in the absence of their

ligands (24-28), while Fas seems to be a trimer in solution (29). The region of the first CRD responsible of TNFR self-association was thus named “Pre-Ligand Assembly Domain” (PLAD) (26). Even if not directly involved in TNF interaction, removal of the PLAD region from TNFRs not only impairs receptors self-association, but also ligand recognition and signal transduction. Two molecular explanations have been proposed for the role of PLADs in TNFR cascade. The first model envisions that pre-assembled receptors could bind ligands with higher affinity compared to monomeric ones (Fig. 4B, top). The second and most credited model assumes that pre-formed TNFR oligomers are recognized by different ligand trimers, this way generating TNF:TNFRs clusters on the cell surface, which initiate productive signalling (Fig. 4B, bottom). This has been shown to occur in the case of Fas activation (30).

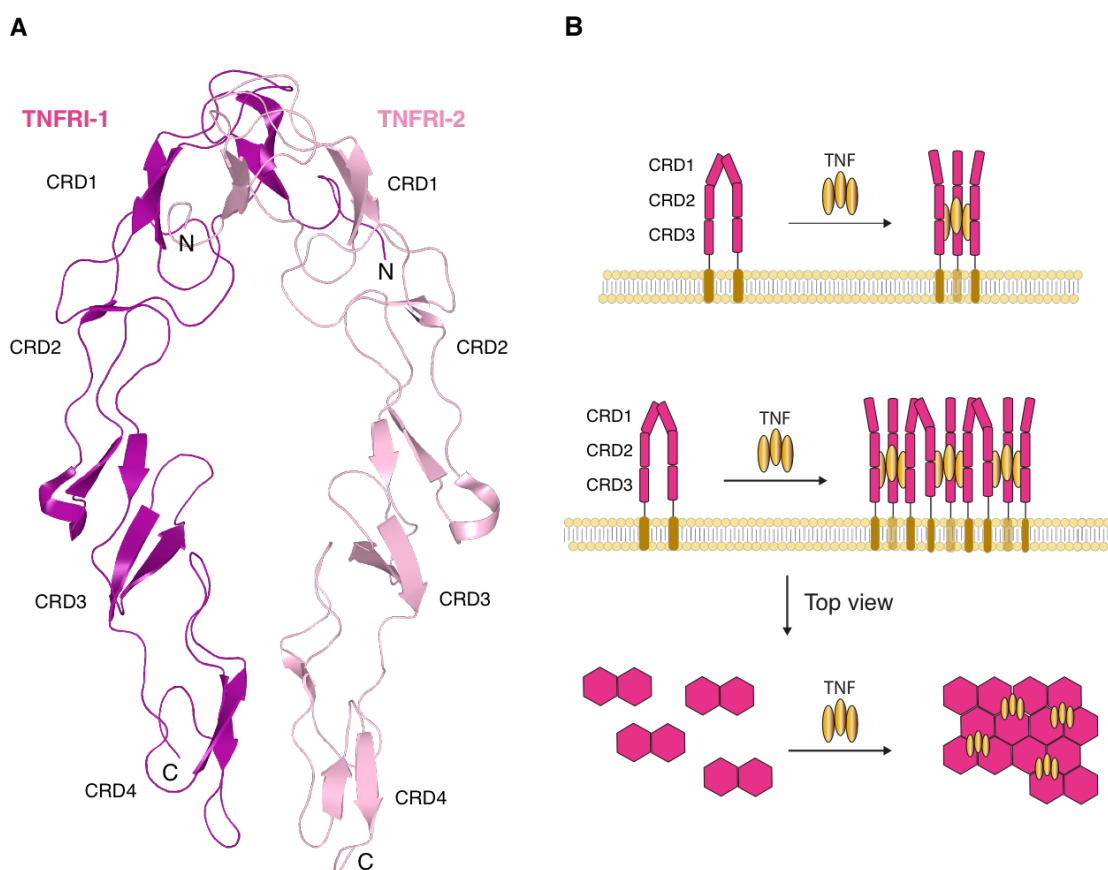


Fig. 4. PLAD determines TNFR oligomerization prior to TNF binding. **A.** Structure of the dimeric ECD of TNFRI (PDB: 1NCF), crystallized without its ligand. The dimeric interface is contributed by the N-Terminal region of the receptor. The two TNFR molecules are showed in magenta and pink. **B.** Graphical representation of the proposed functions of the PLAD domain. Top: The first model proposes that pre-assembled receptors have increased affinity for ligand binding.

Bottom: An alternative model hypothesizes that TNFs bind receptors molecules belonging to different TNFR dimers, creating membrane clusters inducing a stronger signal activation.

TNFRs with single or multiple CRDs interact with their ligands with a different overall topology. Receptors with a single CRD (such as BCMA and Fn14) generally contact a single molecule of TNF within the hetero-hexameric complex via both modules forming the CRD (12, 18, 19, 31), while TNFRs with multiple CRDs (for instance CD40, DR4, and DcR3) usually tie their ligands by the association of a more extended interaction surface comprising two TNF monomers (20-22, 32).

After ligand binding, TNFRs recruit on the intracellular surface adaptor proteins that mediate transduction signal activation. Based on the intracellular features, TNFRs are classified in three major groups (1, 33). The first one is composed by the so called “Death Receptors” (DR), named after the presence of the Death Domain (DD) in their cytoplasmic region. The DD is a globular ~80 residues domain composed by six antiparallel and amphipathic α -helices, with the hydrophobic sides forming the internal core of the domain (34). Upon ligand binding, the DD undergoes a conformational change which allows homotypic interaction with other DD-containing proteins, such as Fas Associated Death Domain containing protein (FADD) (Fig. 5A), creating a platform for the formation of specific complexes inducing cell responses, *e.g.* cell death or inflammation (35, 36). TNFRI, Fas, DR3, DR4, DR5, DR6, EDAR and NGFR belong to the DRs family. Among them, Fas, DR4, and DR5 usually initiate apoptosis upon activation, but also survival and proliferation under certain circumstances (reviewed in (37, 38)). The second TNFR family comprises receptors containing Traf-Interacting Motifs (TIM). Traf proteins are E3 ubiquitin ligases, with an N-terminal RING domain, which is missing in human Traf1 and *Drosophila* Traf3. Traf proteins form trimeric complexes by central coiled-coil interactions and contact the intracellular domain (ICD) of TNFRs with a globular MATH domain (Fig. 5B, top). TNFRs recognize the MATH domain via conserved motifs, that for human Traf1, Traf2, Traf3 and

Traf5 conform to PxQ/EE or PxQ/ExxD or PxQxT sequences, and for Traf4 and Traf6 to RLxA or PxExx sequences (Fig. 5B, bottom) (39, 40). The TNFRs-containing TIM family includes TNFRII, CD27, CD30, CD40, BAFFR, BCMA, TACI, XEDAR, TROY, RANK, OX40, 4-1BB, and LT β R, which, once activated, induce survival, inflammation and proliferation. Finally, decoy receptors, such as DcR1, DcR3, and OPG, contain only the extracellular region, and act by competing with other receptors for the binding to ligands, without inducing intracellular signalling. Notably, the *Drosophila* TNFR Wengen does contain neither the DD nor a known TIM.

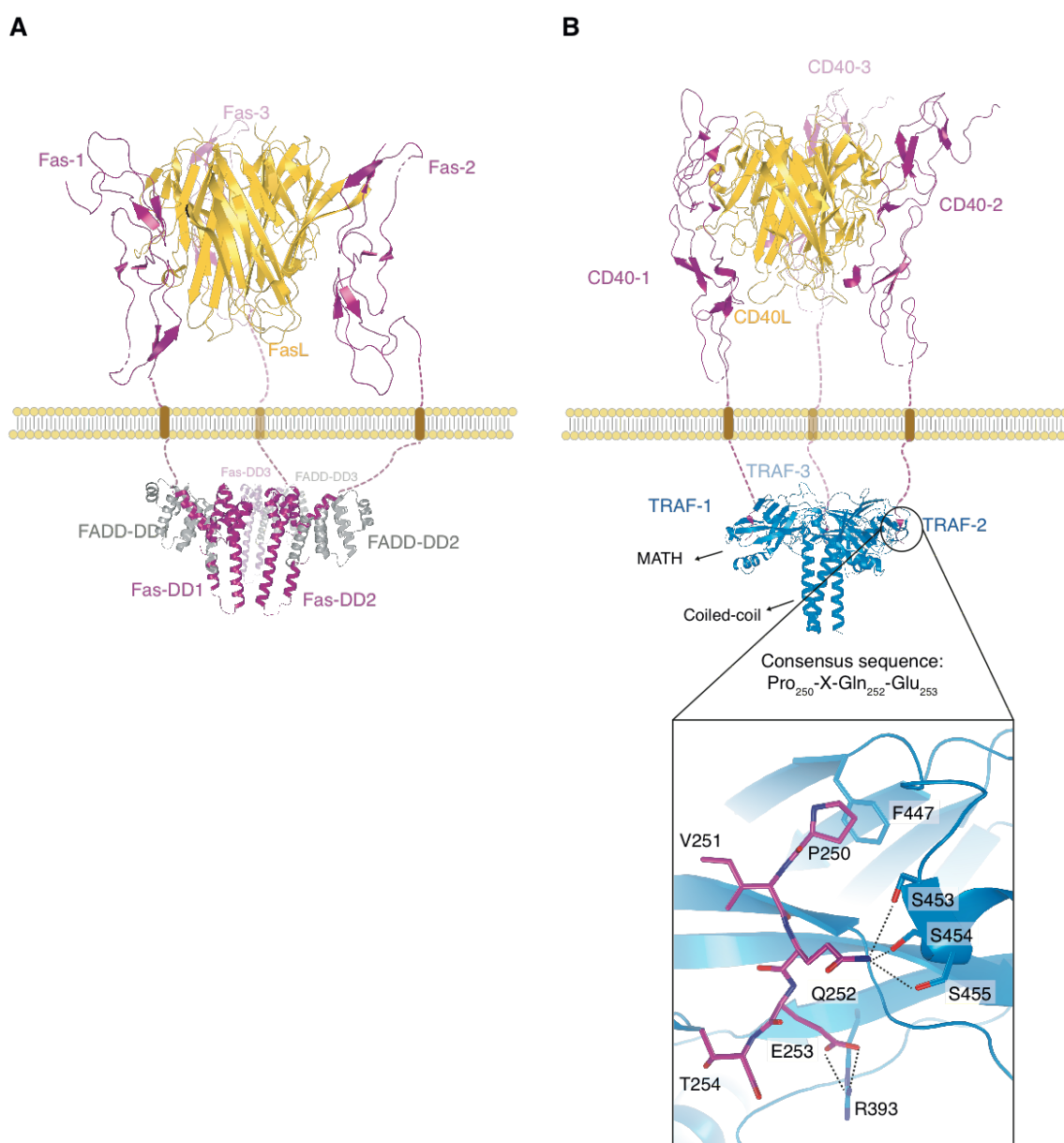


Fig. 5. Signal propagation by TNFRs intracellular domains. A. Fas is an example of DD-containing receptor. Each Fas-DD contacts the DD belonging to a FADD molecule (grey), creating

a platform that triggers signal transduction (PDB: 3THM for Fas; PDB: 5L36 for FasL; PDB: 3EZQ for Fas-DD:FADD-DD). **B.** Top: Upon activation by CD40L ligand binding, Traf2 trimers (in blue) are recruited to the membrane by CD40, and the signal transduction cascade starts. The MATH domain and the coiled-coil of Traf2 are indicated. CD40L and CD40 are depicted in magenta and gold, respectively (PDB: 3QD6 for CD40:CD40L; PDB: 1QSC for CD40:Traf2). Bottom: Close-up on the CD40:Traf2 interaction. CD40 contains a TIM motif in the intracellular region, conforming to the consensus sequence Pro250-X-Gln252-Glu253. Residues involved in stabilizing the interaction between CD40 and Traf2 are indicated.

1.1.5 The *Drosophila* TNF Eiger

The unique *Drosophila* TNF was identified by two independent groups in 2002 by different approaches. Moreno *et al.* analysed the *Drosophila* genome and found a single gene encoding a transmembrane THD-containing protein, that was named “Eiger”, after the Swiss mountain also known as “Wall of death” (41). In the same period, the Miura’s lab was looking for genes able to induce the small-eye phenotype, and found an ORF with this property, that was named with the acronym Eiger that stands for EDA-like cell death trigger (42). Eiger is a type II TNF, consisting of a 36-residues long intracellular region, a transmembrane helix spanning amino acids 37-59, and an ECD containing a C-terminal THD (Fig. 6). Eiger is predominantly expressed in the fly nervous system and in the eye imaginal disc (42, 43). In developing flies and in S2 cells, two isoforms have been detected by RT-PCR: Eiger long (Eiger-L) and Eiger short (Eiger-s), which differ in the presence of the six residues GESLLS on the ECD at position 265, upstream of the THD. Eiger-L is more abundant than Eiger-s during all the developmental stages, but no differences have been detected in their function. In fact, Moreno *et al.* performed experiments using Eiger-L isoform, Miura and his group using Eiger-s, obtaining similar results, showing that both the isoforms are effective in inducing cell death and the small-eye phenotype.

In *Drosophila* hemolymph and in the supernatant of Eiger-transfected SL2 cells, Eiger is present also as soluble protein cleaved between residues 145 and 146 by the metalloprotease

TACE. This soluble form acts also on distant responsive cells, away from the secreting cell, and induce apoptosis (44-46).

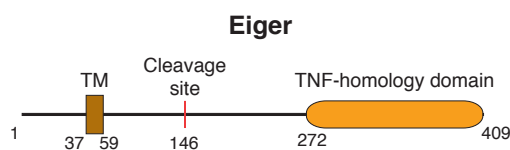


Fig. 6. The *Drosophila* TNF Eiger. Domain structure of Eiger, consisting of a N-terminal intracellular region, a transmembrane domain, followed by an extracellular part characterized by the presence of the TNF domain. The cleavage site at position 146 is indicated.

Eiger plays a role as tumour suppressor in eliminating polarity-deficient cells, such as *scribble* (*scrib*) or *disc large* (*dlg*) mutant, which lose apico-basal polarity and could potentially generate malignant tissues. Eiger-mediated apoptosis requires the activation of the JNK pathway that promote cell death thanks to the expression of the proteins Grim, Hid, and Reaper, suppressors of DIAP1 (*Drosophila* Inhibitors of Apoptosis Protein-1). Once inactivated, DIAP1 is not able anymore to inhibit the Dronc caspase, which can this way initiate apoptosis (47, 48). Eiger overexpression in *Drosophila* imaginal discs causes development defects, due to an increased rate of apoptosis that results in a complete block of organ formation (41, 42).

Eiger endocytosis is required for the JNK cascade to be effective, as demonstrated by the fact that Eiger co-localises with the endosome marker Rab5 during JNK activation, and that apoptotic cells increase the rate of endocytosis, whose inhibition completely impairs cell elimination (49). Furthermore pJNK, the activated form of JNK, is detected in endosomes, suggesting that the JNK signalling propagates from these vesicles (49). On the contrary, knocking-down early endosome proteins, for instance Rab21, reduces Eiger localization in endosomes, and decreases cell death rate as a consequence (50).

TNF ligands have been defined as “double-edged swords” because they can induce cell pro-tumourigenic behaviour under specific condition (1, 51). Indeed, when the loss of Scribble protein is associated to a mutated hyperactive form of Ras (Ras^{V12}), Eiger acts as an

oncogene, with the JNK pathway inducing the expression of the matrix metalloprotease 1 (MMP1) (52-54). MMP1 degrades extracellular membrane components, favouring tumour cell motility malignant behaviour (52).

1.1.6 The *Drosophila* TNFR Wengen

The CG6531 gene was identified by Kanda *et al.* in 2002, that encodes for a protein whose depletion rescued the small-eye phenotype caused by Eiger overexpression (55). The protein encoded by the CG6531 ORF was named “Wengen”, that is a village at the foot of the mountain Eiger. Wengen is a 343-long type I TNFR (44), with an N-terminal signal peptide, followed by a single CRD at residues 99-137, a transmembrane region spanning amino acids 202-224, and a cytoplasmic region characterized by the presence of a predicted coiled-coil (Fig. 7). Contrary to Eiger, Wengen is expressed at low levels before gastrulation. During gastrulation, Eiger mRNA localizes in the epidermal layer, while Wengen is detected in the mesodermal one. Only at later stages, both transcripts are found in the nervous system (44, 56). Wengen and Eiger co-immunoprecipitate (co-IP) in S2 cells, and this interaction is mediated by the CRD of Wengen and the THD of Eiger (55). The small-eye phenotype caused by Eiger overexpression can be reverted by Wengen depletion, suggesting that Wengen transduces pro-apoptotic signals. However, Wengen RNAi does not revert the malignant behaviour of *scrib*^{-/-}; *ras*^{V12} cells in cephalic complexes, indicating that in this system a further activator of the JNK pathway likely exists (53). In spite of lacking a death-domain and a known TIM, Wengen has been shown to co-IP with Traf2 in HEK293T cells transfected with the *Drosophila* proteins (44). If the receptor contains a yet-to-be established new TIM, or if it triggers JNK activation by forming hetero-oligomers with another not yet identified TNFR, remains an open question.

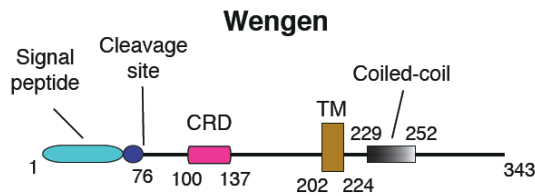


Fig. 7. The *Drosophila* TNFR Wengen. Wengen consists of a N-terminal extracellular domain with a single CRD, a transmembrane region, and a mostly unstructured intracellular domain with a predicted coiled coil.

1.1.7 The human TNFR Fas

In 1989, two separated groups identified mouse monoclonal antibodies against a human cell surface receptor, able to activate the receptor and trigger apoptosis (57, 58). The cDNA encoding this human receptor was subsequently isolated from T cell lymphoma KT-3 cells by Itoh *et al.* in 1991, and named Fas (59). During apoptotic process, activation of caspases drives a series of processes, including the fragmentation of cell components, eventually ending in cell death. Several signs of apoptosis can be observed, *e.g.* cell shrinkage and rounding caused by microvilli degradation, and nuclei and DNA fragmentation. To check if Fas was able to induce apoptosis, mouse WR19L or LB1 cells were transfected with the Fas cDNA and incubated with anti-Fas antibody. DNA fragmentation, membrane blebbing and condensed fragmented nuclei were detected(59), confirming that Fas could be considered a *bona fide* TNFR. After those works, during the latest 30 years Fas has been object of intense studies.

Fas is a 38 kDa type I TNFR. Its ECD spans residues 26-173 and contains three CRDs, followed by a 16 residues-long TM, and an intracellular region comprising a DD at amino acids 225-319 (Fig. 8A). The second and third CRD are engaged in binding the ligand FasL, whereas the first CRD contributes to form PLAD (see paragraph 1.1.4) and generates Fas oligomers. *In vitro* studies showed that the presence of the Fas PLAD leads to formation of receptor dimers, whereas the addition of CRD2 and CRD3 generates trimeric assemblies, suggesting a further role for these CRDs in driving Fas oligomerization (25, 29). Fas:FasL

complexes aggregate in large clusters in lipid rafts, which are internalized in endosomes (60). Upon FasL binding, the death domain of Fas undergoes a conformational change that allows binding to FADD. FADD in turns recruits pro-caspase 8, forming the Death-Inducing Signalling Complex (DISC). DISCs act as proteinaceous platforms, initiating caspase-mediated cell death (61-63). Two types of apoptosis can be distinguished in respect of the activation of different downstream effectors (64). In type I programmed cell death, caspase 8 cleaves and activates effector caspases responsible for the proteolysis of several cell substrates and subsequent apoptosis. In type II apoptosis, caspase 8 processes the pro-apoptotic protein Bid promoting the formation of pores on the mitochondria membrane. As a consequence, SMAC (Second-Mitochondria derived Activator of Caspase) and cytochrome C are released (65, 66), assembly of the apoptosome is triggered and pro-caspase 3 induces cell death, as in type I apoptosis (reviewed in (38)). Interestingly, Fas has been shown also to induce pro-proliferation, invasion and inflammation stimuli by activating the ERK1/2 and JNK MAPKs, and the NF- κ B pathways (Fig. 8B) (67-70). The switch between pro-apoptotic and pro-survival malignant behaviour of Fas is controlled at different levels, reviewed in (37).

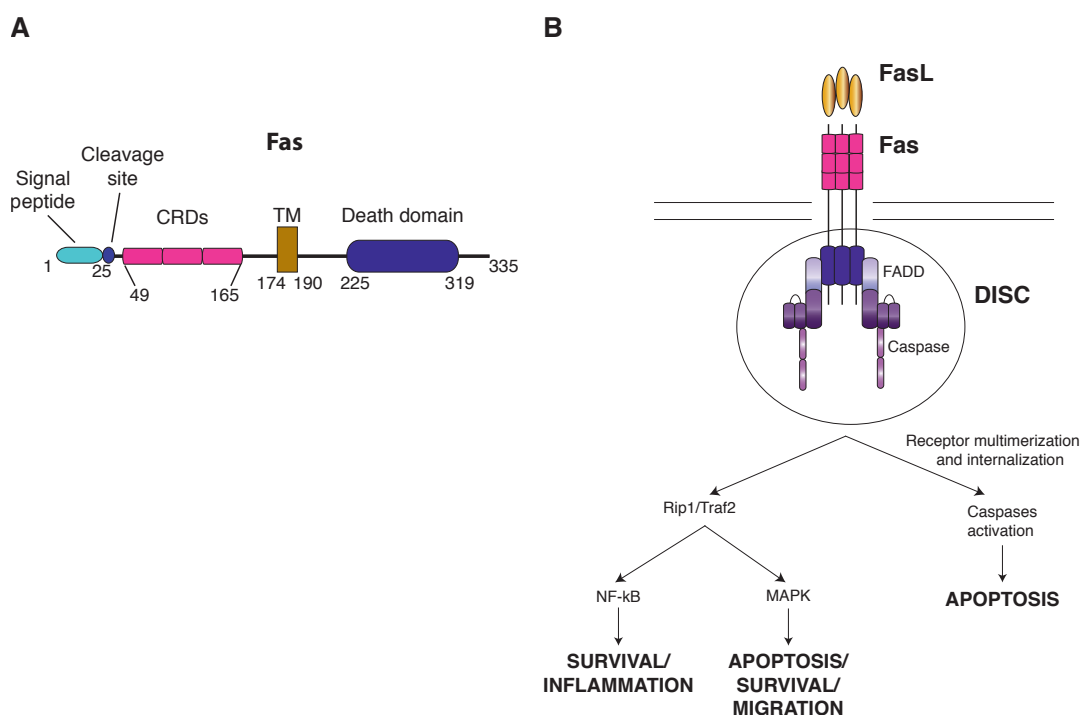


Fig. 8. The human TNFR Fas. A. Domain structure of Fas. Fas contains three CRDs within the N-

terminal extracellular region, followed by a transmembrane domain, and a C-terminal intracellular region showing a death domain. **B.** Signalling pathways induced by Fas activation. Upon binding to FasL, the intracellular DD of Fas interacts with FADD, which in turns recruits pro-caspase 8. The complex formed by Fas, FADD and caspase 8 is named DISC. When endocytosed, DISC leads to apoptosis by effector caspases activation. Under different and yet poorly characterized conditions, Fas can also lead to NF- κ B and MAPKs pathway activation, triggering survival, inflammation, and invasion.

1.2 The *Drosophila* JNK pathway

JNK is a MAPK (Mitogen-Activated Protein Kinase) protein, whose pathway has been implicated in a variety of physiological and pathological processes, including tissue morphogenesis, response to pathogens, longevity, tumour invasion, and apoptosis (71). It is activated in response of a variety of stimuli, resulting in the sequential phosphorylation of a series of kinases, the last of which is JNK. Since the discovery of the first JNK protein in 1993 (72), two additional *jnk* genes have been identified in human genome, causing redundancy issues during analysis of their roles in cells (73). *Drosophila melanogaster* shows high conservation of the pathway, and it possesses a single JNK orthologue, and thus it results to be a powerful tool to dissect the molecular mechanism underlying the JNK cascade.

In flies, one of the major activators for the JNK pathway is Eiger. Upon Eiger binding, the TNFR recruits Traf2 to the intracellular membrane, which in turns activates the kinase complex dTab2/dTAK1 (JNKKK) (74). dTAK1 phosphorylates the JNKK Hemipterous (Hep), whose substrate is JNK, encoded by the *basket* gene (*bsk*) (75). Once activated, JNK phosphorylates Jun and Fos, which hetero-dimerize, forming the transcription factor named AP-1 (76). JNK-mediated phosphorylation of Jun activates AP-1, increases its transcriptional activity, and induces the expression of different targets, leading to opposite cell outcomes, for instance apoptosis or malignancy (Fig. 9). In recent years, several regulatory proteins of this cascade have been identified. A major role seems to be played by

ubiquitination. The ubiquitin carboxyl-terminal hydrolase CYLD deubiquitinates K48-ubiquitinated Traf2, and promotes protein stabilization by inhibiting the proteasomal degradation (77). Traf2 is also K63-ubiquitinated by the ubiquitin-conjugating E2 enzyme Bendless:dUev1a (78, 79). This apparent contradiction between the dual need of a ubiquitinated Traf2 for activation of the JNK pathway, and deubiquitination of Traf2 for protein stability could be justified by the opposite outcomes derived from different ubiquitination types. K63-linked polyubiquitination is involved in kinase signalling activation, protein trafficking, and DNA damage repair, while K48-chains tags substrate for proteasomal degradation (80). Thus, K63-ubiquitination catalysed by Ben:Uev1a could regulate JNK activation, while CYLD could stabilize Traf2 by removal of K48-linked modifications (81, 82).

Another interesting level of regulation of JNK activation in *Drosophila* relies on Eiger endocytosis, needed for the pathway to be activated. Indeed, when the Eiger internalization is inhibited, Eiger-mediated JNK activation cannot be detected (49, 50). Furthermore, AP-1 induces the expression of the phosphatase puckered (*puc*), which dephosphorylates and inactivates JNK proteins, in a negative feedback loop that restrains the signalling cascade extend (83-85). Notably, puckered has been widely used in *Drosophila* as reporter of the JNK activation (Fig. 7).

Depending on the cell context, the Eiger-JNK signalling axis determines different fates and outcomes, that will be illustrated in the next paragraphs.

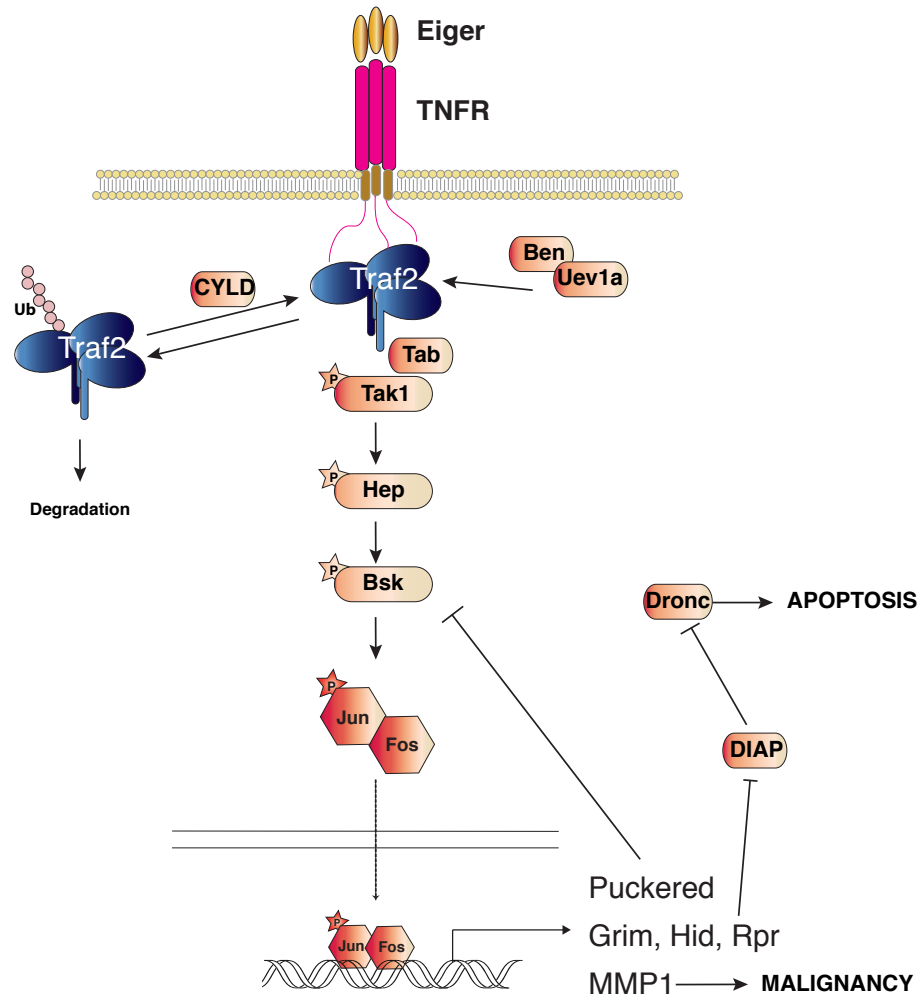


Fig. 9. The *Drosophila* JNK pathway. Eiger:TNFR association determines activation of the JNK pathway, that starts with membrane recruitment of Traf2 trimers. Traf2 activates the JNKKK Tak1, which in turns phosphorylates the JNKK Hemipterous, that activates the JNK Basket. Basket phosphorylates Jun and Fos, which hetero-dimerize forming the AP-1 transcription factors, and activate the transcription of a series of gene. Among them, Grim, Hid, and Reaper block the activity of DIAPs in inducing Dronc degradation, thus stabilizing the *Drosophila* caspase to initiate apoptosis. MMP1 degrades components of the ECM, enhancing cell invasion and malignancy in specific conditions. Puckered is a phosphatase which acts on Basket, limiting the extend of JNK signalling. The role of Traf2 is regulated by polyubiquitination: CYLD deubiquitinates Traf2 to avoid protein degradation, whereas the Ben:dUev1a E2 complex participates in Traf2 K63-polyubiquitination to induce its activation.

1.2.1 Eiger/JNK-mediated apoptosis

JNK is also known as Stress-Activated Protein Kinase (SAPK), as it represents a major player in stress response (86, 87). The pro-apoptotic role of JNK resides in the ability of the active transcription factor AP-1 to induces the expression of *grim*, *hid*, and *reaper*. Grim,

Hid, and Reaper inactivate the inhibitors of apoptosis DIAP1 by inducing its proteosomal degradation, thus stabilizing the *Drosophila* caspase Dronc, which in this way becomes active and starts apoptosis (47, 48). Eiger/JNK-driven cell death is a ubiquitous mechanism exploited to eliminate aberrant cells. This is the case of the loss of the polarity protein scribble (*scrib*), whose mutated gene has been extensively used in *Drosophila* to study the JNK cascade. *scrib* clones are sensed as a harmful pre-malignant signal, that have to be eliminated from the tissue. Surrounding wild-type cells are crucial in this phenomenon. In fact, Eiger is not only produced by *scrib* mutated clones in a cell-autonomous manner, but also by associated cells, such as hemocytes (54). In addition, wild-type neighbouring cells increase the rate of apoptosis by enhancing Eiger internalization and signal transduction (49). Last but not least, normal cells have been shown to activate non-apoptotic Eiger/JNK pathway, which mediates the production of PVR, the PDGF/VEGF *Drosophila* orthologue. PVR promotes premalignant cell elimination by engulfment (88). This mechanism of cell elimination is the result of “cell competition”, a process by which lower fitness-cells are removed from the tissue by surrounding fitter ones. Interestingly, *Drosophila* organ compartments fully composed by *scrib* polarity-deficient cells do not undergo apoptosis, but rather develop tumours (89). These observations confirm the relevance of the surrounding normal cells in eliminating aberrant clones, suggesting a role for the Eiger/JNK cascade in acting as a tumour-suppressing mechanism. Strikingly, these polarity-deficient *scrib* mutants show malignant behaviours, whose maintenance requires the hyperactivation of the JNK pathway, thus depicting the JNK signalling as a tumour promoter pathway. However, the link between loss of cell polarity and JNK activation still remains elusive.

1.2.2 Eiger/JNK-mediated tumour growth

Eiger can function as a tumour-promoter under specific cell conditions. Indeed, when the *scrib*^{-/-} genotype is coupled with the expression of a mutated hyperactive form of Ras (*ras*^{V12}), cells overcome the apoptotic signal and develop malignant invasive tumours, thanks

to the activity of Ras^{V12} in blocking cell death by downregulating Hid expression and to the JNK-driven expression of MMP1 (52-54, 90-92). However, in *scrib*^{-/-};*ras*^{V12} tumours, Jun is dispensable for the JNK-mediated transcription of MMP1, while Fos is necessary, suggesting that Fos might form a homodimer or a complex with another still unknown factor to promote malignancy in this system (93). The immune system plays a controversial role in this system. *scrib*^{-/-};*ras*^{V12} tumours recruit hemocytes at the basement membrane breakdowns, which in turn produce Eiger, stimulating the JNK pathway for MMP1 production (54, 94). In addition, JNK signalling also promotes the secretion of Upd ligands, which bind their receptor Dome on hemocytes and initiate a positive feedback, involving the activation of JAK-STAT pathway and resulting in hemocytes proliferation (95).

1.3 Cell polarity complexes

Cell polarity refers to the asymmetrical distribution of molecules within a cell, resulting in differences in functions and in cell architecture (reviewed in (96-98)). This asymmetry is fundamental for a number of processes, such as differentiation, proliferation, and maintenance of tissue architecture. Epithelial apico-basal polarity is determined by the presence of cellular junctions and polarity complexes. Cell-cell junctions divide the plasma membrane into separated domains, that are different from a biochemical and functional point of view. These structures are named tight junctions (TJ) and adherens junctions (AJ) in human, and subapical region (SAR), zonula adherens (ZA), and septate junctions (SJ) in *Drosophila*.

The three major polarity complexes are the Par complex, the Crumbs complex and the Scribble complex. They organize an apico-basal axis, whose components are nicely conserved among species (Table 1).

Table 1. Protein composition of the polarity complexes in *Drosophila melanogaster* and in human.

	<i>Drosophila</i> protein	Human protein
Crumbs complex	Crumbs	Crumbs1, Crumbs2, Crumbs3
	Stardust	Pal1, Pals2, MPP7
	PatJ	PatJ, MUPP1
	Veli	VeliA, VeliB, VeliC
Par complex	Bazooka	Par3A, Par3B
	Par6	Par6A, Par6B, Par6G
	aPKC	aPKC ι/λ , aPKC ζ
Scribble complex	Scribble	Scribble
	Lgl	Lgl1, Lgl2
	Dlg1	Dlg1, Dlg2, Dlg2, Dlg4, Dlg5

1.3.1 The Crumbs complex

The core components of the Crumbs complex are four proteins: Crumbs (Crb), DmStardust (Sdt)/hPals1, PatJ (Pals1-associated tight junction protein), and Veli (Vertebrate Lin-7 homologue). The first identified protein of the complex was *Drosophila* Crumbs in 1990, which plays a key role in the formation of normal epithelia (99, 100). The Crumbs complex localizes at the subapical region (SAR) in *Drosophila*, and at tight junctions in human. Stardust/Pals1 physically acts as the central scaffold of the complex. In particular, the PDZ domain of Stardust/Pals1 binds the ERLI motif of Crumbs (101, 102), while the first and the second L27 domains recruits PatJ and Veli, respectively (103, 104). The domain structure of the Crumbs complex and the interactions among members are shown in Fig. 10. For simplicity, the represented structures refer only to the *Drosophila* proteins. Any differences with the human counterparts are listed in the caption.

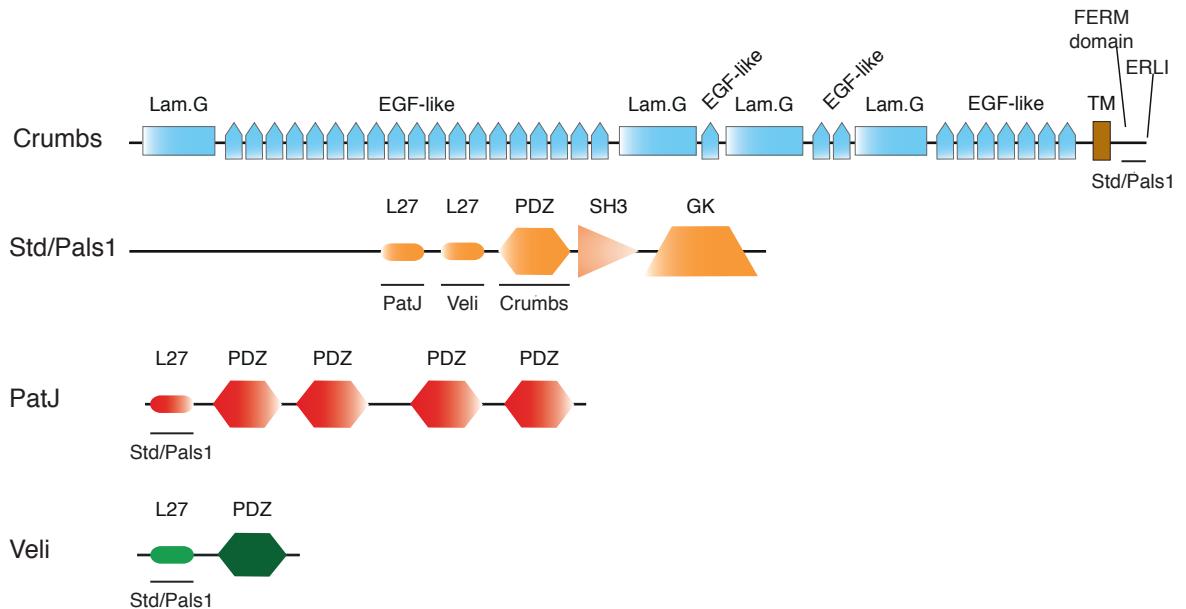


Fig. 10. The Crumbs complex. The Crumbs complex comprises four components: Crumbs, Stardust/Pals1, PatJ, and Veli. *Drosophila* Crumbs is a transmembrane protein, with 29 Laminin-G repeats and 4 EGF-like domains on the extracellular region, followed by a TM, a short intracellular part with a FERM (4.1-Ezrin-Radixin-Moesin) binding domain, and finally an ERLI motif, which binds to the PDZ domain of Stardust/Pals1. The three Crumbs orthologues in human show differences in the organization of the extracellular domain. Stardust/Pals1 has two L27 domains, necessary for the homotypic association with PatJ and Veli, a PDZ domain, which binds the ERLI motif of Crumbs, and an SH3 and a GK domains. *Drosophila* PatJ consists of a L27 domain and four PDZ domains, whereas the human orthologues named PatJ and MUPP1 have 10 and 13 PDZ domains, respectively. Veli presents a L27 and a PDZ domain, with a domain structure shared with the three human Veli counterparts. Protein regions responsible of the interaction with the other members of the Crumbs complex are indicated with black bars.

The Crumbs complex has a role in specify apical cell compartment. Indeed, Crumbs overexpression in *Drosophila* epithelial cells results in spreading of the apical components to the basolateral side of the cell, while *crb* mutants lose apical marker and fail to assemble the zonula adherens (105, 106). The stability of *Drosophila* Crumbs at the cell apical region and its ability in organizing cell polarity depends on phosphorylation of the Crumbs intracellular domain by aPKC. In fact, expression of a not-phosphorylatable form of Crumbs results in cytoplasmic diffusion of PatJ, and in mislocalization of Scribble at the apical membrane (107). Experiments have also shown that in MDCK cells loss of the Stardust

orthologue Pals1 affects PatJ expression, suggesting that PatJ is stabilized by its association with Pals1 (108). On the other hand, PatJ RNAi determines mislocalization of Pals1 and Crumbs in human intestinal cells (109). Veli is necessary for the stability of Pals1 and PatJ in proximal tube epithelia of mice (110). Importantly, members of the Crumbs complex dynamically organize in space and time proteins belonging to the other polarity complexes. For instance, in MDCK cells Veli and the human Stardust orthologue MPP7 interact with Dlg1 at adherens junction (111). Moreover, Par6 binds an internal peptide of Pals1 via its PDZ domain (112), with a mode of interaction which is conserved in *Drosophila* (113).

1.3.2 The Par complex

The Par (Partitioning-defective) complex is composed by Par3, Par6 and aPKC, and localizes at tight junction in human, and at the subapical region of *Drosophila*, slightly basal to the Crumbs complex. The first Par complex components have been identified in 1988 in *C. elegans*, in experiments aimed to study the zygote asymmetric division (114). Mutations in the Par genes determined aberrant symmetric division and defects in partitioning of determinants of the cell daughters' fate. Subsequently, the aPKC (atypical Protein Kinase C) gene was identified, as able to give rise to *par3*-like phenotypes when mutated (115). In *Drosophila*, a single Par3 orthologue has been found, named Bazooka (Baz) (116), sharing a similar structure, as shown in Fig. 11.

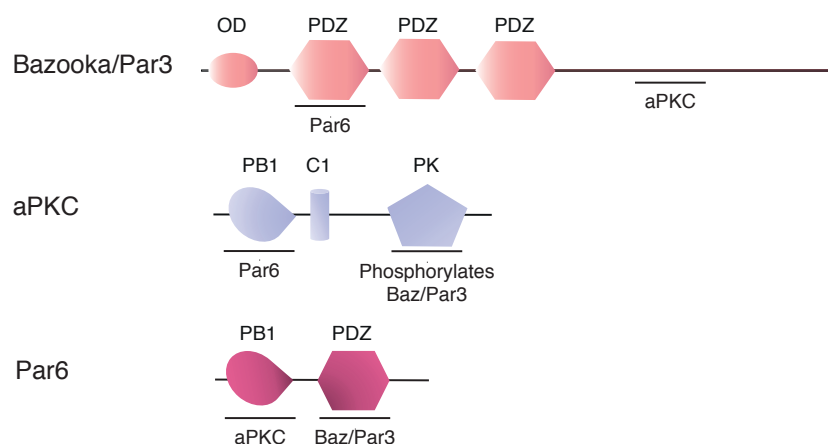


Fig. 11. The Par complex. Baz/Par3 consists of an N-terminal oligomerization domain (OD) necessary for its dimerization, and three PDZ domains. The first PDZ binds Par6 via a homotypic

interaction, whereas the unstructured C-terminus is involved in the association with aPKC, which phosphorylates Baz/Par3 through its protein kinase (PK) domain. aPKC also contains a Phox/Bem-1 (PB1) domain, functional for the binding to the PB1 domain of Par6, followed by a not-functional C1 domain and the protein kinase domain. Par6 has a PB1 and a PDZ domain. The domain structure of the members of the Par complex is conserved in the human orthologues listed in Table 1.

Nevertheless, the interaction between these polarity proteins revealed to be dynamic. In fact, both in mammals and flies, during polarity establishment, Lgl forms a complex with Par6 and aPKC (117, 118). After Lgl phosphorylation by aPKC and its consequent dissociation from the Par6:aPKC dimer (117, 119), Baz/Par3 interacts with Par6 and aPKC to form the Par complex. In turn, Baz/Par3 phosphorylation by aPKC causes dissociation of Baz/Par3 from the Par complex, and self-association via its oligomerization domain. This oligomeric form of Par3 accumulates at the junctions, in a process which is necessary for the establishment of cell polarity and for maturation of the junctions (120-124). Also, the phosphorylation of *Drosophila* Crumbs by aPKC has been shown to be required for Crumbs function and apical localization (107).

1.3.3 The Scribble complex

The Scribble complex is a trimeric complex composed by Scribble, Lgl (Lethal giant larvae), and Dlg1 (Discs large 1), localized at the basolateral membrane at the level of septate junctions in *Drosophila*, and at adherens junctions in human (125, 126). The *Scribble* gene was originally identified in *Drosophila*, in a study designed to find genes involved in epithelial morphogenesis. The domain structure of Scribble, Dlg1, and Lgl is depicted in Fig. 12.

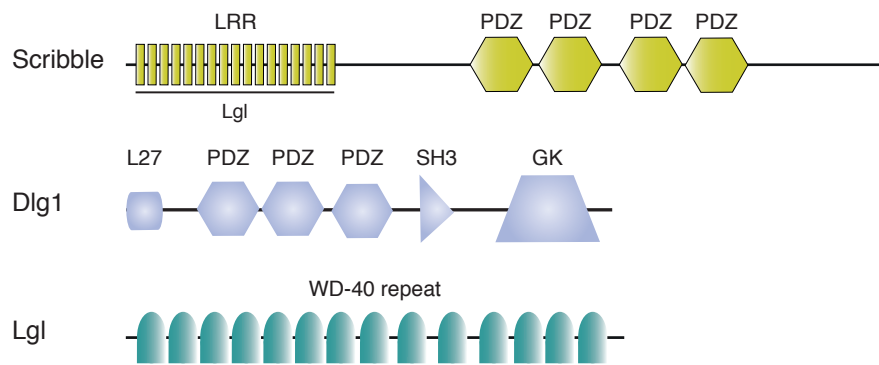


Fig. 12. The Scribble complex. The Scribble complex is composed by Scribble, Dlg1, and Lgl. Scribble contains 16 lysine-rich repeats (LRR) involved in binding to Lgl, and four PDZ domains. Dlg1 is a member of the MAGUK family and consists of an N-terminal L27 domain, three PDZ domain, a SH3, and a GK domains. Lgl has 14 WD-40 repeats, a motif of about 40 amino acids, often ending with the Trp-Asp (WD) dipeptide. The two human orthologues Lgl1 and Lgl2 showing a different number of WD-40 repeats.

In *Drosophila*, mutations of the Scribble complex proteins cause aberrant establishment of polarity during gastrulation, with defects in the formation of the ZA and expansion of apical markers to the basolateral region of the cell (125). This observation suggests a role of the Scribble complex in segregating the apical markers by antagonizing their spreading into the basolateral membrane. Lateral targeting of Scribble depends on the Scribble leucine-rich repeats (LLRs) and on the presence of E-cadherin, since RNAi of E-cadherin in Caco-2 cells impairs the association of Scribble at the basolateral membrane (127). During polarity establishment, Lgl competes with Par3 to form a complex with Par6 and aPKC. Evidence in both humans and flies indicate that after Lgl phosphorylation by aPKC, Lgl is excluded from the Par6:aPKC dimer and from the apical cell side, resulting in its localization at the basolateral membrane and association with Scribble and Dlg1 (117-119). However, the molecular mechanisms of assembly of the Scribble complex and the physical interaction between members in the different epithelial systems still needs to be clarified. To date, Scribble has been shown to co-immunoprecipitate with Lgl2 in MDCK and HEK293T cells (128), while Dlg1 and Scribble seems to associate in *Drosophila* neuronal synapses via a

protein named GUK holder (129). Among the five Dlg proteins identified in human, Dlg1 is the most related to the *Drosophila* one. In intestinal epithelial cells of mammals, Dlg1 is required for proper formation of adherens junctions (130). The L27 domain at the N-terminus of Dlg1 has been shown to interact with Veli and Cask (Calmodulin-associated Ser/Thr kinase) (110), an association required for the Dlg1 basolateral distribution in MDCK cells (131). In renal mice epithelia, VeliC is responsible for the repulsion of Dlg1 from apical regions, and for the basolateral localization (110).

The localization of the three polarity complexes in fly and human epithelial cells are shown in Fig. 13.

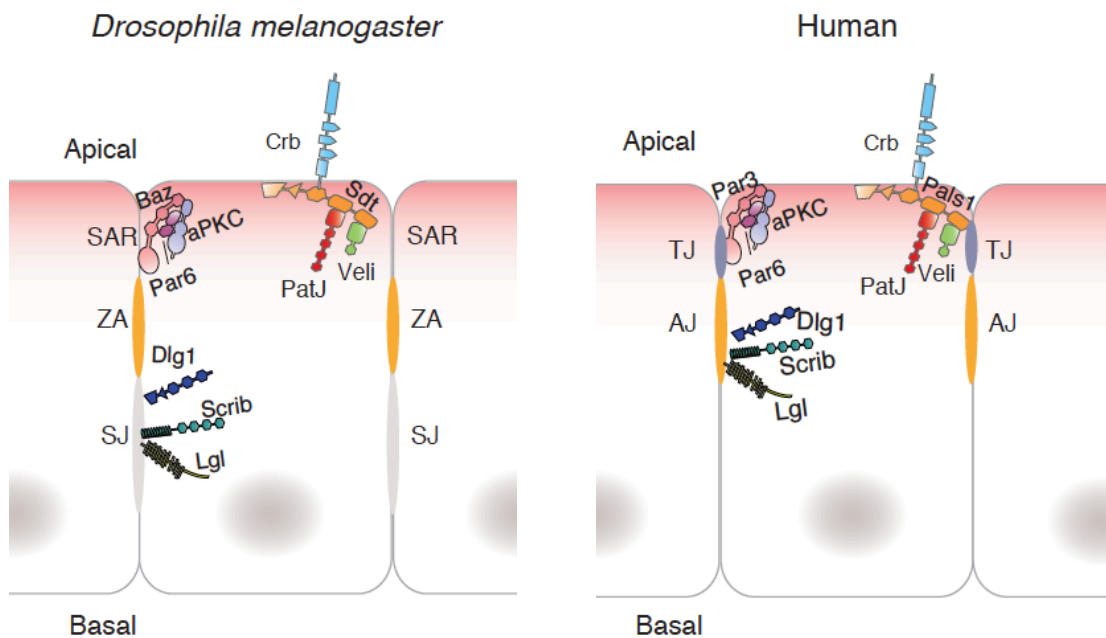


Fig. 13. Polarity complexes in *Drosophila* and human epithelial cells. Three complexes define polarity in epithelial cells. The Crumbs complex is composed by the transmembrane protein Crumbs and the cytoplasmic Stardust/Pals1, PatJ, and Veli, localized at tight junctions (TJ) in human and at the subapical region (SAR) in *Drosophila*. Bazooka/Par3:Par6:aPKC form the Par complex and share a similar localization with the Crumbs complex. The basolateral domain of the membrane is marked by the Scribble complex, whose components are Scribble, Dlg1, and Lgl, positioned at septate junctions (SJ) in flies and at adherens junction (AJ) in human.

Recently, a novel group of proteins with the ability to maintain apico-basal polarity has been found. This group comprises Yurt, Coracle, Neuroxin IV, and the Na⁺K⁺ATPase, localized

at septate junctions in *Drosophila*, and to which has been referred as the “Yurt/Coracle group” (132). Fly embryos lacking the Yurt/Coracle group components do not show defects during gastrulation. During organogenesis, starting by stage 11, the apical membrane is instead expanded, with Crumbs invading the basolateral membrane. However, during terminal differentiation, *i.e.* by stage 14, apical and basolateral markers are correctly positioned, suggesting that the Yurt/Coracle group has a temporary role in maintaining apico-basal polarity limited to the organogenesis step (132). The molecular basis of spatiotemporal polarity control by the Yurt/Coracle group is not completely understood. Yurt encodes a protein containing a FERM and a FA (FERM-associated) domains. During organogenesis, Yurt is recruited at the apical membrane, where it directly binds the FERM binding domain of Crumbs. This interaction antagonizes Crumbs activity and restrains its localization at the apical region of the membrane (133). Oligomerization of Yurt by both the FA and the FERM domains appears to be necessary for apical localization and binding to Crumbs (134). Phosphorylation of the FA domain of Yurt by aPKC prevents premature apicalization of Yurt, thus preserving the formation of a correct apico-basal axis. In turn, during organogenesis Yurt binds aPKC and inhibits its phosphorylating activity (135), suggesting a reciprocal antagonistic regulation between Yurt and aPKC. Yurt is not necessary for the integrity of the structure of septate junctions, but it is crucial for their barrier function (132), while both *coracle* and *neuroxin IV* mutants show defects in the structure of the septate junctions (136). The binding interface between Coracle and Neuruxin has been defined, and it resides on the N-terminal domain of Coracle and the intracellular region of Neurexin. These two proteins are mutually necessary for their proper localization at SJ (137).

The function of Yurt as a basolateral polarity protein seems to be conserved in mammalian, since the knockdown of the EHM2 orthologue in MDCK cells causes enlargement of the apical region and loss of lateral markers such as E-cadherin and Scribble (132). Moreover,

the interaction between the FERM binding domain of Crumbs1 and the FERM domain of the Yurt orthologue YMO1 is maintained in human HEK293T cells (132).

1.3.4 Loss of polarity proteins: consequences

As described, the Crumbs, Par, and Scribble complexes are crucial to define apical and basolateral membrane domains in epithelial cells. They are also necessary for the maintenance of the epithelial structures, *i.e.* the junctions, since loss of polarity proteins causes defects in the junction maturation, as detailed in the paragraphs above.

Thus, it is not surprising that loss of cell polarity results in tissue disorganization and contributes to epithelial-to-mesenchymal transition (EMT) (138, 139). Tumorigenic cells that lose polarity, acquire dynamic properties, disrupt the basal membrane, and invade other tissues, forming metastasis. This is the reason why loss of cell polarity and EMT has been defined as hallmark of cancer (139). During oncogenic processes, polarity proteins deregulation occurs in terms of altered expression and aberrant localization (98, 140). Since loss of Scribble, Dlg1, and Lgl leads to neoplastic growth in *Drosophila* discs, these proteins have been referred to as tumour suppressors (89). Further evidence confirmed that they can play a tumor suppressive role also in mammals. In fact, components of the Scribble complex are usually downregulated or mislocalized in human cancer cells, for instance Scribble in breast and liver cancer (141, 142), Dlg1 in adenocarcinoma (143), and Lgl in melanoma (144). Interestingly, viruses responsible for cancer onset take advantage of the requirement of polarity proteins for preserving normal cell architecture, and act by targeting polarity components. An example is the human papillomavirus E6 protein, which targets PatJ for proteosomal degradation, causing loss of polarity and disruption of TJ, thus enhancing the progression and malignancy of cervical cancer (145).

1.3.5 Polarity proteins and membrane proteins

In polarized epithelia, maintenance of distinct separated domains of the plasma membrane is fundamental not only for tissue integrity, but also for processes such as asymmetric cell division, cell migration, and actin cytoskeleton organization (146, 147). In recent years, a number of evidence highlighted a cross-talk between polarity proteins and membrane proteins, mostly receptors. Shelli *et al.* showed that in MDCK cells Veli is involved in localization and stabilization of HER2 receptor through two different regions. The N-terminus of Veli is involved in the ER-to-Golgi translocation to basolateral membrane, while Veli-PDZ stabilizes the HER2 receptor at the basolateral membrane by binding the C-terminus of the receptors, thus inhibiting HER2 endocytosis for lysosomal degradation (148). The association of Veli to *C. elegans* EGFR-like Let23 receptor not only blocks degradation after endocytosis, but also enhances the basolateral sorting of the receptor after transcytosis from the apical membrane (149). VeliB-PDZ has been implicated in basolateral retention of the potassium channel Kir 2.3, determining an increase in its conductance activity (150). Also, human Dlg1 participates in controlling NMDA receptor membrane delivery (151). *Drosophila* Crumbs anchors in the extracellular region Notch at the apical membrane preventing ligand-independent endocytosis, which would lead to the activation of the Notch pathway (152).

Despite a well-documented role for TNFRs in regulating the fate of polarity-deficient cells, the molecular mechanism linking polarity and TNFRs still remains elusive.

1.4 Identification of the *Drosophila* TNFR Grindelwald

Recently, the group of Pierre Léopold at Institute of Biology Valrose (iBV) performed a genome-wide tumour-suppressor screen in *Drosophila*, aimed at finding new genes involved in regulating cancer progression. In details, the RNAi of the syntaxin *avalanche* was performed in wing discs to look for genes reverting aberrant phenotypes, including 2-days

delay in puparation, ectopic Wg expression, and tumour overgrowth. A library of 10100 RNAi lines was tested to find genes necessary for the generation of these defects. Among them, only 8 genes were able to rescue all the mentioned phenotypes, with 6 genes belonging to the JNK cascade (*traf2*, *bendeless*, *tab2*, *tak1*, *hemipterous*, *basket*), and two predicted ORFs. Of them, one is not yet annotated, the other was classified with the name CG10176 ORF. Sequence analysis revealed that the CG10176 gene codes for a 241-residues protein, containing a single cysteine-rich domain with 8 cysteines in the predicted extracellular region. Interestingly, CRDs are hallmark of TNFRs, and the predicted one shares significant homology with the known vertebrate counterparts. For this reason, the CG10176 gene was named “Grindelwald” (Grnd), from a small village at the foot of the Eiger mountain, where “Eiger” is also the name of the unique fly TNF (153).

1.4.1 Grnd activation by Eiger leads to apoptosis or malignancy

Based on previous literature, in flies the TNF Eiger is able to induce JNK signalling, leading to apoptosis or malignancy in a cell context-dependent manner (49, 53, 90). As a consequence of an increased rate of apoptosis, overexpression of Eiger in the *Drosophila* eye caused the “small eye” phenotype (41, 42, 153). Grnd knockdown partially rescued the small-eyes, giving rise to a “hanging-eye” phenotype, likely because a diffusible form of Eiger is able to act at a distance and induce the activation of the JNK pathway. Overexpression of the only extracellular domain of Grnd (Grnd-extra), lacking the intracellular region, traps Eiger and prevents its diffusion and binding to endogenous full-length Grnd, thus blocking the activation of the JNK pathway and apoptosis. As a consequence, the small-eye phenotype caused by Eiger overexpression is completely reverted by the concomitant expression of Grnd-extra (153). On the contrary, Wengen knockout does not rescue the Eiger-induced small-eye phenotype. Moreover, neither the “hanging-eye” phenotype caused by the Grnd RNAi in Eiger-overexpression background is further rescued in Wengen knockout flies, suggesting that Wengen is not required for the

Eiger-induced small-eye in this system (153). These results are in contrast with previous findings, that indicated Wengen as involved in the generation of the small-eye phenotype caused by Eiger-overexpression (55). Additional experiments will be necessary to solve this apparent discrepancy. As expected, the RNAi of Traf2, or the overexpression of Puckered, completely reverted the aberrant phenotype (153). Taken together, these results indicate that the newly identified Grnd is a *Drosophila* TNFR, mediating the Eiger-induced apoptosis. Interestingly, TNFs are “double-edged sword” with the ability to switch from a pro-apoptotic to a pro-tumorigenic behaviour depending on cell conditions (1, 51). Data from the Xu lab showed that eye-antennal discs depleted of the polarity protein Scribble (*scrib*), but overexpressing a mutated active form of Ras (*ras*^{V12}), exhibit a tumour-like behaviour, resulting in overgrowth and metastasis (53, 90). Leopold group showed that metastatic cells carrying the *scrib*^{-/-}; *UAS-ras*^{V12} genotype in eye-brain complexes display MMP1 expression, which is driven by JNK activation, and invade the ventral nerve cord (153). As a consequence of *grnd* down-regulation, tumour invasion is suppressed, and MMP1 is not expressed. The same outcome is achieved knocking-out Eiger, suggesting that Eiger-mediated Grnd activation of the JNK pathway determines malignancy in polarity-deficient cells expressing the hyperactive form Ras^{V12} (153). Overgrowth of these tumours is partially dependent on Yorkie, which is activated by the JNK signalling and induces cell-cycle progression (154). These results are in line with previous findings: in 2006 Igaki *et al.* showed that the metastatic behaviour of *scrib*^{-/-}; *ras*^{V12} clones is not suppressed by Wengen RNAi in cephalic complexes, and it is only partially reverted in ventral nerve cord (53), suggesting the existence of an additional mediator of cells’ malignant behaviour driven by the JNK pathway, not yet identified at that time. Nevertheless, data from the Milan group argued against the relevance of Eiger and Grnd in driving JNK activation and consequent neoplastic growth in *dlg1-RNAi*; *ras*^{V12} tumours (155), in contrast with Léopold lab observations. Whether these discrepancies in JNK activation and malignancy are caused by the different polarity defects remains to be tested.

1.4.2 Grnd activation independently by Eiger

Léopold group also showed that the activation of the JNK pathway caused by the *rn>avl*-RNAi relied on Grnd, but is Eiger-independent (153). It is well known that polarity-deficient cells activate the JNK signalling and undergo apoptosis or malignancy (refer to paragraphs 1.2.1 and 1.2.2). A possible link between loss of polarity and JNK activation was hypothesised by looking at the positioning of Grnd, which co-localizes with the components of the Crumbs polarity complex Veli and Crumbs (153). However, the molecular connection between Grnd, JNK activation, and loss of polarity is poorly understood.

1.5 Aim of the project

Over the past years, major efforts have been spent to identify cellular pathways and external stimuli contributing to tumour development. Despite the number of discoveries on the working principles of oncogenic machineries, the molecular mechanisms underlying tumour initiation and progression are far from being understood.

In order to gain better understandings of the mechanisms driving cancer onset, the group of Pierre Léopold recently identified Grnd as a novel TNFR by exploiting a genome-wide screening in *Drosophila* (refer to paragraph 1.4) (153). *Drosophila melanogaster* serves as a model organism to study neoplastic growth, because it offers two main advantages: the availability of sophisticated genetics and molecular tools, and the conservation across evolution of pathways controlling cell survival and invasiveness (156, 157).

The ability of Grnd to balance apoptosis and malignancy prompted us to investigate the molecular mechanisms underlying this dual activity. We thus started a collaboration with Pierre Léopold's lab.

In this context, the aim of my PhD project was to characterize Grnd structurally and functionally in order to understand how it is activated by Eiger, and consequently linked to apoptosis or over-proliferation in flies. Furthermore, I also aimed at highlighting the connection between Grnd, JNK pathway activation, and loss of polarity, and to look for Grnd orthologue receptors in vertebrates likely involved in cancer onset.

2 RESULTS

2.1 Grnd is a novel *Drosophila* TNFR

2.1.1 Grnd interacts with Traf2 and activates the JNK pathway

As the other TNFRs, Grnd was predicted as a type I transmembrane protein, with an extracellular N-terminus consisting of a signal peptide followed by a single cysteine-rich domain (henceforth CRD) sharing homology to the known vertebrate counterparts (Fig. 14A), a transmembrane helix and an intracellular part, that is predicted to be largely unstructured (Fig. 14B, top). Further analysis of the Grnd sequence highlighted the presence of a Pro-Ala-Glu-Asn-Ser amino acids motif at position 200-204, which conforms to the Pro-X-Glu-XX motif used by some human TNFRs to interact with Traf6 (39, 158). Thus, I tested the ability of Grnd to interact directly with Traf2, the *Drosophila* orthologue of Traf6, via a GST pull-down assay. Traf2 is classified as an E3 ubiquitin ligase, and contains an N-terminal RING domain, a central coiled coil and a C-terminal MATH domain responsible of the recognition of TNFRs' intracellular domain (Fig. 14B, bottom) (159). On these bases, for the pull-down assay, I designed a Grnd fragment encompassing the entire intracellular region (residues 125-241, ICD hereon), which I fused to a GST-moiety, and I purified the MATH domain of Traf2, encompassing amino acids 319-475. I immobilized the GST-Grnd¹²⁵⁻²⁴¹ on glutathione-sepharose (GSH) beads at 2 μ M concentration, and then I incubated the beads with 10 μ M of Traf2-MATH in solution. To test if the Pro200-X-Glu202-XX motif of Grnd was responsible for the association with Traf2, in the GST pull-down I included Grnd-ICD constructs carrying double and single mutations on the PXEXX motif. After washes, species retained on beads were separated by SDS-PAGE and visualized by Coomassie staining, which revealed that Grnd and Traf2 interact directly, and that the single mutation Glu202Ala on Grnd is sufficient to completely impair the binding (Fig.

14C). To determine if the JNK pathway was activated in *avalanche*-KD condition, Julien Colombani from Pierre Léopold's group used a technique widely used to monitor the JNK cascade in *Drosophila*, which exploits the *puckered* enhancer trap (*puc-lacZ*) in specific discs (42, 85). With this method, he showed that the JNK pathway is upregulated in *rn>avl*-RNAi discs, but the signalling is silenced upon KD of both *grnd* and *tak1*, confirming that Grnd is required to activate the JNK pathway in *Drosophila* (Fig 14D).

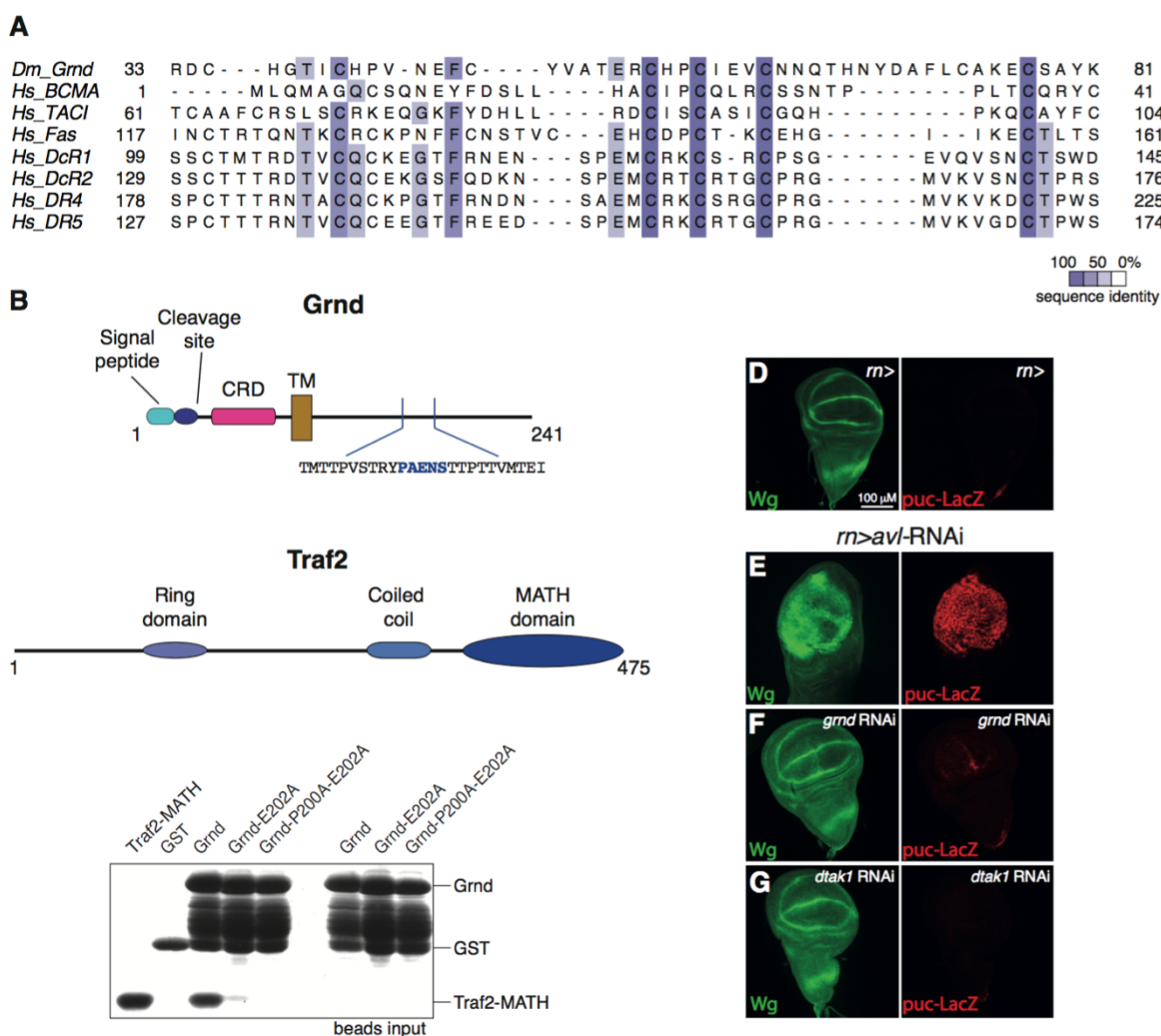


Fig. 14. Grnd interacts with Traf2 and activates the JNK pathway. **A.** Sequence alignment of the CRD of Grnd with the human TNFRs Fas (ID: P25445), DcR1 (ID: 014798), DcR2 (ID: Q9UBN6), DR3 (ID: Q93038), and DR4 (ID: O00220). Residues are coloured based on conservation. **B.** Domain structure of Grnd (above) and Traf2 (below). Grnd contains an extracellular region with an N-terminal signal peptide and a predicted cleavage site at residue 27, an extracellular CRD, followed by a transmembrane domain and an intracellular region predicted to be mostly unstructured. The sequence containing the PXEXX motif is indicated. The domain structure of Traf2 consists of an N-terminal RING domain, a coiled-coil region and a MATH domain. **C.** GST pull-down between Grnd-ICD and Traf2-MATH shows that Grnd-ICD interacts with Traf2-MATH, and that the binding is abolished by the point mutants E202A and P200A-E202A. Proteins retained on

beads were separated by SDS-PAGE and stained with Coomassie blue. Grnd-ICD undergoes degradation, as indicated by the presence of bands below the Grnd-ICD band. **D-E-F-G.** *Drosophila* wing discs stained for Wg (left) and puc-LacZ (right) under WT conditions and in RNAi-avalanche discs. JNK hyper-activation shown in E is reverted by silencing the expression of both Grnd and Tak1 (F-G).

2.2 Biochemical characterization of Eiger-ECD and Grnd-ECD

The molecular events leading to the activation of human TNFRs by their cognate TNF ligands have been elucidated by structural and functional studies (1, 2). TNFRs with a single CRD usually bind TNFs in a one-to-one stoichiometry, for instance TALL:BCMA, whereas the ones with multiple CRDs generally associate with two TNF molecules, including CD40:CD40L. To shed light on the mechanism of Grnd activation by Eiger, I set out to characterize their interaction with a biochemical and structural approach.

2.2.1 Grnd-ECD and Eiger-ECD interact with nanomolar affinity, independently of Grnd glycosylation

The presence of cysteines predicted to form disulphide bridges within the extracellular domains of Grnd required to express the domain under conditions suitable for disulphide bonds. In particular, I used SHuffle B cells (NEB), a specific *E. coli* strain carrying mutations in the *gor* and *trxI* reductases genes that allow an oxidative intracellular environment, and expressing a cytoplasmic variant of the disulphide bond isomerase *DsbC*, which ensures the generation of native-folded proteins (160). Bacterial expression of Grnd-ECD in Shuffle cells was successful, and yielded a good amount of pure protein.

Grnd is also predicted to be N-glycosylated at Asn63, which conforms to the Asn-X-Thr/Ser motif (Fig. 15A, top). Thus, to study the effect of Grnd glycosylation on its interaction with Eiger, I decided to express Grnd-ECD also in High Five insect cells, which allow eukaryotic posttranslational modifications, using the baculovirus system. Specifically, we engineered the baculovirus vector pFastBac1 inserting the mellitin secretion signal upstream of the

multiple-cloning-site (MCS). This way, we were able to express a glycosylated Grnd-ECD, secreted to the extracellular oxidative environment. Thus, I produced unmodified Grnd-ECD encompassing residues 30-97 wild-type or carrying the Asn63Ala mutation expressed in SHuffle cells, and purified glycosylated Grnd-ECD in High Five insect cells. To produce samples of the putative Grnd ligand Eiger, I adopted a strategy similar to the one described for Grnd-ECD. Briefly, I designed an Eiger construct on the short isoform of Eiger, spanning amino acids 146-409 (referred to as Eiger-ECD in the following), which is the Eiger portion found in the *Drosophila* hemolymph after cleavage by TACE proteinase (Fig. 15A, bottom) (44, 45). Eiger-ECD was expressed in SHuffle cells as a strep-tagged construct, and used to test binding to Grnd-ECD in pull-down experiments. 2 μ M of Eiger-ECD absorbed on streptactin beads were incubated with 10 μ M of unmodified Grnd-ECD-WT, Asn63Ala or glycosylated Grnd-ECD. After washes, species retained on beads were separated by SDS-PAGE (Fig. 15B). The experiment revealed that Eiger-ECD interacts directly with Grnd-ECD WT, Asn63Ala and glycosylated, indicating the interaction is not affected by Grnd glycosylation. To quantify the binding affinity more accurately and check whether the binding was modulated by Grnd glycosylation, I decided to measure the strength of the binding between Eiger-ECD and Grnd-ECD by Isothermal Titration Calorimetry (ITC). ITC is a technique that allows the determination of the thermodynamic parameters of a binding reaction, including the dissociation constant (K_d), the enthalpy and entropy, and the binding stoichiometry. Specifically, this method is based on the measurement of the heat exchange occurring during the formation of a complex, when receptor molecules are titrated to saturation into a cell containing the ligand. The heat released or absorbed upon binding is then plotted as a function of time and fitted with a non-linear model, generating for a binary interaction a sigmoidal curve from which thermodynamic parameters can be extrapolated. I started characterizing the interaction between Eiger-ECD and unmodified Grnd-ECD. The ITC experiment was performed by subsequent injections of 2 μ l of Grnd-ECD at 100 μ M concentration into a cell containing 8 μ M Eiger-ECD. The experiment yielded a K_d of 30.7

± 5.8 nM. I then repeated the titration using Grnd-ECD glycosylated or the Grnd-Ans63Ala mutant, and discovered that they both displayed a similar affinity for Eiger-ECD as the wild-type unmodified protein, with a K_d of 25.2 ± 6.2 nM for glycosylated Grnd-ECD, and 38.8 ± 5.6 nM for Grnd-ECD-Asn63Ala (Fig. 15C). I concluded that Grnd glycosylation is not necessary nor affects the binding to Eiger. This result is not in line with the hypothesis proposed by Bilder's lab, which claimed that the glycosylation of Grnd could affect the affinity for Eiger (161), as reasoned in details in the Discussion (chapter 3).

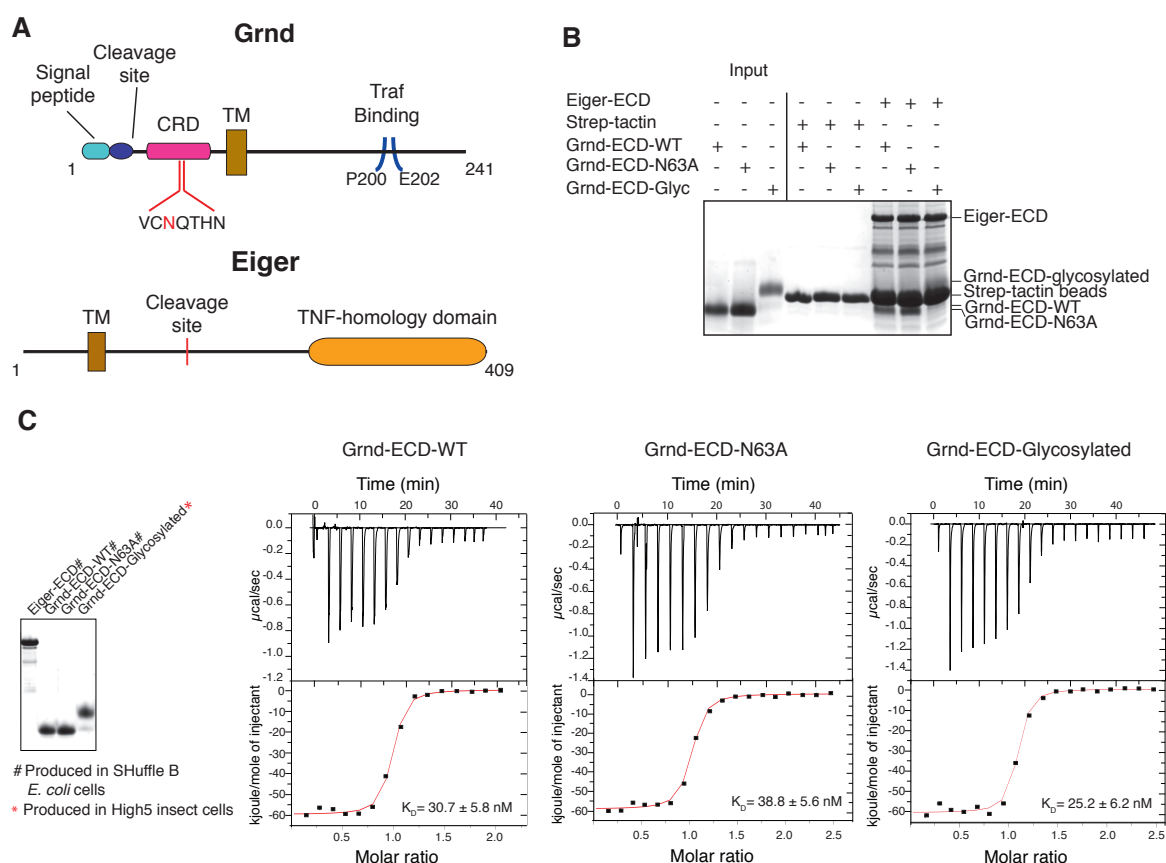


Fig. 15. Eiger-ECD and Grnd-ECD interact with nanomolar affinity, independently of Grnd glycosylation. **A.** Top: Domain structure of Grnd highlighting the presence of a consensus sequence for glycosylation at Asn63. Bottom: Domain structure of Eiger, comprising an intracellular N-terminal region, a transmembrane domain and a C-terminal TNF-homology domain. The cleavage site recognized by TACE is indicated as a red bar. **B.** Pull-down assay between Eiger-ECD on Strep-tactin beads and Grnd-ECD wild-type, Asn63Ala or glycosylated. The assay shows that all Grnd-ECD constructs bind similarly to the receptor. **C.** Left: Samples of Eiger-ECD and Grnd-ECD wild-type, Asn63Ala and glycosylated used for the ITC experiment. Right: ITC isotherms in which 100 μ M of Grnd-ECD were injected in a cell containing 8 μ M of Eiger-ECD. The measured K_d are 30.7 ± 5.8 nM for Grnd-ECD-WT, 25.2 ± 6.2 nM for glycosylated Grnd-ECD, 38.8 nM for Grnd-ECD-Asn63Ala. K_d is reported as mean $\pm$ error of fitting of the ITC data with the isotherm (red line).

2.2.2 Trimeric Eiger forms hetero-hexamers with Grnd-ECD

To check the homogeneity of the Eiger-ECD: Grnd-ECD samples, I conducted analytical size-exclusion chromatography (SEC) experiments by loading the pre-assembled complex on a Superdex 200 5/150 column. The sample elutes as a single peak slightly earlier than the 158 kDa globular molecular weight marker (Fig. 16A), indicating that Eiger-ECD and Grnd-ECD likely associated as oligomers. Grnd-ECD in isolation elutes around the 17 kDa molecular weight marker, in line with its molecular weight of about 8 kDa, indicating that it is likely a monomer in solution. Conversely, Eiger-ECD elutes in fractions compatibles with a trimeric assembly.

TNFs:TNFRs usually associate in 3:3 complexes, with TNFs natively produced as trimers (12, 19, 162-164). Interestingly, some TNFRs, for instance Fas and TNFRI, have been found pre-assembled in oligomers thanks to the presence of an N-terminal region, named pre-ligand assembly domain (PLAD) (23, 25, 26). To determine the oligomerization state of Eiger-ECD and Grnd-ECD in isolation and in complex, we used a size-exclusion column coupled to static light scattering instrument (SLS). SLS is an optical technique that measures absolute molecules' molecular weight by recording the intensity of light scattered by the sample in solution. Larger proteins scatter proportionally more light than smaller ones, according to the Zimm equation. SLS analysis confirmed that Grnd-ECD is a monodispersed monomer in solution, with a measured molecular weight of 8.2 kDa (Fig. 16C), while Eiger-ECD is trimeric, with a 91 kDa mass (Fig. 16B). SLS analysis of the pre-assembled Grnd-ECD:Eiger-ECD complex revealed that the two proteins form a hetero-hexamer with a measured MW of about 114,8 kDa (Fig. 16D). Based on the SEC and SLS data of the proteins in isolation and in complex, we conclude that trimeric Eiger-ECD associates with three monomeric Grnd-ECD.

In addition, the biochemical analyses described indicated that the Grnd and Eiger constructs produced in bacteria are folded and soluble, and interact with nanomolar affinity with a 3:3

stoichiometry. I therefore decided to use these samples for crystallization experiments of Grnd-ECD in isolation and in complex with Eiger-ECD.

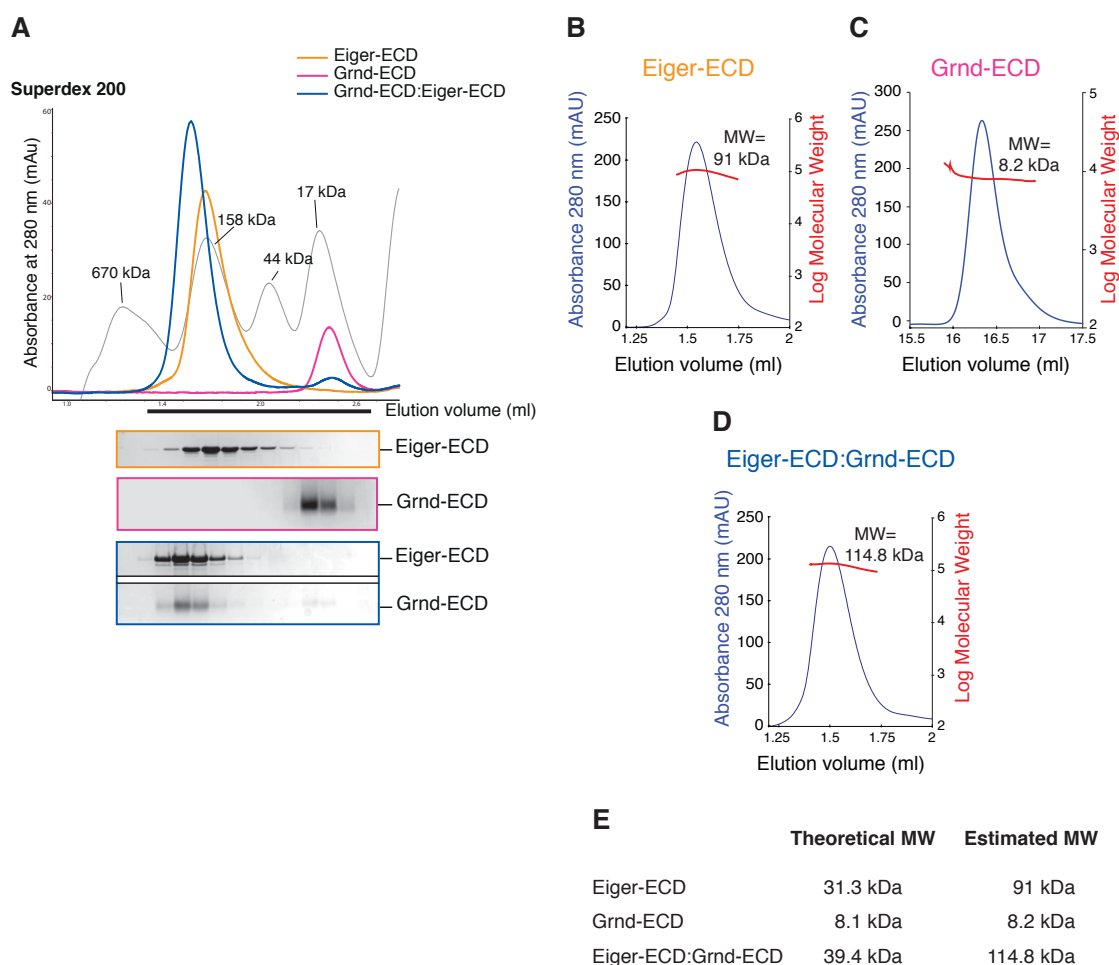


Fig. 16. Trimeric Eiger-ECD binds monomeric Grnd-ECD with a 3:3 stoichiometry. **A.** SEC elution profile of the complex formed between Eiger-ECD and Grnd-ECD mixed at 30 μ M concentration, with Coomassie-stained SDS-PAGE of the peak fractions corresponding to the horizontal black bar. The elution profile of globular markers is reported as a dashed grey line. Individual runs of Eiger-ECD and Grnd-ECD are shown for comparison. **B-C-D.** Static-Light-Scattering profile of Eiger-ECD (B), Grnd-ECD (C), and the Eiger-ECD:Grnd-ECD complex (D). The UV absorbance trace is shown in blue (left y axis) and the measured MW in red (right Y axis). The analyses show an average molecular mass of 91 kDa, 8.2 kDa, and 114,8 kDa along the peaks, respectively, which are consistent with a trimer for Eiger-ECD, with a monomer for Grnd-ECD, and with a hetero-hexamer for the TNF:TNFR complex. **E.** Table comparing the theoretical molecular weight of Eiger-ECD, of Grnd-ECD, and of the Eiger-ECD:Grnd-ECD complex, and the molecular weights measured by SLS.

2.3 Crystal structure of Grnd-ECD

I first tried to crystallize bacterially produced Grnd-ECD spanning residues 30-97. Screenings of crystallization conditions were performed combining several strategies, *e.g.* hanging and sitting drop methods, different temperatures and protein concentrations, various ratios of reservoir to protein volume. However, all attempts were unsuccessful. We then reasoned that the construct could contain a disorganized region possibly impairing crystallization. Thus, to trim experimentally the domain boundaries, I decided to exploit limited proteolysis, a method based on the use of limited amounts of proteases cutting at exposed regions of a protein such as loops and flexible portions, this way producing stable sub-domains. I treated Grnd-ECD with trypsin at 1:50 = weight:weight ratio for 16 hours at 4 °C (Fig. 17A), and obtained a homogenous sample, that I further purified by SEC (Fig.17B).

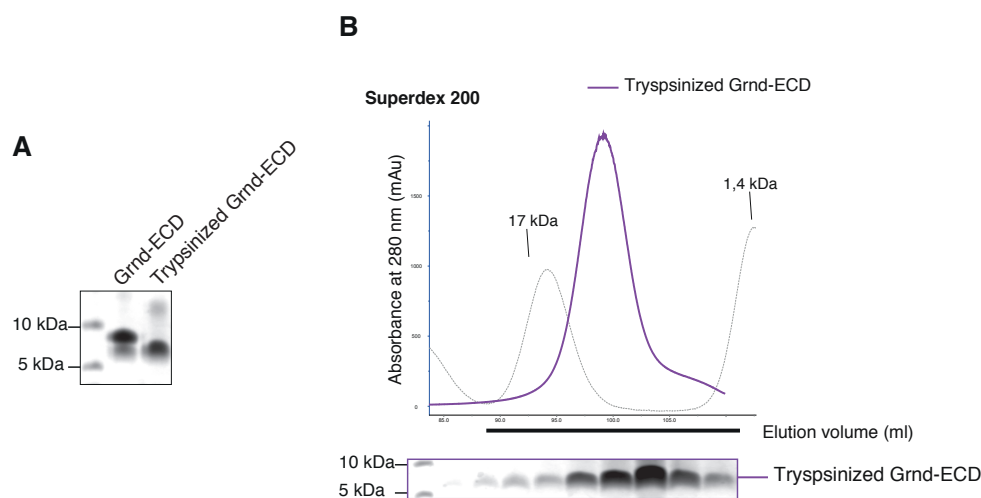


Fig. 17. Trypsinization of Grnd-ECD. **A.** Coomassie blue-stained SDS-PAGE showing Grnd-ECD before and after trypsinization. **B.** Grnd elutes as a homogenous sample. Top: SEC elution profile of trypsinized Grnd-ECD run on a Superdex 200 16/60 at 2 mM concentration. Molecular weight markers are indicated as a dashed grey line. Bottom: SDS-PAGE of the fraction indicated by the horizontal black bar, visualized by Coomassie blue.

2.3.1 Crystallization of Grnd-ECD

I conducted initial crystallization experiments with the trypsinized Grnd-ECD by using commercial sparse-matrix screens, as detailed in Mat. & Meth. section. Bipyramidal crystals

grew in about 30% of conditions, most of them containing PEG and MPD (Fig. 18A-C). Manual optimization was not necessary, since initial crystals were already single with dimensions suitable for diffraction studies.

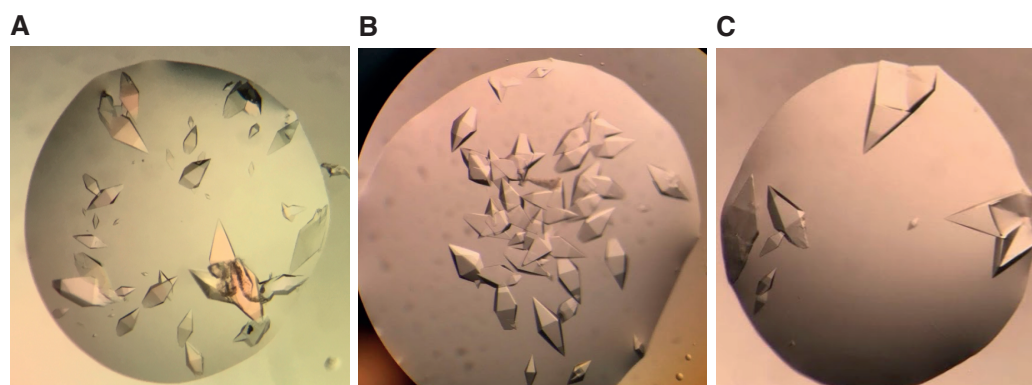


Fig. 18. Crystals of Grnd-ECD. **A-C.** Crystals of Grnd-ECD grown by sitting-drop vapour diffusion after trypsinization. Bipyramidal crystals appeared after 2 days of crystallization mixing the protein sample with 0.1 M Hepes pH 7.5, 45 % MPD, and 0.2 M ammonium acetate (A), 0.1 M Bis-Tris pH 5.5, 45 % MPD, and 0.2 M calcium chloride dehydrate (B), and 0.1 M Na Hepes pH 7.5, 28 % PEG 400, and 0.2 M CaCl_2 (C).

Crystals grown in 0.1 M Hepes pH 7.5, 45 % MPD and 0.2 M ammonium acetate were fished and flash-cooled in liquid N₂ without additional cryo-protection. X-ray diffraction data were collected at the Swiss Light Source (SLS). Crystals diffracted to 0.92 Å resolution (Fig. 19A), and belonged to the P4₁2₁2 space group, and yielded a high-quality electron density map that could be interpreted automatically (Fig. 19B).

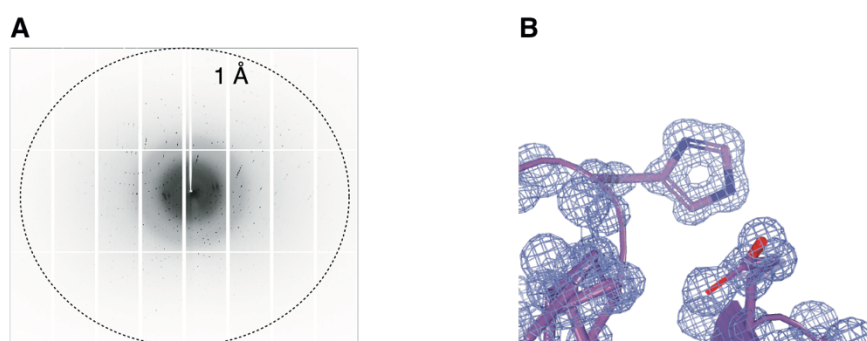


Fig. 19. Diffraction of Grnd-ECD crystals. **A.** 0.92 Å resolution X-ray diffraction pattern of Grnd-ECD obtained using a synchrotron-radiation source on beamline PXIII at Swiss Light Source. **B.** Electron-density map displaying His36 at 0.92 Å resolution contoured at 1.8 sigma level.

Experimental phases were determined with a S-SAD experiment conducted on the same crystals at a wavelength of 2.075 Å. Refinement was performed with Phenix (165) with a final R_{free} of 15.6 % and R_{work} of 13 %. The final model contains residues 34-81 of Grnd. Data collection and refinements statistics are given in Table 2.

Table 2. Data collection and refinement statistics for Grnd-ECD.

	Grnd-ECD	
	Native	S-SAD
Data collection	PXIII-SLS	PXIII-SLS
Space group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Wavelength	0.9256 Å	2.075 Å
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	31.48, 31.48, 97.76	31.45, 31.45, 97.75
α , β , γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	29.967-0.926 (0.942-0.926)	48.88-1.92 (5.21-1.92)
R_{sym} or R_{merge}	0.064 (0.412)	0.073 (0.040)
CC _{1/2}	0.999 (0.849)	1.000 (0.998)
I/ σ I	21.5 (2.4)	163.4 (11.8)
Completeness (%)	98.2 (83.8)	96.4 (43.2)
Unique reflections	33869 (1382)	4015 (89)
Multiplicity	10.6 (3)	221.8 (5.7)
Refinement		
Resolution (Å)	29.967-0.926	
$R_{\text{work}}/R_{\text{free}}$	0.130 / 0.156	
No. atoms		
Protein	412	
Water	83	
B-factor protein/water	11.25/33.41	
R.m.s. deviations		
Bond lengths (Å)	0.017	
Bond angles (°)	2.46	
Ramachandran values		
Favoured (%)	100	
Allowed (%)	0	
Outliers (%)	0	

Values in parenthesis are for highest-resolution shell

2.3.2 Architecture of Grnd-ECD.

The structure of trypsinized Grnd-ECD consists of an N-terminal β -hairpin followed by two C-terminal antiparallel α -helices, with the 8 cysteines engaged in four disulphide bonds (Fig. 20A). TNFRs CRDs are generally organized in two regions, named “modules”,

distinguished by characteristic tertiary fold and consensus sequence (13). The N-terminus of Grnd spans residues 35-56 and is defined as a X2 module, described before in the TNFR BAFFR (12). X2 modules are composed by two antiparallel β -strands connected by a β -turn, forming a β -hairpin stabilized by two intercalated disulphide bonds. In Grnd the disulphides are between Cys35 and Cys47, and between Cys40 and Cys54. The C-terminal portion of trypsinized Grnd-ECD is consistent with a C2 helix-loop-helix module, present also in the CRD of the human Fn14 (Fig. 20D) and in the forth CRD of TNFRI (16, 18). The C2 module consists of 21 residues with four cysteines organized in a nested pattern of disulphide bridges, according to a consensus sequence that for Grnd-ECD is Cys57 – X₃ – Cys61 – X₆ – X₅ – Cys73 – X₃ – Cys77 (where X is any given amino acids and the subscript n indicates the number or residues). In Grnd the C2 helical module comprises two helices extending from Cys57 to Cys61 and from Cys73 to Cys77, respectively (Fig. 20B). The overall topology of Grnd resembles the fold of the human single-CRD receptors Fn14 and BCMA, and the second CRD of TACI, all consisting of a β -hairpin and a helix-loop-helix motif (Fig. 20C-E). These receptors interact with their ligands via both modules of the CRDs, with major contacts from the β -hairpin. Indeed, BCMA and TACI share a common Asp-X-Leu motif located on the hairpin tip, then replaced by a Ser-X-Asp-Leu sequence in Fn14, which contact the human TNF partners. The binding between the TNFR and their cognate ligands is further strengthened by contribution of amino acids residing on the helix-loop-helix domain (12, 14, 17, 19, 166). Notably, the Asp-X-Leu motif is not conserved in Grnd, therefore I expected that Grnd interacts with Eiger via a novel and non-canonical stretch of residues.

2.4 Crystal structure of the Eiger-ECD:Grnd-ECD complex

2.4.1 *In vitro* reconstitution of the complex

Initial crystallization attempts with the purified Eiger-ECD:Grnd-ECD sample resulted in multiple tiny crystals, unsuitable for diffraction analysis (Fig. 21A). Strategies to obtain single and larger crystals, including slowing down nucleation by decreasing the temperature, or changing the seeding time or the protein concentration while screening reservoirs, were all unsuccessful. Intriguingly, I observed that crystals took 15 to 45 days to grow, thus I reasoned that maybe proteins were undergoing a natural degradation before packing in well-ordered three-dimensional arrays. To check if the tiny crystals of Eiger-ECD:Grnd-ECD contained an intact complex, I fished some crystals and dissolved them in water before running the sample on a SDS-PAGE. Coomassie-staining revealed that Eiger-ECD degraded from a 31 kDa construct to about 15 kDa, whereas Grnd-ECD was mostly undegraded, but was also giving rise to a second band running at slightly lower molecular weight (Fig. 21B, left). Since Eiger-ECD was purified with an N-terminal His-tag, to determine if the degradation was at the N- or C-terminus, I performed a Western blot using an anti-His antibody. The antibody did not recognize the Eiger band at 15 kDa (Fig. 21B, right), suggesting that Eiger-ECD was degraded at the N-terminus. This result is consistent with the presence of a well folded TNF domain at the C-terminus of the construct. We initially thought to purify shorter Eiger constructs, trimming Eiger-ECD at the N-terminus. However, such constructs turned out to be poorly soluble in bacteria. Therefore, I tried to express them in High Five insect cells, and obtained only small soluble amounts of the proteins, which were not suitable for crystallization purposes. Thus, I also explored the possibility of producing recombinant trimmed Eiger-ECD constructs in mammalian HEK293T cells using a pHLSEC vector (167), that contains a signal peptide for secretion. However, also this strategy turned out to be unsuccessful. For this reason, I decided to use a limited proteolysis approach again. I treated the Eiger-ECD:Grnd-ECD sample with trypsin in a 1:20=w:w ratio for 2 hours at 4 °C. The proteolysed sample was analysed by SDS-PAGE, which revealed

the presence of species running at the same molecular weight of the ones observed in the dissolved crystals (see panel 21B). I then investigated the stoichiometry of the trypsinized sample by analytical SEC, which revealed that the proteolysed complex still elutes at high MW, indicating that the 3:3 stoichiometry of the assembly is preserved (Fig. 21C). To obtain information about the sequence of the trypsinized 15 kDa band of Eiger, I combined N-terminal sequencing analysis with intact molecular weight determination by mass spectrometry. N-terminal sequencing identifies the sequence of amino acids starting from the N-terminal end by Edman degradation, whereas molecular weight determination provides the exact molecular weight of a protein construct. Combining information of the starting amino acid of a construct with its precise molecular weight allows the identification of the exact boundaries of the construct. To apply this strategy, I treated the Eiger-ECD:Grnd-ECD sample with trypsin, and purified the cleaved sample on a SEC column pre-equilibrated with a buffer without glycerol, which is not compatible with the intact molecular weight study by proteomic. Peak fractions were collected and subjected to proteomic analysis and N-terminal sequencing, which revealed that the proteolysed Eiger fragment spanned residues 267-409, comprising the whole TNF domain (Fig. 21C and 21D, top). Similar proteomic analyses showed that Grnd-ECD remained mostly uncleaved, with only a minor portion degraded to a fragment comprising residues 30-81 (Fig. 21C and 21D, bottom).

Taken together this evidence indicated that Eiger₂₆₇₋₄₀₉ engages with Grnd₃₀₋₈₁ in stable hetero-hexamers. I therefore decided to use this sample for crystallization experiments.

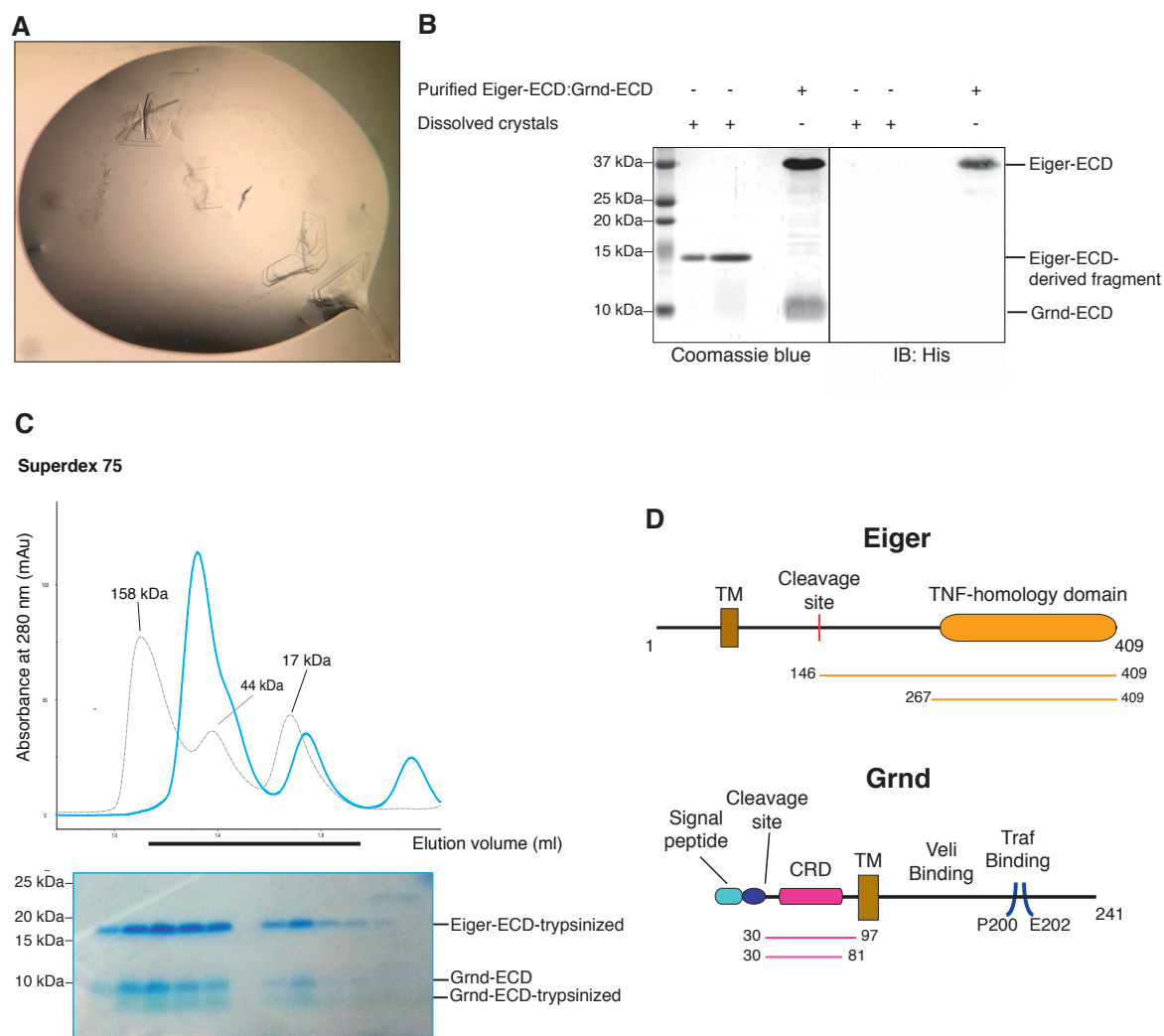


Fig. 21. Trimming of the minimal Eiger and Grnd extracellular domains forming hetero-hexameric complexes **A.** Initial multiple crystals of Eiger-ECD:Grnd-ECD grown manually by hanging-drop vapour diffusion in 0.1 M Tris-HCl pH 8.5, 8 % PEG 8000. **B.** Analysis of Eiger-ECD degradation occurring during crystallization by anti-His immunoblot on dissolved crystals. Left: Coomassie blue staining of the purified Eiger-ECD:Grnd-ECD complex used for initial crystallization screening and of crystals dissolved in water. Eiger-ECD runs below the 37 kDa MW marker, whereas Grnd-ECD runs as an 8 kDa protein. In crystals, Eiger-ECD degrades to a 15 kDa band. Right: Anti-His immunoblot on the same sample shown in the left panel. The His-tag is detected only on the intact Eiger-ECD molecule, but not on the degraded band, suggesting that the 15 kDa fragment may be an N-terminal degradation. **C.** Analytical SEC of the trypsinized Eiger-ECD:Grnd-ECD at 75 μ M concentration showing that the complex still forms hetero-hexamers. Peak fractions were separated on an SDS-PAGE and Coomassie-stained. **D.** Domain structure of Eiger (top) and Grnd (bottom) with a scheme of the original constructs and of the fragments identified by N-terminal sequencing and intact molecular weight determination.

2.4.2 Crystallization of the Eiger-ECD:Grnd-ECD complex

I first scaled-up the limited proteolysis approach used to identify a stable Eiger:Grnd complex to prep a sample suitable for crystallization experiments. Sample concentration is a crucial variable during protein crystallization. For this reason, I decided to define which concentration was the best one to perform crystallization experiments with the proteolysed sample using the Pre-Crystallization Test kit (PCT, Hampton Research). This test is performed with precipitating agents containing high amounts of salt or polymers, which should drive the protein supersaturation within the drop. Upon mixing these precipitants with the sample to be crystallized, proteins too concentrated will form a heavy amorphous precipitate, whereas samples too diluted will give rise to clear drops. Performing the pre-crystallization test with proteolysed Eiger-ECD:Grnd-ECD, I observed that small crystals grew after a few hours in a precipitant containing 0.1 M Tris-HCl pH 8.5, 0.2 M MgCl₂, and 15 % w/v PEG 4000. To obtain larger single crystals, I decided to try to reproduce manually the hit but performing grid screens, changing the PEG 4000 concentration from 13 % to 20 %, the pH values from 8 to 9, and the protein concentration from 15 to 22 mg/ml. I also tested several seeding protocols varying the seeding time between 4 hours and 16 hours, both at 20 °C and 4 °C (Fig. 22).

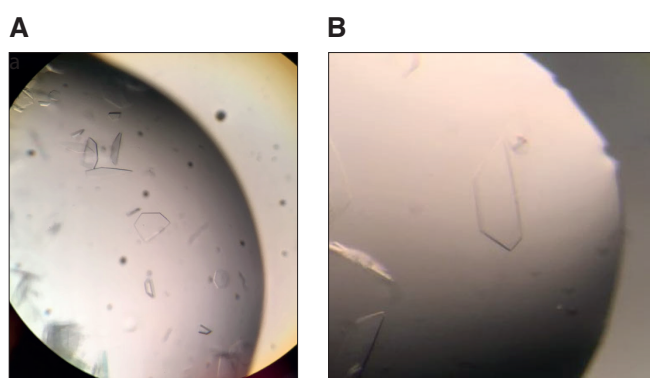


Fig. 22. Crystals of proteolysed Eiger-ECD:Grnd-ECD. **A.** Crystals of Eiger-ECD:Grnd-ECD after proteolysis grown in hanging drops mixing the protein sample at a concentration of 22 mg/mL with a reservoir containing 0.1 M Tris-HCl pH 8.5, 14 % PEG 4000, and 0.2 M MgCl₂. **B.** Screening of initial crystallization conditions yielded single crystals appearing after few hours of crystallization

mixing the protein sample at a concentration of 15 mg/mL with a reservoir consisting of 0.1 M Tris-HCl pH 8.5, 18 % PEG 4000, and 0.2 M MgCl₂.

Crystals of trypsinized Eiger-ECD:Grnd-ECD were used for X-ray data collection at the European Synchrotron Radiation Facility (ESRF). Crystals diffracted to a resolution of 2.02 Å and belonged to the space group P321, indicating a trigonal symmetry with a three-fold rotation axis. The Matthews coefficient of 2.24 was compatible with a single copy of Eiger-ECD and Grnd-ECD in the asymmetric unit. Since no structure of homologous complexes was available, to solve the structure of the Eiger:Grnd complex, we performed molecular replacement in *Phaser* (168), using a model of the Eiger TNF domain threaded with Phyre2 (169) on the TNF domain of EDA-A2 (PDB ID: 1RJ8), from which the unconserved loops were removed. After placing one copy of Eiger in the asymmetric unit, we added by molecular replacement one molecule of Grnd using as a search model the coordinates of the Grnd determined before (see chapter 2.3). The Eiger:Grnd structure was refined in *Phenix* to a final R_{free} of 21.4 % and R_{work} of 19.2 %. The final model includes residues 269-409 of Eiger-ECD and amino acids 31-81 of Grnd-ECD. Data collection and refinement statistics are reported in Table 3.

Table 3. Data collection and refinement statistics of the Eiger-ECD:Grnd-ECD complex.

Eiger-ECD:Grnd-ECD	
Data collection	ID23-2-ESRF
Space group	P321
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.7, 78.7, 57.66
α , β , γ (°)	90.0, 90.0, 120.0
Resolution (Å)	57.66-2.02 (2.02-2.05)
R _{sym} or R _{merge}	0.207 (0.888)
CC _{1/2}	0.997 (0.569)
I/ σ I	8.6 (2.2)
Completeness (%)	99.9 (98.5)
Unique reflections	13860 (663)
Multiplicity	11.7 (10.1)
Refinement	
Resolution (Å)	44.0-2.02
R _{work} /R _{free}	0.192 / 0.214
No. atoms	
Protein	1554
Water	118
B-factor protein/water	37.70/42.99
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.91
Ramachandran values	
Favoured (%)	95.74
Allowed (%)	4.26
Outliers (%)	0

Values in parenthesis are for highest-resolution shell

The biologically relevant hetero-hexamer can be visualized by applying the three-fold crystallographic symmetry to the binary Eiger:Grnd model that we built and refined. The architecture of the 3:3 complex consists of a central compact Eiger trimer bound to Grnd receptors positioned distally (Fig. 23A), with an overall topology resembling the one of other TNFs in complex with TNFRs with single CRDs, for instance the TALL:BCMA complex (Fig. 23B).

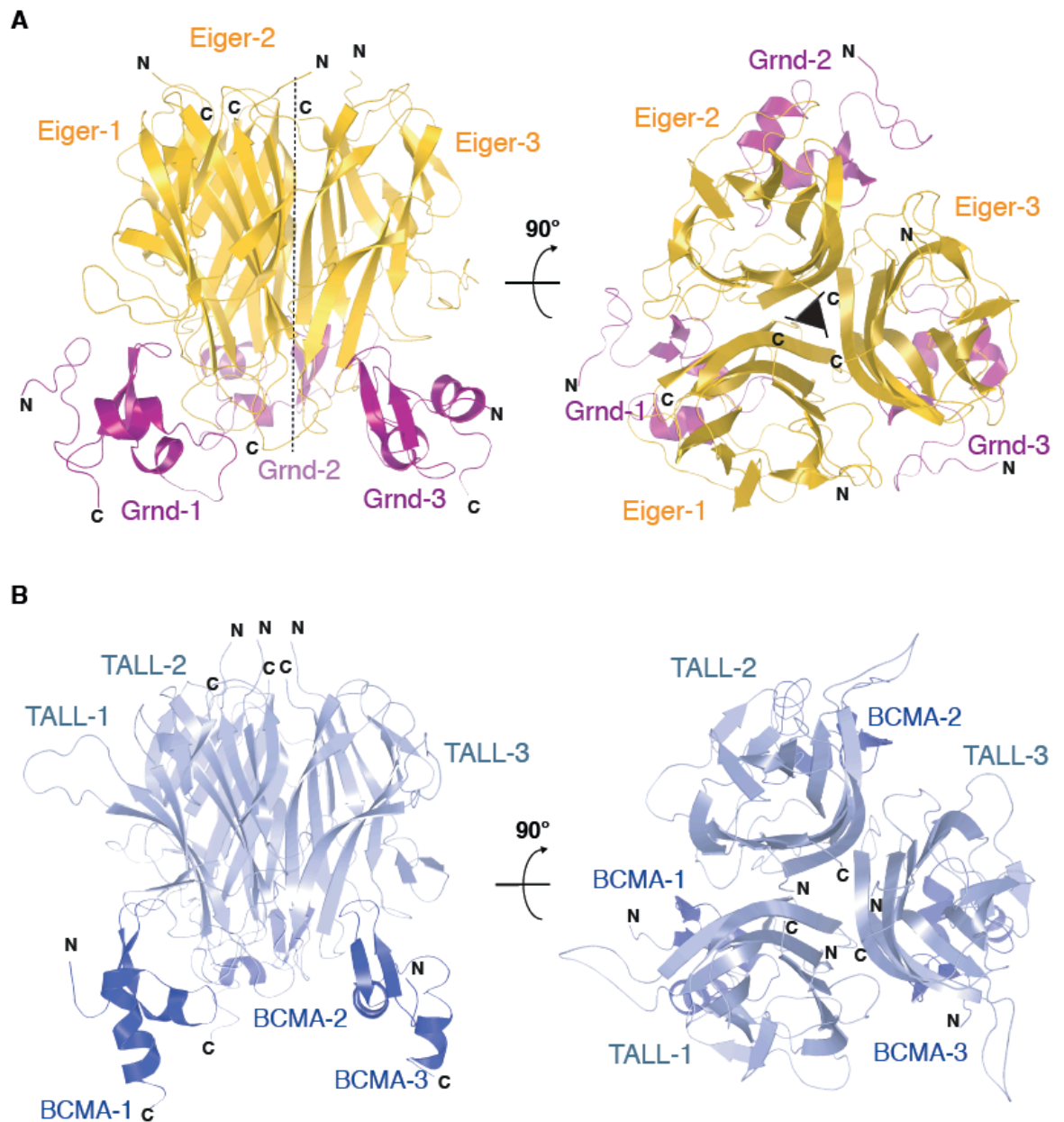


Fig. 23. Packing of the Eiger:Grnd complex. **A.** Cartoon representation of the Eiger:Grnd complex at the indicated orientations. Eiger molecules are shown in gold, whereas Grnd monomers are depicted in magenta. On the right, the three-fold axis symbol is indicated **B.** TALL:BCMA heterohexamers are visualized by light blue and blue cartoons, respectively. The overall fold resembles the one of the Eiger:Grnd complex.

2.4.3 Architecture of the Eiger-ECD:Grnd-ECD complex

The structure of the Eiger:Grnd complex shows that the TNF domain of Eiger adopts a typical “jelly-roll” fold (Fig. 24A), with an inner and an outer β -sheets composed by antiparallel β -strands, named A’AHCF and B’BGDE, respectively (Fig. 24B). The inner β -sheet is involved in trimer formation across an extended hydrophobic interface, whereas the

outer one creates the exposed surface of the molecule. Each Grnd contacts two Eiger molecules (see below for more details), with a conformation that well superposes to the unbound receptor, indicating that Grnd does not undergo significant conformational reorganization upon binding to Eiger. Small differences can be appreciated only in Grnd loops not involved in binding to Eiger (Fig. 24C).

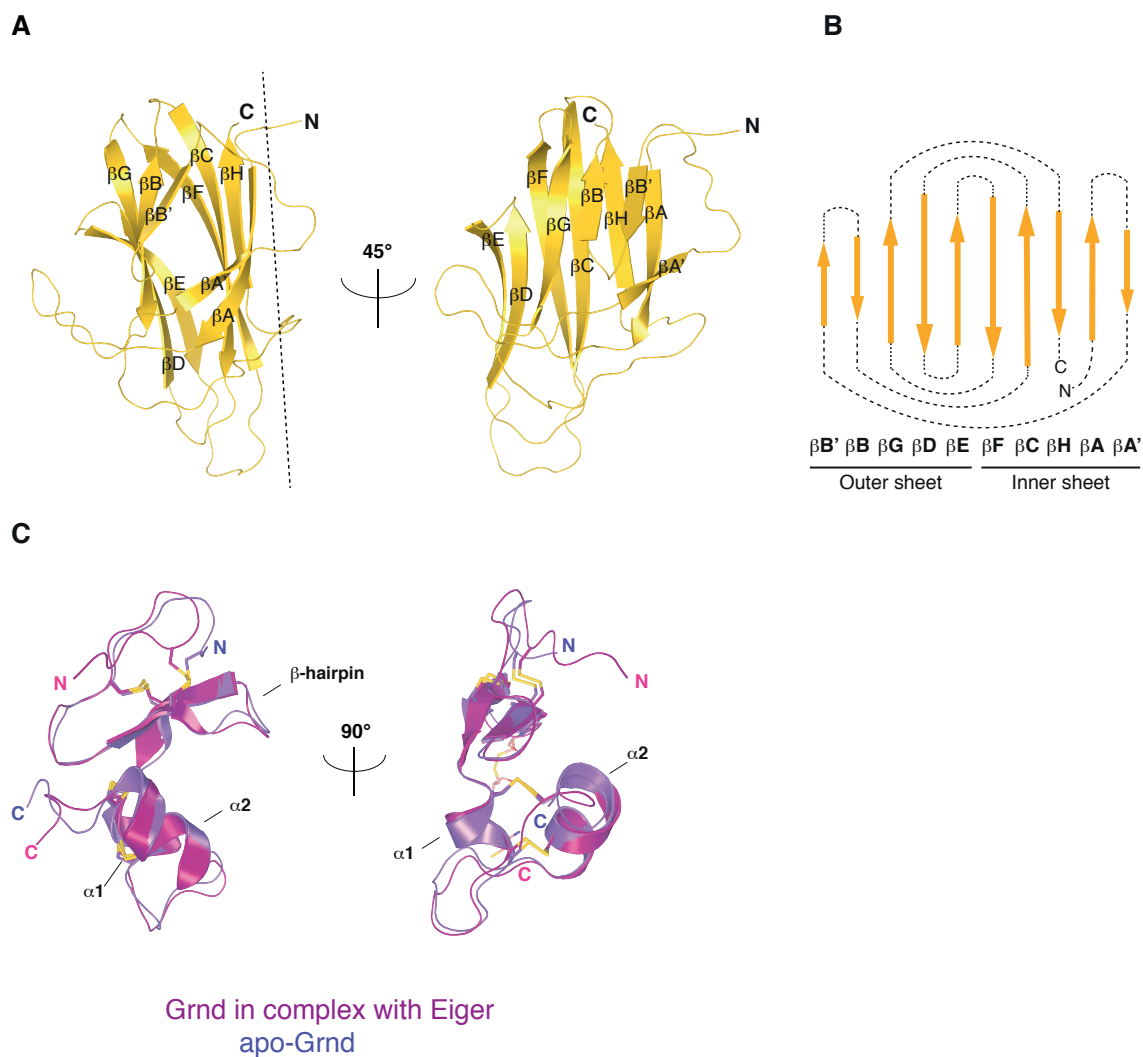


Fig. 24. Fold of Eiger-ECD and Grnd-ECD. **A.** Cartoon representation of Eiger TNF domain. Schematic organization of β -strands forming the jelly-roll fold, in which each strand is indicated by a letter. **B.** Scheme of the jelly-roll fold of Eiger TNF domain. Connections between β -strands and belonging to the inner or outer sheets are indicated. **C.** Structural superposition of Grnd in complex with Eiger (magenta) and in isolation (purple). Not major arrangements occur in the Grnd structure upon hetero-hexamers formation.

The overall Eiger:Grnd assembly is reminiscent of the fold of other human TNF:TNFR complexes in which the receptor has a single CRD (12, 31). In particular, Grnd is arranged

in a close conformation, in which the N- and C-terminal modules are in close proximity, and both modules contact different loops of Eiger. A peculiar feature of the Eiger:Grnd assembly is that the hexamer formation is promoted by a “one-to-two” interaction, in which one monomer of Grnd contacts two different molecules of the trimeric ligand (Fig. 25A), while human TNFRs with single CRDs usually bind their ligand in a one-to-one conformation. Interestingly, this one-to-two mode of interaction is reminiscent of the way in which TNFRs with multiple CRDs recognize their TNFs (21, 22, 32). In the case of the Eiger:Grnd interaction, the binding surface is organized in three surface patches. The first one is centred on Glu52 on the β -hairpin tip of Grnd, which makes polar interactions with the positively-charged Arg369 and Arg401 of Eiger in the loop connecting strands β G and β H. On the same β -hairpin tip, Grnd-Thr51 maintains the correct orientation by hydrogen bonding with the main chain nitrogen of Grnd-Arg53. Notably, the Grnd-Thr51 main chain strengthens the interaction with Eiger by forming a second hydrogen bond with the side chain of Eiger-Arg332, located in the β C- β D loop (Fig. 25B). The second interacting patch comprises Grnd-Tyr48 and Grnd-Asp69, facing the loop β G- β H of an adjacent Eiger molecule and making hydrogen bonds with His388 and Arg391, respectively (Fig. 25C). Eiger-Asp377 on the nearby β C- β D loop of the second Eiger molecule is engaged in hydrogen bonds and polar interactions with Grnd-His66 and Grnd-Asn56, in a third interacting patch (Fig. 25D).

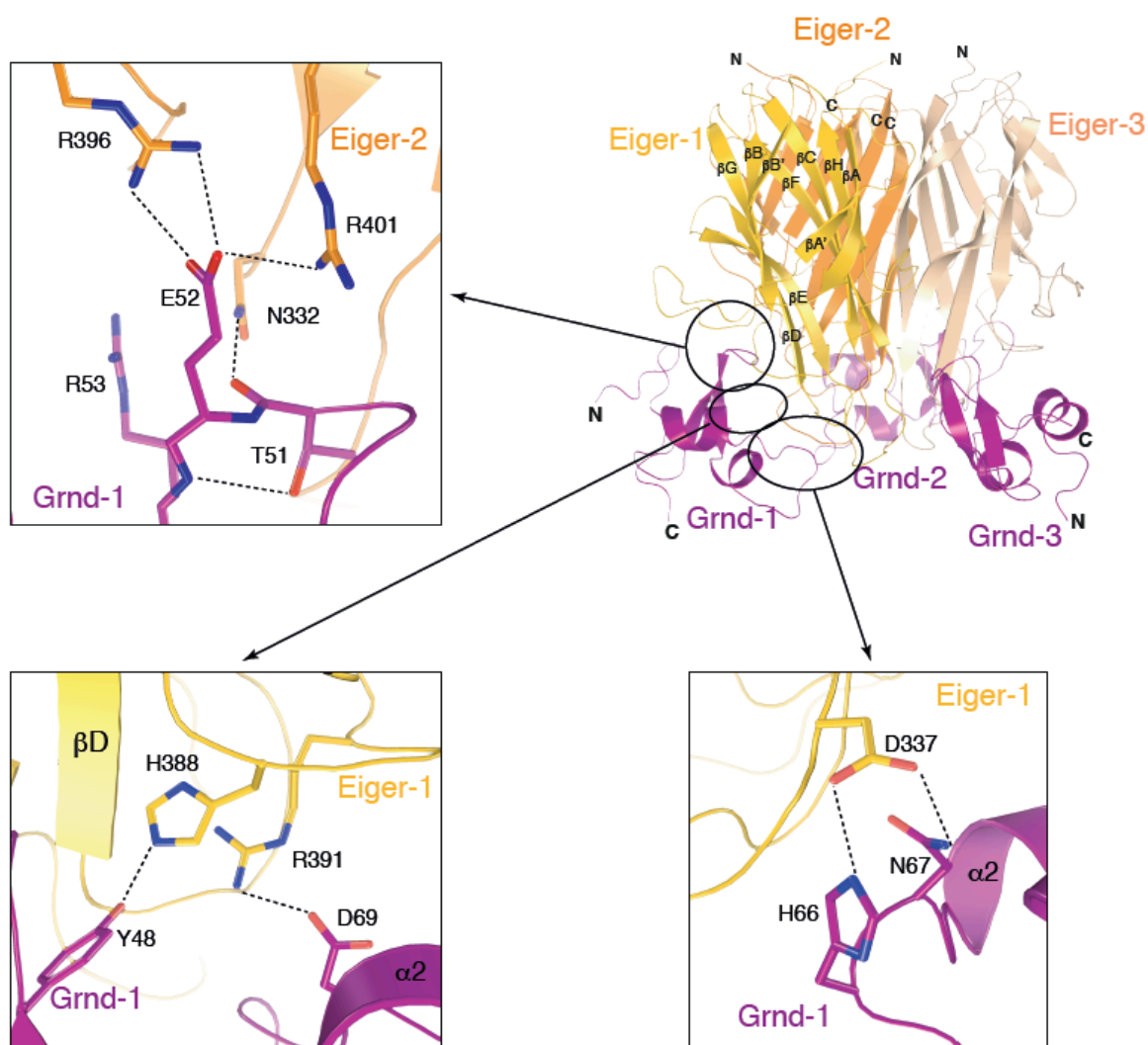


Fig. 25. Architecture of the Eiger:Grnd interface. **A.** Cartoon representation of the Eiger:Grnd complex, with the three Eiger molecules coloured in yellow and orange shades, and the Grnd molecules in magenta. **B-D.** Enlarged views of three patches of the interacting interface between Eiger-ECD and Grnd-ECD. **B:** Glu52 on the β -hairpin tip of Grnd points towards Arg396 and Arg401 on the β G- β H loop of one Eiger molecule, while Grnd-Thr51 secure the β -hairpin orientation by a hydrogen bonding to the backbone amide of Grnd-Arg53. The Grnd-Thr51 main chain further stabilizes the interaction via the formation of a hydrogen bond with Eiger-Asn332 side chain. **C:** Grnd-Tyr48 and Grnd-Asp69 contact Eiger-His388 and Eiger-Arg391 on the β G- β H loop of a second Eiger molecule. **D:** Grnd-His67 and Grnd-Asn67 on the loop between Grnd helices of the C2 module engage in polar interactions with Eiger-Asp337 on the loop β C- β D of the second molecule of Eiger.

2.4.4 Biochemical analysis of the Eiger:Grnd interface

The topology of the interaction suggests that the three patches work together to organize the Grnd:Eiger binding interface. To identify the key residues of the interaction, I designed point

mutants on both sides of the interface, and tested the residual binding in strep pull-down assays conducted with purified proteins. I first checked the effect of Grnd mutations (Fig. 26A). Double replacement of Grnd-His66-Asn67 with Ala and single substitution of Grnd-Thr51 completely abrogated the interaction between Grnd and Eiger, whereas single mutations Grnd-Glu52Ala, Grnd-His66Ala and Grnd-Asn67Ala impaired only partially the association. These results are consistent with the notion that the Grnd β -hairpin tip and the loop between helices plays a prominent role in the ligand-receptor recognition, as observed in the structure. Intriguingly, we observed that the Grnd sequence alignment in Fig. 14A shows the presence of a conserved phenylalanine at position 46. In the case of BCMA, the conserved Tyr13 is involved in forming hydrogen bonds with TALL-1-Tyr206 (12). Based on our structural data, Grnd-Phe46 is not exposed to the solvent, but buried within the hydrophobic core of the Grnd-ECD domain (Fig. 26B). Nevertheless, we decided to test whether mutation of Grnd-Phe46 could impair binding to Eiger in the same strep pull-down assay described above. The Grnd-Phe46Ala mutant was produced and purified as a soluble protein (not shown). Surprisingly, it was not able to bind Eiger in the pull-down assay (Fig. 26A, last lane). This result suggests that Grnd-Phe46 could be involved in maintaining the fold of Grnd, ensuring a compact conformation of the C2 and X2 modules, this way preserving the correct reciprocal orientation of the Grnd patches interacting with Eiger trimers. Interestingly, mutation of the conserved TACI-Phe78 causes loss of affinity of TACI for the TNF April, even if the Phe78 does not contact the surface of April (19), similarly to Grnd (see also the Discussion).

To confirm that the Grnd-ECD mutations abrogating binding with purified Eiger-ECD are sufficient to disrupt the interaction between full-length Grnd and Eiger in cells, we performed co-IP experiments using S2 cells lysate expressing HA-Eiger and Flag-tagged versions of Grnd. Immunoprecipitation of HA or Flag was performed, and bound species were visualized by immunoblot using anti-HA and anti-Flag antibodies. As shown in Fig. 26C, both His66Ala and Asn67Ala Grnd mutations completely impair association with

Eiger, to the same extent of the Phe46Ala Grnd mutation. Conversely, the Grnd-His66Ala mutant displays only a reduced affinity, as expected for a minor contact. Immunoprecipitation of V5 was used as negative control (Fig. 26C). To obtain quantitative information about the binding affinities of the mutant, I conducted ITC experiments analogous to the ones described in paragraph 2.2.1. ITC experiments revealed that the binding strength of the Grnd mutants Glu52Ala, His66Ala and Asn67Ala for Eiger decreases about 30 to 100-folds as compared to wild-type Grnd with K_d of $1.20 \pm 0.220 \mu\text{M}$, $3.20 \pm 0.009 \mu\text{M}$, and $4.34 \pm 0.007 \mu\text{M}$, respectively, whereas mutation Phe46Ala, Thr51Ala, and the double mutation His66Ala-Asn67Ala completely abrogate the binding (Fig. 26D). We next tested the effect of mutating Eiger residues at the interface with Grnd on the ligand-receptor binding affinity, in a strep pull-down analogous to the one described before. The assay was performed using Eiger-ECD carrying specific mutations on residues expected to contact Grnd-ECD, then maintaining Grnd-ECD WT in solution. The experiment revealed that substitution of Eiger-Asp337, or double substitutions of Eiger-His388-Arg391 or Eiger-Arg396-Arg401 impaired binding to Grnd-ECD (Fig. 26E). On the contrary, the Eiger-Asn332Ala mutant associates to Grnd-ECD to the same extent of the wild-type ligand, indicating that mutation of this side chain is not sufficient to disrupt receptor recognition. Importantly, Asn332 is one of the predicted glycosylation sites of Eiger. The result of the pull-down showed above does not rule out the possibility that glycosylation of this residue affects the binding affinity between Eiger and Grnd (see also paragraph 2.4.6 for more details).

Collectively, these biochemical analyses revealed that the key residues for the Eiger:Grnd interaction are Thr51, His66-Asn67 on the Grnd side, and Asp337, His388-Arg391, and Arg396-Arg401 on the Eiger side.

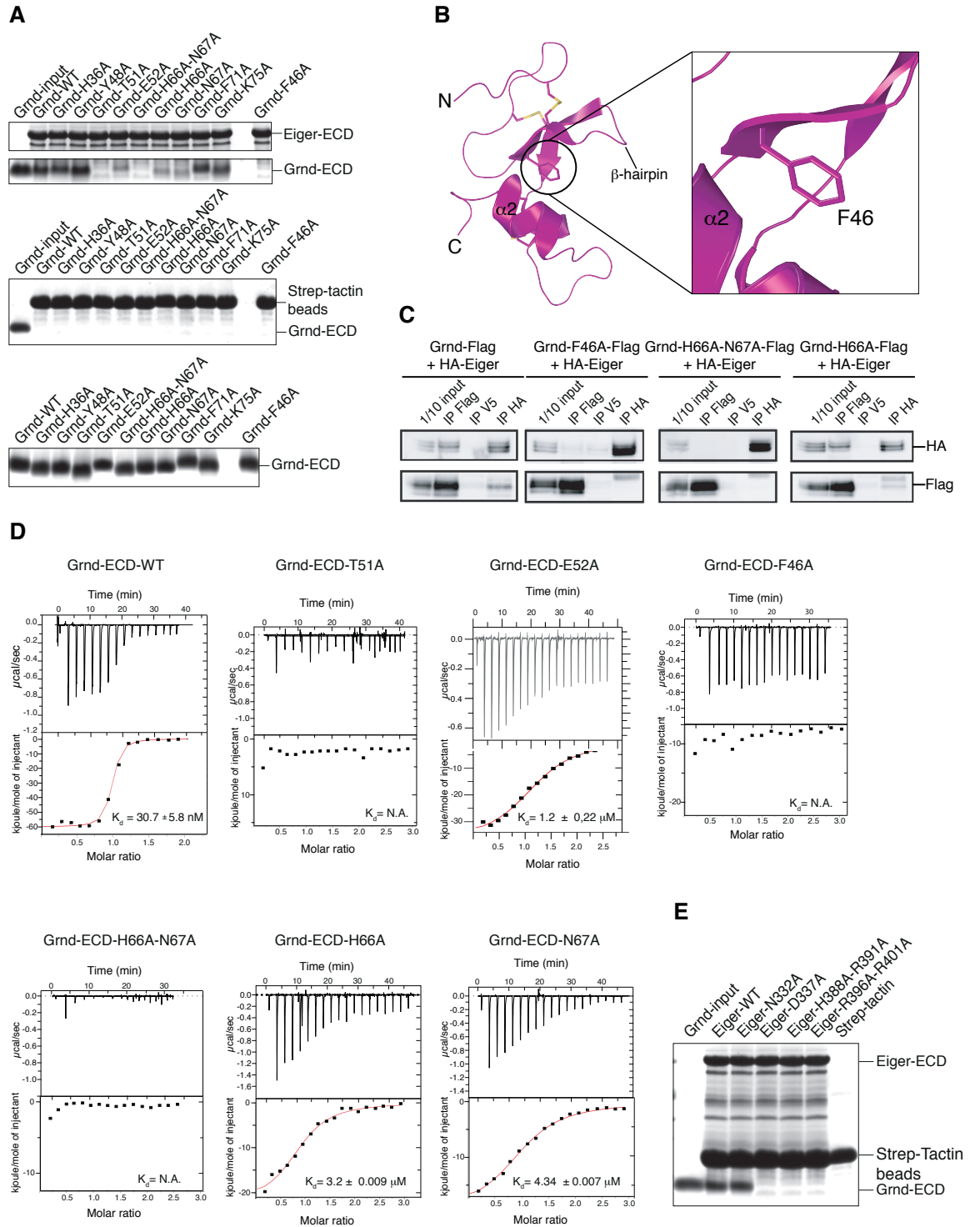


Fig. 26. Analysis of the Eiger:Grnd binding interface. **A.** Top: Strep pull-down assay using Eiger-ECD absorbed on strep-tactin beads and Grnd-ECD in solution, either wild-type or carrying the indicated alanine substitutions. Bottom: As negative controls, strep-tactin beads were incubated with the same Grnd-ECD species used in the pull-down described before. Grnd-ECD proteins used in the pull-down were separated on SDS-PAGE and Coomassie-stained. **B.** Cartoon representation of Grnd-ECD, with Phe46 depicted as sticks, showing that Phe46 is not exposed, but buried within the hydrophobic core of the domain. **C.** Co-IP experiments between Flag-Eiger and HA-Grnd, wild-type or carrying the Phe46Ala, the His66Ala-Asn67Ala, or the His66Ala mutations. Bound molecules are visualized by anti-HA and anti-Flag antibodies. Results of the assay confirmed the results obtained

in vitro with purified proteins. Immunoprecipitation of V5 was used as specificity control. **D.** ITC measurements of the binding affinity between Eiger-ECD and wild-type or mutated Grnd-ECD. The binding is completely impaired by Grnd mutation of Thr51Ala on the β -hairpin and His66Ala-Asn67Ala on the loop between helices. **E.** Strep pull-down analogous to the one described in (A), performed with Eiger-ECD mutants and Grnd-ECD-WT. The binding is disrupted by mutating Eiger amino acids Asp337, His388-Arg391, Arg396-Arg401.

The structure of the Eiger:Grnd complex also showed that Asn63 of Grnd, that can be glycosylated, does not reside on the binding interface with Eiger, but points outwards (Fig. 27). This observation is consistent with the results of our ITC analysis, which did not detect any difference in the binding affinity between Eiger and wild type or glycosylated Grnd (see Fig. 15).

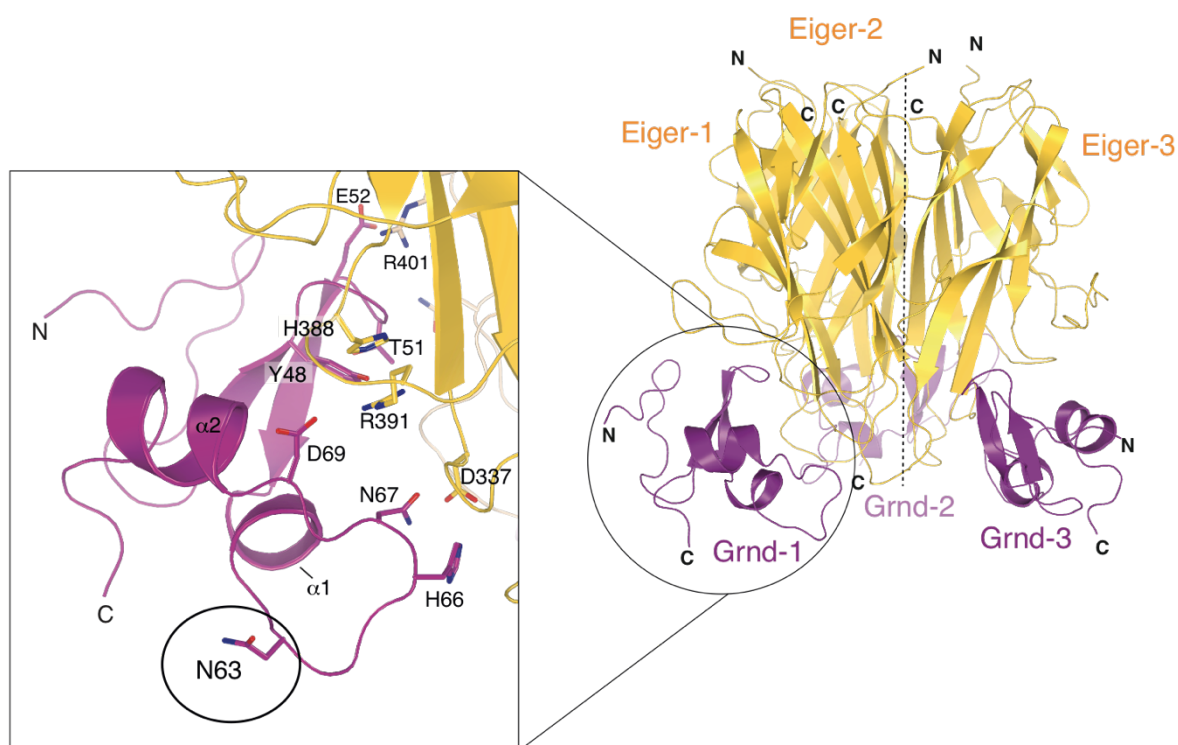


Fig. 27. Asn63 of Grnd does not contact Eiger. Close-up of the Grnd-Asn63 in the Eiger:Grnd complex. Asn63 is at the opposite site with respect to the Eiger:Grnd interface.

2.4.5 Grnd stabilizes Eiger trimers

Sequence analysis revealed that Eiger is predicted to be glycosylated on Asn332 and on Asn400. Looking at the localization of these two amino acids on the Eiger:Grnd structure

that we determined, I noticed that Asn400 is located at the interface between Eiger copies in the trimer (Fig. 28A). To test whether glycosylation of Eiger could affect its oligomeric state, I purified the TNF domain of Eiger (Eiger-TNF hereon) spanning residues 270-409, produced in High Five insect cells as a secreted protein, and studied the topology of Eiger-TNF through analytical gel-filtration. Eiger-TNF appears as a three-bands molecule on SDS-PAGE, which is compatible with the presence of a not glycosylated and two glycosylated forms. Consistently, upon PNGase-F treatment Eiger-TNF migrates as a single unmodified specie (Fig. 28B). Unexpectedly, Eiger-TNF elutes as a multiple glycosylated monomer from a SEC column. To test whether glycosylation could hamper trimer formation, I produced an Eiger TNF domain carrying the Asn400Ala mutation and analysed its oligomerization state by SEC. The elution profile of Eiger-TNF-Asn400Ala superposes with the elution profile of glycosylated wild-type Eiger-TNF domain and is consistent with a monomer (Fig. 28C, light blue trace), suggesting that the lacks of glycosylation is not sufficient to drive oligomerization of the TNF domain of the ligand. Unfortunately, we could not test the effect of genetically removing both the glycosylation site of Eiger because the resulting Eiger-TNF-Asn332Ala-Asn400Ala construct could not be secreted by insect cells upon over-expression. However, the second glycosylation site predicted to be at Asn332 is

not at the trimer interface (Fig. 25B), therefore most likely it is not implicated in modulating trimerization of Eiger.

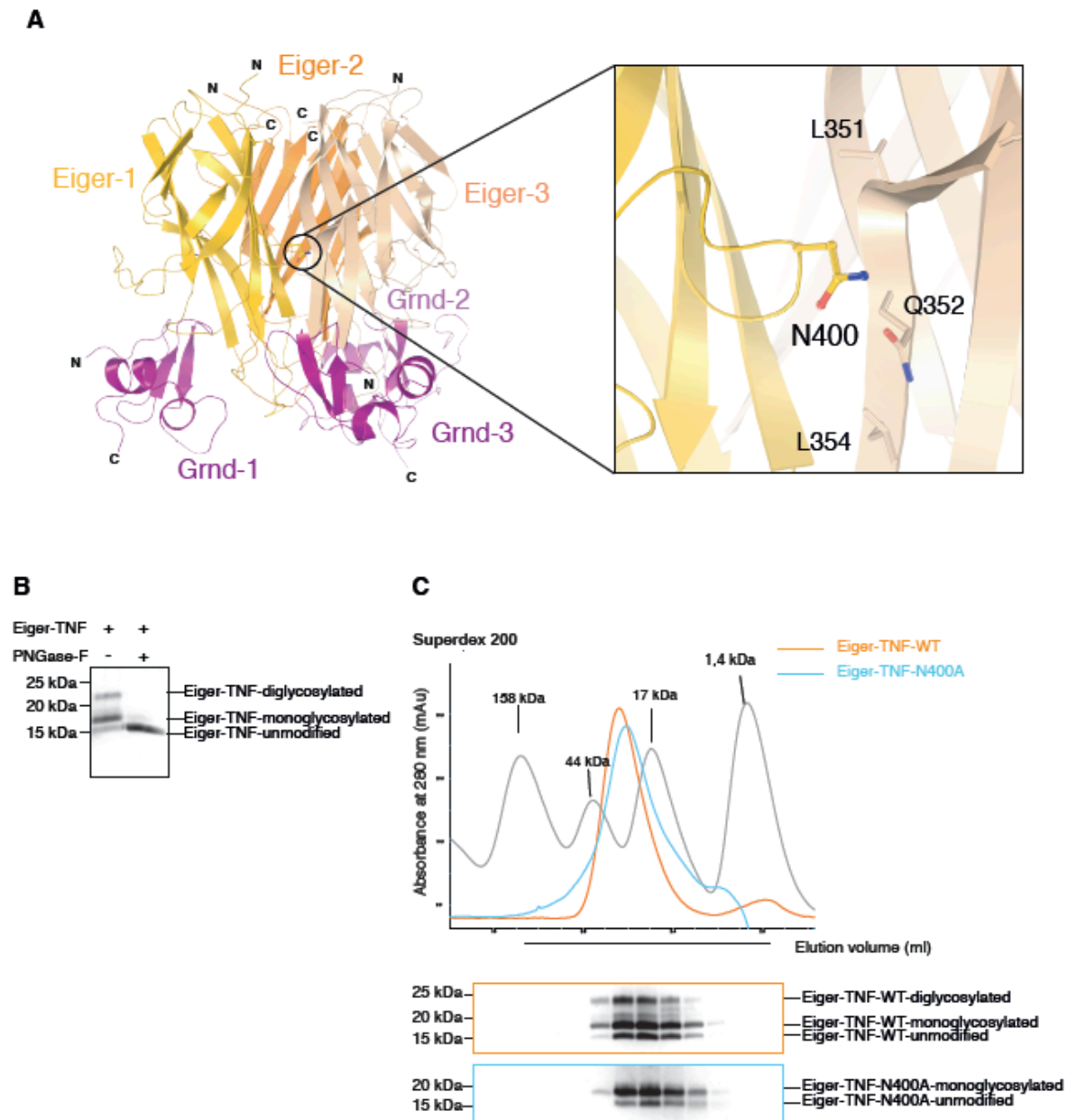


Fig. 28. Eiger trimerization is not affected by Asn400 glycosylation. **A.** Left: Cartoon representation of the Eiger:Grnd complex. Right: Close-up on Asn400 localization on Eiger. Eiger monomers are shown in gold, orange and wheat. Asn400 and facing residues are indicated as sticks. **B.** Eiger-TNF produced in insect High Five cells runs as three molecular species on SDS-PAGE. PNGase-F treatment leads to the appearance of a single band at lower MW. **C.** SEC analysis showing Eiger-TNF wild-type or carrying the Asn400Ala mutation eluting as a monomer, regardless of the presence of glycosylation on Asn400. Top: Elution profiles of Eiger-TNF wild-type (orange trace) and Asn400Ala (light blue trace) at 30 μ M concentration from a Superdex-200 15/150 column. The run of globular molecular-weight standards is shown as a grey dotted trace. Bottom: Peak fractions were loaded on SDS-PAGE and stained with Coomassie blue. Multiple bands for Eiger-TNF are

visualized, corresponding to the differently glycosylated forms of Eiger-TNF, as indicated in the figure.

To gain further insights into the Eiger oligomerization process, I reasoned that the monomeric Eiger-TNF I used for the glycosylation analysis lacked 120 residues at the N-terminus compared to the bacterial construct Eiger-ECD utilized to determine the hetero-hexameric crystallographic structure. Therefore, I decided to test if this N-terminal region could be responsible for Eiger trimerization. To this aim, I subjected a sample of Eiger-ECD produced in bacteria to trypsin digestion for 2 hours at 4 °C (Fig. 29A), followed by SEC analysis. The SEC elution profile of the trypsinized sample shows that Eiger-ECD deprived of its N-terminal region is monomeric, indicating that the flexible N-terminal portion of the extracellular region of Eiger preceding the TNF domain is necessary for the protein to oligomerize (Fig. 29B). When the Eiger-ECD:Grnd-ECD sample is subjected to an analogous trypsin treatment, the complex maintains a 3:3 stoichiometry (refer to Fig. 21C). Similarly, monomeric Eiger-TNF obtained by trypsinization trimerizes upon incubation with Grnd (Fig. 29C). Collectively, these data suggest that Grnd both stabilizes and induces the trimerization of the TNF domain of Eiger, likely by bridging between two different Eiger-TNF domain copies.

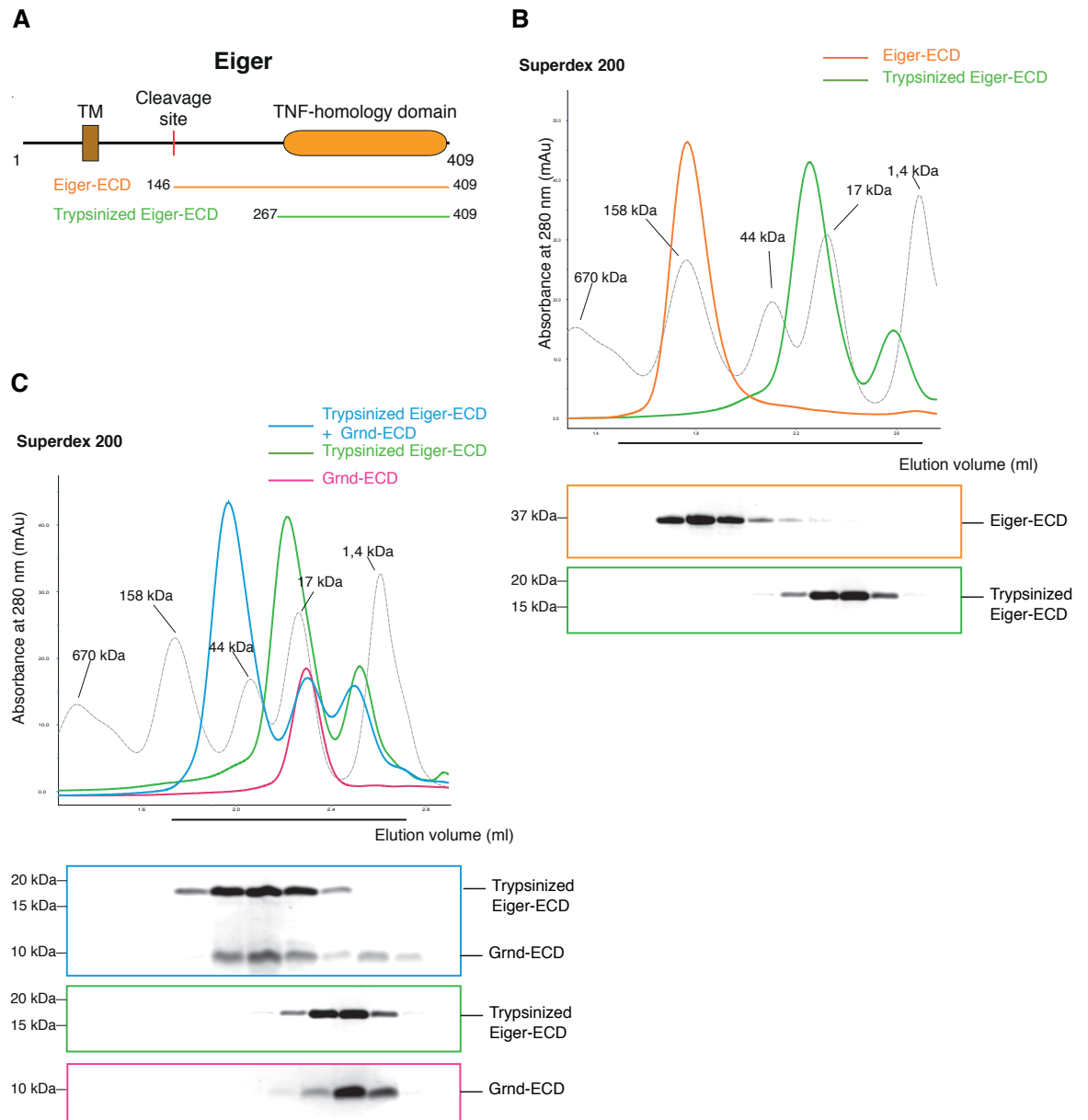


Fig. 29. Grnd stabilizes Eiger trimerization **A.** Domain structure of Eiger, showing the purified Eiger-ECD construct (orange) and the trypsinized one (green). Boundaries are indicated. **B.** Eiger trimerization is driven by a region upstream of the TNF domain. SEC analysis showing that Eiger-ECD elutes as a trimer, whereas the trypsinized sample runs as a monomer. Top: Elution profiles of 30 μ M Eiger-ECD intact (orange trace) and trypsinized (green trace) from a Superdex-200 15/150. Molecular-weight standards are shown as a grey dotted line. Bottom: Fractions-containing peak were separated on SDS-PAGE and Coomassie-blue stained. **C.** Grnd drives trimerization of monomer Eiger. Top: SEC elution profiles of trypsinized Eiger-ECD (green trace), Grnd-ECD (magenta trace), and the complex formed by trypsinized Eiger-ECD and Grnd-ECD (light blue trace) at 30 μ M concentration. Bottom: SDS-PAGE visualized by Coomassie blue staining of the fractions indicated by the black bar. MW markers are represented by a grey dashed line.

2.4.6 Eiger glycosylation affects the binding to Grnd

Analysis of the Eiger:Grnd structure revealed that Asn332 is close to the interaction surface between the ligand and the receptor (Fig. 25B). We previously showed that the Eiger-Asn332Ala mutation does not affect the binding affinity between the ligand and the receptor (Fig. 26E). To assess whether the presence of a bulky sugar on the Asn332 side chain could reduce the strength of the Eiger:Grnd interaction by steric hindrance, we reconstituted the Eiger-TNF:Grnd-ECD complex using a glycosylated Eiger-TNF sample produced in insect cells, and studied by analytical SEC its ability to bind Grnd-ECD in the presence of glycosylation. Glycosylated Eiger-TNF shows an impaired ability in forming a complex with Grnd-ECD as compared with the trypsinized Eiger-ECD (compare Fig. 30, and Fig. 29C).

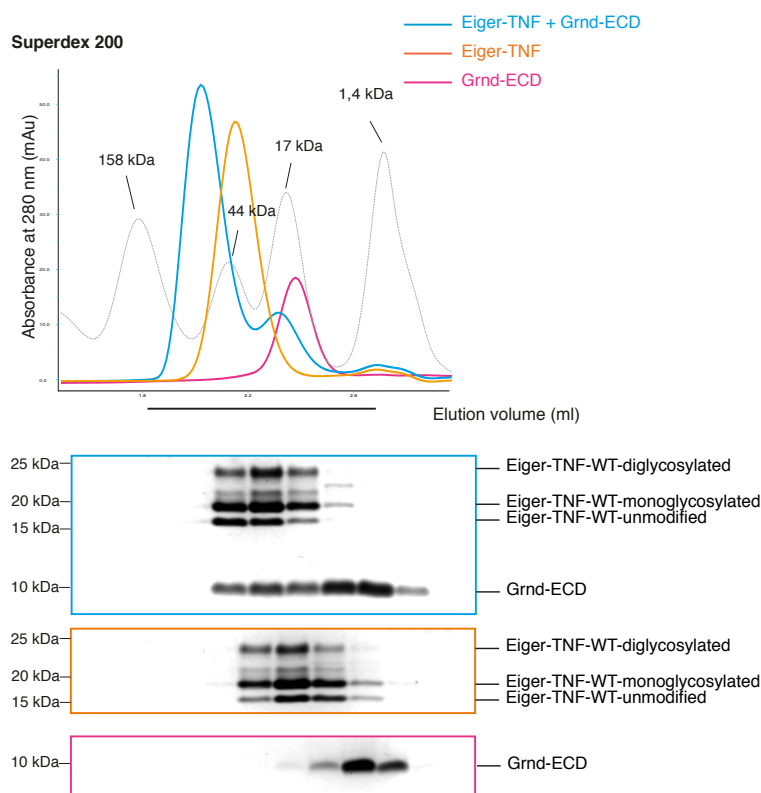


Fig. 30. Glycosylated Eiger-TNF binds Grnd-ECD with lower affinity. Top: Elution profile of glycosylated Eiger-TNF in complex with Grnd-ECD, mixed at 30 μ M concentration (light blue trace). Single runs of Eiger-TNF (orange trace) and Grnd-ECD (magenta trace) are showed for comparison. Bottom: Coomassie blue-stained fractions indicated by the black bar and separated on SDS-PAGE. Eiger-TNF does not form a complex with all the available Grnd-ECD.

To obtain more accurate information about the role of Eiger glycosylation in modulating the binding affinity to Grnd, I plan to compare the K_d of the interaction between Grnd and glycosylated or unmodified Eiger-TNF, measured by ITC.

Collectively, this evidence demonstrate that Eiger glycosylation may affect the ability of Eiger to bind Grnd, but not the oligomeric state of Eiger. Instead, trimerization of Eiger is driven by a portion located at the N-terminal region of the TNF domain, which is maintained upon cleavage of the metalloproteinases TACE, thus preserving the topology of Eiger. In turn, Grnd plays a role in stabilizing this trimeric conformation of Eiger.

2.5 Regulation of the TNFR signalling by polarity proteins

2.5.1 Grnd activates the JNK pathway by interacting with Veli, independently of Eiger

Strikingly, in the original *rn>avl*-RNAi screening performed by Leopold group, Eiger was not required to induce tumorigenesis (153). Moreover, previous studies showed that organ compartments fully composed of polarity-deficient cells do not undergo apoptosis, but rather develop tumours through the JNK pathway, contrary to what happens when polarity is disrupted in single clones (see paragraph 1.2.1 and 1.3.4). For these reasons, we decided to study how polarity was linked to Grnd and JNK activation, independently of Eiger. In *Drosophila* discs, Grnd co-localizes with the polarity proteins Crb and Veli (153). Interestingly, a previous two-hybrid screen scored an interaction between the CG10176 gene and Veli (170). *Drosophila* Veli consists of an N-terminal L27 domain, necessary for the oligomerization with Stardust and PatJ, followed by a PDZ domain (Fig. 31A). To test the direct binding between Grnd and Veli, I performed a GST pull-down analogous to the one described in Fig. 14C, using GST-Grnd-ICD adsorbed on GSH beads, and Veli constructs comprising the FL protein, the L27 domain or the PDZ domain. As a specificity control, I decided to include in this assay the first PDZ domain of the polarity protein Bazooka (Baz). The experiment revealed that only Veli-FL and its PDZ domain are retained on beads, while

the L27 region and Baz-PDZ domain are not (Fig. 31B). I next asked which was the minimal binding region of Grnd to Veli. To this end, I designed a battery of Grnd truncation mutants, including a fragment close to the transmembrane helix and two C-terminal constructs (Fig. 31C, top), and I used them in a pull-down experiment with Veli. Binding of Grnd to Veli is recapitulated by the membrane proximal stretch of Grnd (Fig. 31C, bottom). This result was unexpected, because PDZ domains generally interact through their carboxylate binding loop with C-terminal peptides, accommodated in their hydrophobic pocket (171), as shown for Par6-PDZ and the C-terminal peptide of Cdc42 (Fig. 31D, left). However, the association of PDZ domains with internal peptides is less common, but not unique. An example is the binding between the PDZ domain of human Par6 and the internal peptide of Pals1, in which the conformational change of the carboxylate loop allows fitting of the peptide into the pocket (Fig. 31D, right) (112). Therefore, we reasoned that the JNK pathway could be activated independently of Eiger by the binding of Grnd to the Veli subunit of the Crb complex. To test this hypothesis, Julien Colombani from Pierre Léopold's lab generated the same aberrant morphology observed in the *rn>avl*-RNAi genotype by expressing the intracellular region of Crb in discs (*rn>crb-intra*) (Fig. 31E) (172), and checked whether Grnd and Veli have a role in this phenotype. Interestingly, reducing levels of Grnd and Veli by RNAi completely rescued the discs' defects (Fig. 31G, H), to the same extent of overexpressing puckered (Fig. 31F), suggesting that Grnd links JNK signalling to the Crb polarity complex by interacting with Veli.

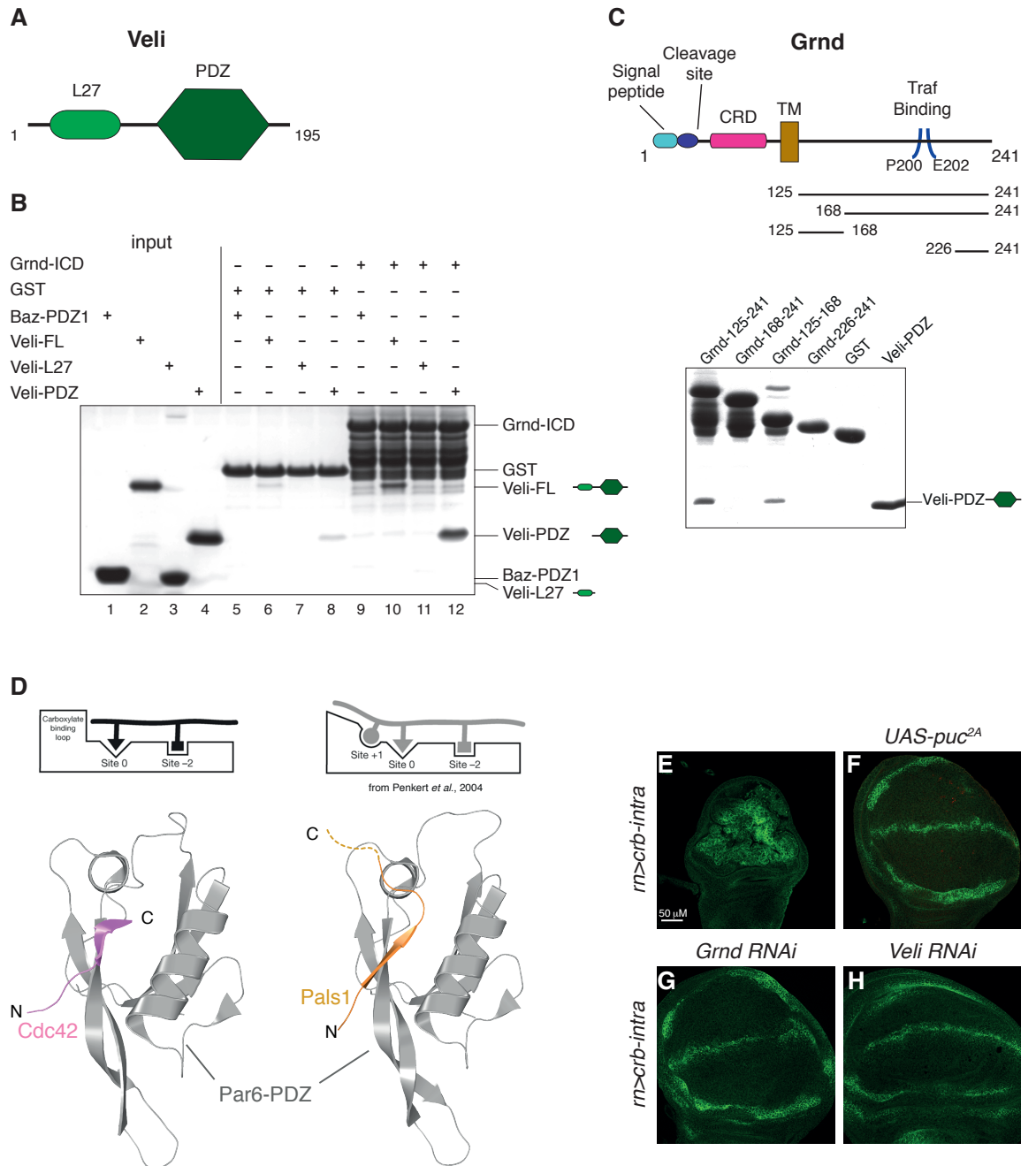


Fig. 31. The direct interaction between Grnd and Veli regulates JNK signalling activation. **A.** Domain structure of Veli, consisting of an N-terminal L27 domain and a C-terminal PDZ domain. **B.** GST pull-down between Grnd-ICD and Veli-FL, or its complimentary fragments Veli-L27 and Veli-PDZ. The Coomassie-stained SDS-PAGE shows that Grnd and Veli interact directly, and that the binding is recapitulated by the PDZ domain of Veli. Baz-PDZ is used as a specificity control. **C.** Top: domain structure of Grnd, and scheme of the constructs used to map the binding surface between Grnd and Veli. Bottom: GST pull-down assay between complimentary constructs of Grnd-ICD and Veli-PDZ. The experiment shows that the membrane-proximal stretch of Grnd binds Veli-PDZ. **C.** Top: schematic representation of PDZ binding of C-terminal and internal peptides. Bottom: Par6-PDZ interacting with the C-terminal region of Cdc42 (left, PDB: 1RZX) or an internal peptide of Pals1 (right, PDB: 1X8S). The carboxylate-binding loop of the PDZ domain undergoes a conformational change which allows the arrangement of the internal peptide into the binding pocket.

E-H. Discs overexpressing Crb-intra display Wg ectopic expression and neoplastic growth (**E**). These aberrant phenotypes are reverted by the RNAi of both Grnd and Veli, or the overexpression of Puckered (**F, G, H**), suggesting that Grnd couples the activity of the JNK complex to epithelial polarity by interacting with Veli.

Grnd is endowed with the unique property of coupling cell polarity with the JNK pathway thanks to its ability to interact directly with the Crumbs complex protein Veli and with the JNK component Traf2 (153). This observation raised the question as to whether a Grnd TNFR orthologue related to epithelial polarity exists in humans.

2.5.2 Fas interacts with Veli and Dlg1

Sequence analysis revealed poor conservation between Grnd and vertebrate TNFRs. Thus, I decided to search for a human Grnd orthologue experimentally by testing the binding ability of human TNFRs to the orthologue of Veli. In human, three Veli proteins exist, with a high level of conservation, especially in the PDZ domain (Fig. 32A). To search for a Grnd orthologue, I decided to test human TNFRs binding to both VeliB and VeliC, which gave similar results. For simplicity, here only assays with the PDZ domain of VeliC will be presented. As a collection of human TNFR, I focused at first on death receptors, because of the ability of some of them to trigger apoptosis or cell proliferation depending on the cell context, a feature that is shared with Grnd (33). In my initial screen, I also included CD40, as it contains a TIM which directly binds Traf6 (39), and TNFRIB. I thus performed RT-PCR on the RNA isolated from HeLa and HaCaT cells to obtain the ICD of human TNFRI, Fas, EDAR, DR4, DR5, TNFRIB, and CD40, and I sub-cloned them in a pGEX-6PI vector as GST-fusion proteins. A preliminary expression test showed that some TNFR-ICDs were not soluble in bacteria. Thus, I tested the binding to VeliC-PDZ only of Fas, EDAR, TNFRIB and CD40. I decided to test the binding of TNFRs only to the PDZ domain of Veli because Grnd associates with the PDZ domain of Veli (see paragraph 2.5.1). 5 μ M of TNFR-ICDs were immobilized on GSH beads and then incubated with 10 μ M of VeliC-PDZ in solution.

Species retained on beads were separated by SDS-PAGE and visualized by Coomassie blue. The GST pull-down revealed that only Fas-ICD directly interacts with Veli-PDZ (Fig. 32B). Human Fas consists of an ECD with three CRDs, a transmembrane region and an ICD, spanning residues 191-335, characterized by the presence of the DD, recognized by the FADD protein and acting as a platform for the DISC assembly (Fig. 32C). To further trim the Veli-binding site, I repeated the pull-down experiment with shorter complimentary Fas-ICD fragments, showing that the very C-terminal residues of Fas suffice to engage Veli-PDZ (Fig. 32D). This result is consistent with the presence of a type I PDZ-binding motif on Fas C-terminus, conforming to the consensus sequence S/T-X- ϕ , where ϕ is a hydrophobic amino acid, and X is any given residue.

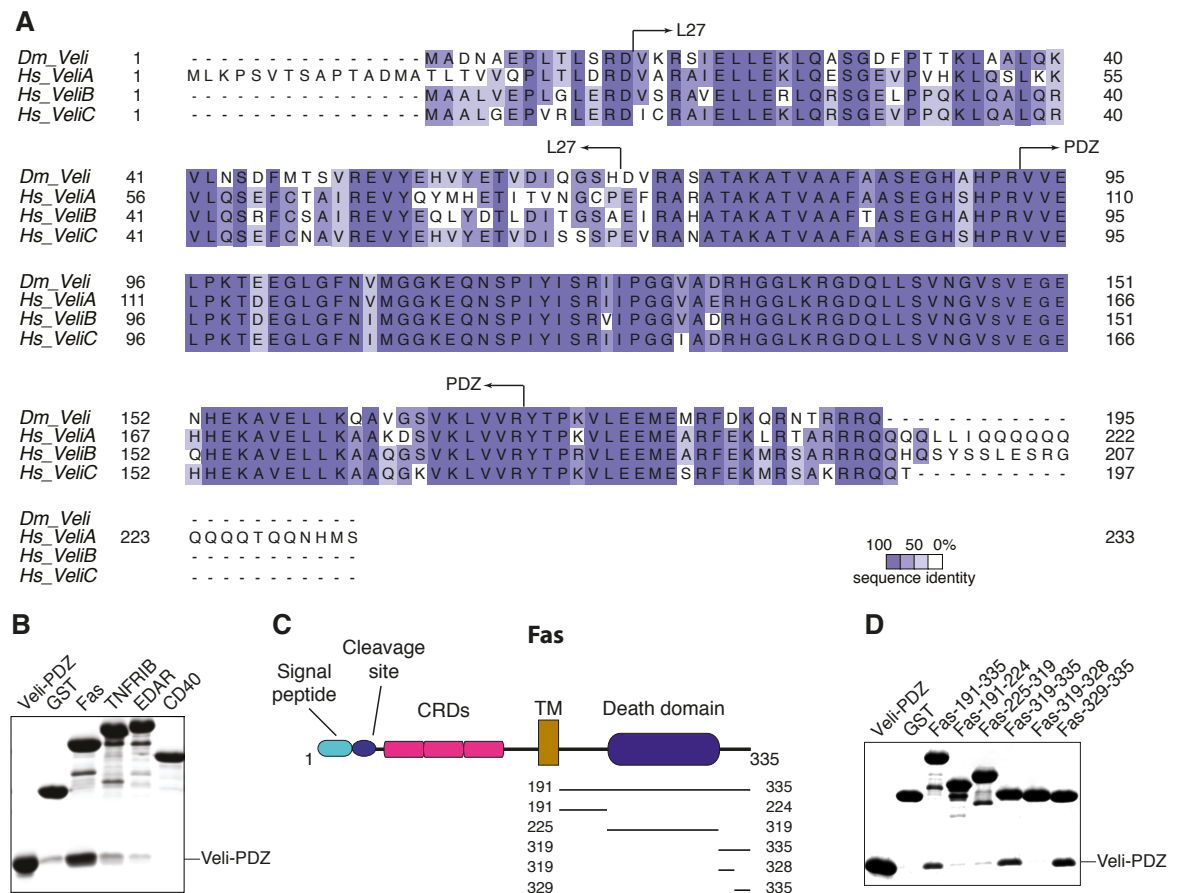


Fig. 32. Fas is the biochemical orthologue of Grnd. **A.** Sequence alignment of *Drosophila* and human Veli. Positioning of L27 and PDZ domain are indicated. Conserved residues are coloured based on sequence conservation. **B.** GST pull-down assay with GST-fusions of intracellular domains of human Fas, TNFRIB, EDAR and CD40 adsorbed on GSH beads and purified VeliC-PDZ in solution. The species retained on beads were separated by SDS-PAGE and visualized by Coomassie

staining. VeliC-PDZ binds only Fas-ICD. **C.** Domain structure of Fas, consisting of an N-terminal signal peptide, followed by three CRDs, a transmembrane region TM, and an intracellular death domain. Complimentary fragments used for the pull-down in **D** are indicated. **D.** Pull-down experiment as in **B** with Fas-ICD deletion mutants, showing that VeliC-PDZ interacts with the last 7-residues of Fas.

In epithelial cells of the intestinal and biliar tract, Fas localizes basolaterally (173-175). In turn, Veli forms a trimeric complex with CASK and Dlg1 (110, 176), the latter of which is also a polarity protein containing three PDZ domains. Thus, I next asked whether also Dlg1 could interact with Fas. I performed a GST pull-down using 5 μ M of Fas-ICD absorbed on beads, which were then incubated with 2 mg of HEK293T cell lysate. The presence of Dlg1 retained on beads were visualized by immunoblot using an anti-Dlg1 antibody produced in-house. The assay showed that Fas-ICD binds Dlg1, and that the last seven amino acids of the receptor are minimal binding region to Dlg1 (Fig. 33A). Dlg1 consists of an N-terminal L27 domain, followed by three PDZ domains, a SRC homology-3 (SH3) one and the C-terminal guanylate kinase (GK) region (Fig. 33B). To test whether the PDZ domains of Dlg1 were responsible of the interaction with Fas, I then performed an *in vitro* pull-down analogous to the one described in Fig. 32D, using the Fas fragments retained on GSH beads and the purified PDZ domains of Dlg1 in solution, which revealed that the C-terminus of Fas directly associate with the PDZ domains of Dlg1 (Fig. 33C). I then repeated the binding assay with the last 7-residues of Fas and a battery of different combinations of the PDZ domains of Dlg1, showing that the PDZ1 and the PDZ2 of Dlg1 recapitulate the binding to Fas C-terminus (Fig. 33D).

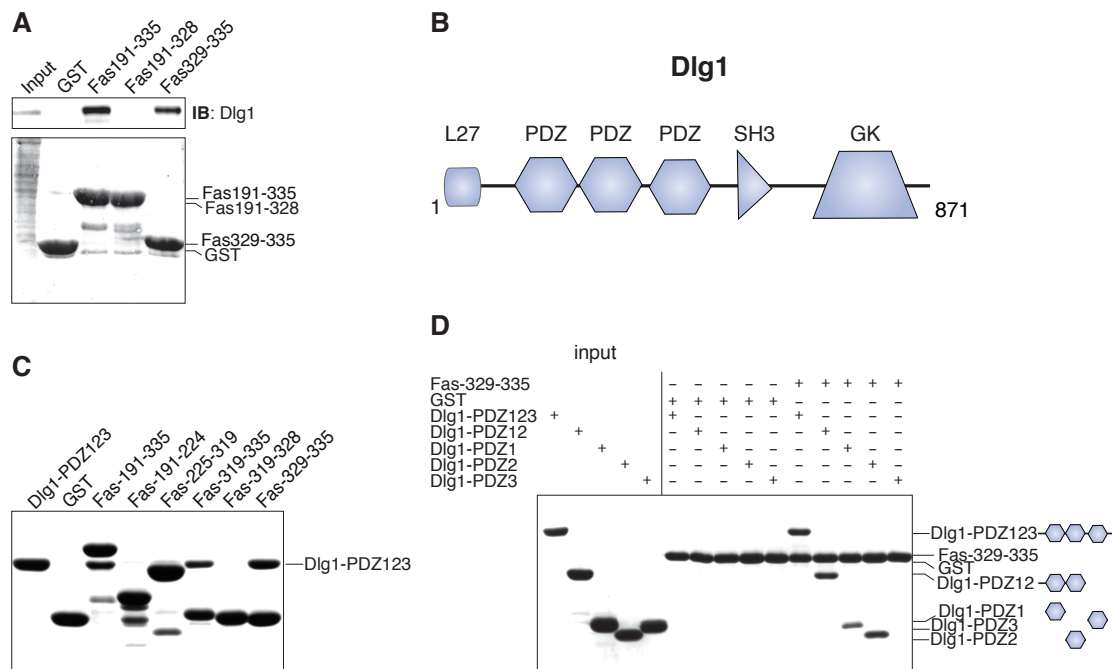


Fig. 33. Fas directly interacts with Dlg1. **A.** Pull-down assay with complementary fragments of Fas-ICD immobilized on GSH beads and HEK293T lysates showing that endogenous Dlg1 binds to Fas329-335. Top: Dlg1 is visualized by immunoblotting with a monoclonal anti-Dlg1 antibody generated in-house. Bottom: Membrane was stained with ponceau to visualize the amount of the GST-fusion proteins. **B.** Domain structure of Dlg1, comprising an N-terminal L27 domain, three PDZ domains, a SH3 domain and one GK domains. **C.** GST pull-down performed using Fas-ICD fragments absorbed on GSH beads and PDZ domains of Dlg1 in solution. Species retained on beads were separated by SDS-PAGE and visualized by Coomassie blue. Only the C-terminus of Fas is necessary for binding the PDZ domains of Dlg1. **E.** GST pull-down assay with Fas-329-335 on GSH beads and various combinations of Dlg1-PDZ domains in solutions, visualized as in C. Dlg1-PDZ2 recapitulates the binding of Dlg1 to the 7-residue C-terminal tail of Fas.

2.5.3 Veli and Dlg1 are competitive ligands for Fas

The evidence that Veli and Dlg1 share the same Fas-ICD binding region (Fig. 32D and 33C), which is only a 7-residue long stretch, raised the question as to whether the two Fas polarity proteins can associate to the ICD of Fas concomitantly. To test this possibility, I performed a competition pull-down assay, incubating 3 μ M of Fas-319-335 adsorbed on GSH beads with constant 7.5 μ M of Veli-PDZ and increasing concentrations of Dlg1-PDZ123, from 0 to 50 μ M. The assay revealed that Veli and Dlg1 do not form a trimeric complex with Fas, but rather compete for the binding (Fig. 34).

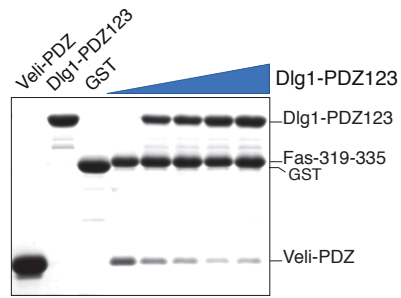


Fig. 34. Veli and Dlg1 are competitive ligands for Fas. In *vitro* competition binding assay of Veli and Dlg1 to Fas. Increasing amounts of Dlg1-PDZ123 (from 0 to 50 μ M) were titrated with 3 μ M Fas-319-335 at constant 7,5 μ M Veli-PDZ concentration. Species on beads were separated by SDS-PAGE and Coomassie-stained. The binding of the two polarity proteins to Fas is mutually exclusive.

3 DISCUSSION

Under stress conditions, TNFRs act as master regulators of cell fate. During my PhD studies, we provided structural and functional evidence for the existence of a new TNFR in *Drosophila melanogaster*, that we named Grindelwald (Grnd). We demonstrated that Grnd associates with the unique *Drosophila* TNF Eiger and with Traf2, thus activating the JNK pathway, which leads to apoptosis or malignancy *in vivo*. With biochemical and crystallographic analyses we uncover the architecture of the Grnd:Eiger complex, and identified on both subunits key residues important for the complex formation. We also showed that Grnd directly binds to the polarity protein Veli, and this interaction is required for activation of the JNK pathway in polarity-deficient cells. Interestingly, our biochemical analyses indicate that Fas is the only human TNFR interacting with both the polarity proteins Veli and Dlg1, suggesting Fas as the human biochemical orthologue of Grnd. Our studies are in part presented in the paper entitled “The *Drosophila* TNF receptor Grindelwald couples loss of cell polarity and neoplastic growth”, published in Nature in June 2015 (153).

***Drosophila melanogaster* TNFR Grnd**

My studies started from the sequence analysis of the identified *grnd* gene, in which was scored the presence of a single CRD, that is a hallmark of TNFRs. On the intracellular region of Grnd, we also recognized the presence of the Pro-X-Glu-X-X motif, that represents the consensus sequence for binding of TNFR to Traf2, the most upstream component of the JNK pathway. In an *in vitro* binding assay, we demonstrated that the association between Grnd-ICD and Traf2-MATH is direct and mediated by the consensus motif on Grnd-ICD (Fig. 14C). This stretch of amino acids is evolutionary conserved, as it has been identified also in human TNFRs, such as CD40, as mediating the interaction with Traf6, which is the human orthologue of *Drosophila* Traf2 (158). Interestingly, the *Drosophila* TNFR Wengen

previously identified does not contain a death domain nor a known TIM, but it was shown to co-immunoprecipitate with Traf2 (44). One hypothesis to explain how this interaction could take place is that Wengen may form hetero-complexes with Grnd, thus indirectly associating with Traf2. Alternatively, it is possible that Wengen recognizes Traf2 via a non-canonical TIM. Targeted experiments will address the functional link between Grnd and Wengen.

It is known that Eiger leads to Traf2/JNK activation inducing the expression of Hid, Grim, and Reaper, that cause apoptosis, and of MMP1, which supports malignancy (48, 52), and of the phosphatase Puckered (83, 85). The interaction between Grnd and Traf2 results in the induction of the JNK cascade, as we demonstrated with *in vivo* experiments performed using Puckered as a readout for the JNK activation (Fig. 14D-G).

A feature of TNFRs is the presence of CRDs on the ECD, whose fold is stabilized by cysteines engaged in disulphide bridges. Each CRD is composed by two modules, classified based on the tertiary fold and the pattern of cystines (2, 9, 13). Crystallographic analysis carried out on the extracellular portion of Grnd revealed that it contains a unique CRD organized in two modules, named X2 and C2, consisting of a β -hairpin and a helix-loop-helix motif, respectively (Fig. 20A). The eight cysteines of Grnd-ECD are organized in an intercalated pattern of disulphide bonds connecting Cys35 with Cys47, and Cys40 with Cys54 in the X2 module, and in a nested pattern in the C2 module, with disulphides between Cys57-Cys77 and Cys61-Cys73 (Fig. 20B). In spite of this unique disulphide arrangement, the overall fold of Grnd conforms to other known TNFRs carrying single CRDs, including BCMA and Fn14 (12, 18), and also to the second CRD of TACI (19) (Fig. 20C-E).

These TNFRs contact their ligands with two separated regions located on the β -hairpin tip of the first module, and on the second helix-loop-helix module. The crystallographic structure of the Eiger:Grnd complex that we determined revealed that the two proteins form a hetero-hexameric complex (Fig. 23A), with an overall topology resembling the one of single CRD-containing TNFRs with their cognate TNF (Fig. 23B). The assembly is

organized around a central trimer of the TNF domains of Eiger, each of which folds in a jelly-roll conformation, with the 10 antiparallel β -strands forming two β -sheets (Fig. 24A, B). As for human TNFs, the inner β -sheet is involved in forming the trimeric surface of the THD, whereas the outer one is engaged in binding to Grnd. Notably, the structural superposition of Grnd in complex with Eiger or in isolation showed that Grnd does not undergo substantial conformational changes upon binding Eiger, but rather behaves as a compact rigid scaffold (Fig. 24C). Structure analysis revealed that each monomer of Grnd contacts two Eiger copies, resembling the one-to-two mode of interaction already described for some TNFRs with multiple CRDs, for instance CD40 and DR4 (21, 22). This is a peculiar feature of the Eiger:Grnd complex differing from the one-to-one mode of interaction generally shown by TNFRs with single CRDs. In details, the Eiger and Grnd interface is formed by three distinct patches of interaction (Fig. 25A). The first involves Thr51 and Glu52 on the β -hairpin tip of Grnd facing Arg396-Arg401 of the β G- β H^{Eiger} loop and Asn332 of the β C- β D^{Eiger} loop (Fig. 25B). A further region is defined by Grnd-His66-Asn67 on the α 1- α 2^{Grnd} loop contacting Asp337 on the β C- β D^{Eiger} loop of a second molecule of Eiger (Fig. 25C). On the same loop of the second Eiger monomer, the His388-Arg391 couple binds Tyr48 and Asp69 of Grnd (Fig. 25D). *In vitro* pull-down assays defined Grnd-Thr51 and Grnd-His66-Asn67 as the major determinants of the association with Eiger (Fig. 26A, C). On the Eiger side, mutation of Asp337, His388-Arg391, or Arg396-Arg401 completely disrupt the binding to Grnd (Fig. 26E).

The qualitative results on the Grnd:Eiger interface obtained by pull-down assays were confirmed by more quantitative ITC analyses, which revealed that Grnd binds Eiger with a K_d in the nanomolar range, fully in line with the binding affinities measured for other TNFs:TNFRs interactions (177-179). Calorimetric measurements of the binding affinity of the Grnd mutants identified in the pull-down assays showed that Grnd-Thr51Ala and Grnd-His66Ala-Asn67Ala do not bind, while mutations Glu52Ala, His66Ala, and Asn67Ala display a reduced but still detectable affinity (Fig. 26D).

We also addressed the role of Grnd-Phe46, which is conserved among human TNFRs, and it has been found as necessary in ligand binding (Fig. 14A). Specifically, the corresponding BCMA-Tyr13 is at the interface with TALL-1 (Fig. 20E), while the TACI-Phe78 does not show any contact with the ligand April. However, mutation of TACI-Phe78 into alanine completely impairs the binding affinity between TACI and April (12, 19), possibly because of its role in organizing the two TACI modules in the correct orientation for the interaction with its ligand. Structural analysis of Grnd showed that Grnd-Phe46 is buried within the hydrophobic pocket of the receptor fold (Fig. 26B). Nonetheless, Grnd-Phe46Ala does not bind to Eiger (Fig. 26A, 26C, 26D). This evidence suggest that Grnd-Phe46 has a structural role similar to the TACI-Phe78 in maintaining the correct arrangement of the X2 and C2 modules of Grnd, enabling the interaction with Eiger.

We think that the information obtained on the Grnd-Eiger interface will be useful to investigate the relevance of the Grnd:Eiger interaction *in vivo* because it will allow the generation of mutant flies with Grnd variants totally impaired in Eiger-dependent JNK activation, or able to transduce Eiger signals with reduced efficiency. Specifically, to test the relevance in a physiological context of the Grnd residues identified *in vitro*, we will perform *in vivo* analyses in *Drosophila* in collaboration with Ditte Andersen and Julien Colombani. To generate Grnd mutant flies, we are currently carrying out gene editing at the *grnd* locus, which results in the deletion of the first coding exon of the endogenous *grnd* locus to generate a *grnd* null mutants, and in the subsequent replacement of endogenous *grnd* with: i) Grnd-His66Ala-Asn67Ala gene as a representative of a Grnd mutant completely impaired in the binding to Eiger; ii) Grnd-His66Aala that is a mutant partially disrupting the Eiger:Grnd association; or iii) Grnd-Phe46Ala, to confirm that this residue is required for the correct folding of Grnd and hence for its activities *in vivo*. As described above, the small-eye phenotype generated by Eiger overexpression is completely suppressed in *grnd* interfered conditions. Similarly, one would expect the small-eye phenotype to be suppressed in animals where endogenous Grnd has been replaced with mutant Grnd receptors that no

longer bind Eiger. Conversely, *scrib*^{-/-};*ras*^{V12} mutant cells exhibit a dramatic tumour-like overgrowth and metastatic behaviour, that critically relies on the Eiger and Grnd interaction, and results in the expression of MMP1. To explore the role of the Eiger:Grnd interaction under pathological conditions, we will test in collaboration with Lèopold's lab the ability of Eiger to induce MMP1 expression and *scrib*^{-/-};*ras*^{V12} tumour progression in Grnd-His66Ala-Asn67Ala, Grnd-His66Ala, and Grnd-Phe46Ala mutant backgrounds. These analyses will shed light on the role of Grnd in transducing Eiger signals under normal and pathological conditions.

Most of TNF ligands are trimeric, and assemble in 3:3 complexes with their receptors. Consistently, the variant of Eiger found in the hemolymph, spanning residues 146-409, that we used for biochemical analyses, is a trimer in solution (Fig. 16A, 16B). However, we found that a shorter construct encompassing only the TNF domain (residues 270-409) is monomeric according to SEC analysis (Fig. 29B). We conclude that the N-terminal portion of Eiger promotes trimerization in the absence of Grnd, with a mechanism that still remains unclear.

The fragment of Eiger that we crystallized after limited proteolysis encompasses amino acids 267-409, and comprises the whole TNF domain, which form hetero-hexamers with Grnd (Fig. 21C, 29C). The evidence that Eiger-TNF domain, which is monomeric in isolation, is sufficient to form hetero-hexamers with Grnd indicate that Grnd stabilizes and drives Eiger trimerization *in vitro*. Whether these observations are relevant for the spatial organization of ligand-receptor complexes at the membrane and for Eiger-mediated activation of Grnd and the JNK pathway still remains to be investigated.

Eiger is predicted to be glycosylated at Asn332 and Asn400. Indeed, when expressed in insect cells as a secreted protein, the TNF domain of Eiger is doubly glycosylated (Fig. 28B). Our Eiger:Grnd structure revealed that Asn400 is located near the interface among Eiger monomers (Fig. 28A), although it does not have effect on the oligomerization state of the

ligand (Fig. 28C). The second predicted glycosylation site of Eiger, at Asn332, is instead at the binding interface with Grnd (Fig. 25B). *In vitro* analytical gel-filtration seems to suggest that glycosylated Eiger has a lower affinity for Grnd (Fig. 30). To provide a more quantitative information on the binding affinity between glycosylated Eiger-TNF and Grnd-ECD, we will compare the ability of Grnd-ECD to interact with unmodified Eiger-TNF (obtained by trypsinization of the bacterially-produced Eiger-ECD, thus lacking glycosylations) and with the glycosylated Eiger-TNF domain produced in insect cells. Based on the preliminary SEC experiments, we speculate that Eiger glycosylation could possibly weaken the interaction between the ligand and the receptor, this way modulating signal transduction. Notably, this hypothesis could also provide a molecular explanation for the phenotype recently observed upon ablation of the mannosyltransferase ALG3 in *Drosophila* imaginal discs (161) (see the next paragraph for more details).

Also Grnd is predicted to be glycosylated at Asn63. A recent study shows that disruption of the gene encoding for the mannosyltransferase ALG3 determines incomplete glycosylation of Grnd, and leads to hyperactivation of the JNK pathway (161). The authors hypothesize that this post-translational modification could modulate the JNK cascade by affecting the affinity of the interaction between Grnd and Eiger. Our quantitative analyses of the association between Eiger and Grnd revealed that Grnd glycosylation does not have any impact on the ability of the receptor in binding Eiger (Fig. 15C). This is consistent with the Eiger:Grnd crystallographic structure showing that Grnd-Asn63 resides on the loop between helices, pointing away from the Eiger:Grnd interacting surface (Fig. 27). The evidence that Grnd glycosylation does not alter the binding affinity with Eiger is not a unique feature of Grnd because also the glycosylation of the human TNFRs DR5 and TRAIL-R1 does not affect binding to their TNF ligands (180, 181). Nonetheless, receptor glycosylation may contribute to regulation of TNFR-mediated activation of the JNK pathway by controlling receptors' aggregation. Studying whether the reduced JNK activation associated to Grnd glycosylation is caused by altered receptor clustering would be of great interest.

Polarity-deficient *scrib* mutant cells show strong JNK activation and eventually develop tumours (89). However, the link between loss of polarity and JNK cascade remains mostly unclear. Strikingly, in the original *rn>avl*-RNAi screen for JNK activation, and consequent malignant cell behaviour, Grnd was necessary, but Eiger was dispensable (153). Therefore, we asked whether epithelial polarity could be directly linked to Grnd activity. Grnd co-localizes at the apical region of the membrane with the polarity protein Veli, which belongs to the Crumbs complex (153)

In agreement with results of a previous two-hybrid screen (170), we showed that Grnd directly binds the PDZ domain of Veli (Fig. 31B) with an internal membrane-proximal stretch (Fig. 31C), in a non-canonical manner compared to the more common and well-known association between PDZ domains and C-terminal peptides (171) (Fig. 31D). Importantly, the direct association of Grnd-ICD to Traf2 and Veli is mediated by disjointed portions of Grnd, suggesting that the two interactors can associate simultaneously on the same Grnd molecule, this way integrating cell polarity with JNK signalling (Fig. 35A). Studying how clusterization regulates Grnd-mediated induction of the JNK pathway in this system would better clarify the molecular mechanism of Grnd activation.

Further supporting the connection between Grnd activities and tissue polarity, we found that JNK activation and tissue overgrowth caused by Crb-ICD overexpression can be reverted by both Grnd and Veli RNAi, and by Puckered overexpression (Fig. 31E-H). Collectively, these results depict Grnd as the first example of a TNFR directly coupling cell polarity with neoplastic growth via the JNK pathway (Fig. 35B).

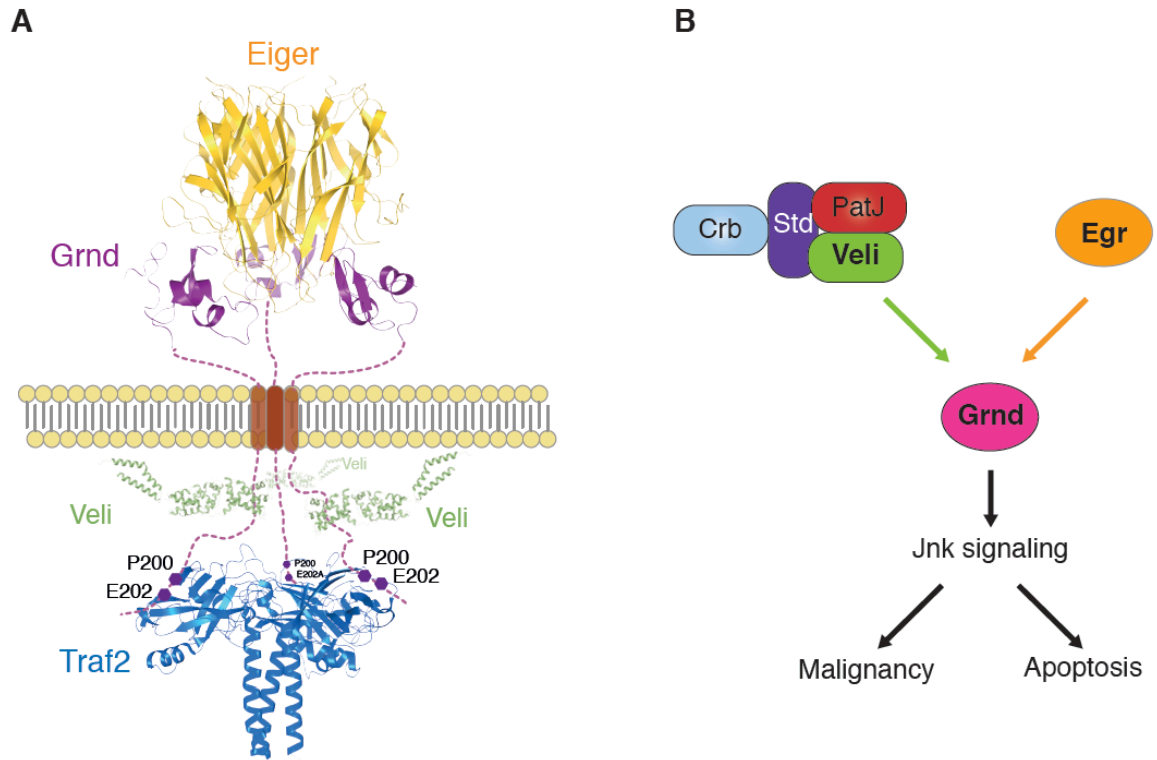


Fig. 35. Schematic representation of the role of Grnd in integrating polarity and the JNK pathway. **A.** The binding region of Grnd to Veli resides nearby the Grnd transmembrane domain, whereas the Traf2 interacting motif conforming to the consensus sequence Pro-X-Glu-X-X is closer to the C-terminal region of the receptor. This organization is compatible with the formation of a trimeric complex between Grnd, Veli, and Traf2. **B.** Grnd binding to Eiger induces activation of the JNK signalling, leading to cell death or malignancy depending on the cell context. Moreover, Grnd is able to couple JNK activity to polarity via a direct interaction with the polarity protein Veli, which is part of the Crumbs complex, and is compatible with the concomitant binding with Traf2.

An open question which still needs to be addressed is defining the mechanism by which the Grnd:Veli interaction is connected to the JNK activation and malignancy in polarity-deficient cells. An increasing body of evidence highlights the ability for polarity proteins to regulate membrane proteins localization, stability, and recycling. Consistently, one hypothesis is that Veli could stabilize Grnd at the apical membrane, thus inhibiting its endocytosis and apoptotic signalling. It is known that, upon binding to its receptor, Eiger needs to be endocytosed for the apoptosis to start, since blocking its internalization completely impairs cell death (49). In line with this hypothesis, in our original screen, in the absence of Avalanche Grnd accumulates in a wider apical region, together with the Crumbs complex (153). In addition, stabilization of Grnd at the membrane can result in Grnd

clustering, and some human TNFRs have been shown to require cluster formation at the membrane for correct signal transduction (30, 35). The role of Veli in stabilizing membrane proteins has been already described in MDCK cells for the HER2 receptor (148). In fact, binding of HER2 by the PDZ domain of Veli blocks the endocytosis of the receptor and its consequent proteosomal degradation (148). A future line of investigations should address the mechanism by which Veli controls Grnd activity.

Human TNFR Fas

Part of my research activities dealt with the identification of a human TNFRs able to bind the human orthologue of dVeli. In fact, we reasoned that a TNFR which shares the same biochemical properties with Grnd in interacting with this polarity protein may show similar activities in cells.

In humans, the TNFR superfamily consists of 29 members, 26 of each containing an intracellular domain (Fig. 3). Sequence analysis revealed poor homology between Grnd and human TNFRs, making difficult to predict *in silico* if a Grnd orthologue exists. For this reason, we performed *in vitro* binding assays to identify a TNFR capable of binding Veli-PDZ. At first, we focused on TNFRs that can switch between pro-apoptotic and pro-malignant cell behaviour, as Grnd does in flies. This way we identified Fas as the only TNFR able to bind the PDZ domain of human Veli (Fig. 32B). In contrast with Grnd, the interaction surface of Fas with Veli resides in its C-terminal 7-residues fragment (Fig. 32D), whose sequence is consistent with the class I PDZ-binding motif, according with the consensus sequence S/T-X- ϕ , where ϕ represents a hydrophobic residue. Moreover, we noticed that Fas localizes at the basolateral membrane of epithelial cells of the intestinal and biliar tracts (110, 175, 176). In turn Veli has been detected at the basolateral membrane forming a trimeric complex with Dlg1 and CASK (182). Thus, we asked whether Fas was able also to associate with the PDZ-containing protein Dlg1. We found that Fas interacts with the second PDZ domain of Dlg1 through the same C-terminal region involved in binding Veli (Fig. 33A-

33C, Fig. 32D). Interestingly, Dlg1-PDZ2 has been found to be responsible for the association also with other receptors, *e.g.* GluR-A and 5HT2A, but the relevance and function of these interactions *in vivo* has not been defined yet (183). As expected because of the overlapping binding surface, an *in vitro* competition binding assay revealed that the binding of Dlg1 and Veli to Fas is mutually exclusive (Fig. 34). While this thesis was under preparation, a work from Hueber's lab further shed light on the role of Dlg1 in regulating Fas activity (175). Specifically, they showed that Fas interacts with Dlg1 by performing an *in vivo* pull-down experiment using Fas (WT or lacking the last three amino acids) adsorbed on beads and HCT15 cell lysates. Dlg1 was among the 53 proteins identified by proteomic analysis of the co-IP. This study also identified VeliC and CASK among the Fas interactors, which are known to be part of a trimeric complex with Dlg1 (182). The authors also demonstrate that Dlg1 knockdown in HCT15 cells does not alter Fas localization at basolateral cell junctions, indicating that Dlg1 is not necessary for Fas positioning in cells. Interestingly, Dlg1 RNAi causes increased activation of caspase 8 and apoptosis induced by Fas in both HCT15 and SW480 cells, but the molecular mechanism underlying this phenotype still remains elusive. How does binding of Dlg1 to Fas inhibit cell death? One possibility is by steric hindrance due to the presence of Dlg1 close to the death domain of Fas, where the DISC should assemble and activate caspase (62, 63). A second hypothesis impinges on the ability of polarity proteins to modulate receptors stability and recycling, as discussed for Grnd above. In fact, it is known that also Fas needs to be internalized in endosomes for apoptosis to take place (60, 184). Binding of Dlg1 could stabilize Fas at the membrane, blocking its endocytosis and subsequent cell elimination by caspase activation. Clarifying whether the ability of Dlg1 to inhibit Fas-dependent cell death is compatible with its ability to promote non-cell death pathways triggered by Fas will be a very interesting direction for future investigations.

Most importantly, all these results highlight that the role of polarity proteins in the control of TNFR signalling is evolutionary conserved among species.

4 MATERIALS AND METHODS

4.1 Retro-transcription

Total RNA was extracted from HaCaT and HeLa cells using the Quick-RNA miniprep kit (Zymo Research) according to manufacturer's instructions. Reverse-transcription of RNA was performed using ImProm-II Reverse Transcription system (Promega). Briefly, the procedure consists of mixing 1 µg of isolated RNA with Oligo (dT)₁₅₋₁₈ primers at 0.5 µg/µl concentration in a total volume of 5 µL. Incubation of this reaction mix at 70 °C for 5 minutes denatures the secondary structure of RNA potentially formed in the samples. The mix is then chilled on ice for 5 minutes. The reverse transcription mix was prepared on ice, composed by nuclease-free water, 0.5 M dNTPs, recombinant RNasin Ribonuclease inhibitor, 1.5-8 mM MgCl₂, ImProm-II reaction buffer and ImProm-II reverse transcriptase, and was then incubated with 5 µL of the RNA-oligo mix for 5 minutes at 25 °C, one hour at 42 °C and 15 minutes at 70 °C. The cDNA obtained was used as a template to amplify the coding sequence of the ICD of human TNFRs Fas, CD40, TNFRIB and EDAR, and the polarity protein VeliB. To this aim, the used primers for Fas, TNFRIB, CD40, EDAR, and VeliB amplification are listed in Table 4. VeliC DNA was a gift from Laurent Gagnoux-Palacios (iBV, Nice).

Table 4. Primers used for Fas, TNFRIB, CD40, EDAR, and VeliB amplification.

Gene	Oligo Forward	Oligo reverse
Fas	CGCGCGGAATTCAAGAGAGAAAGG AAGTAC	CGCGCGGTCGACCTAGACCAAGCT TTGGAT
TNFRIB	CGCGCGGGATCCAAAAAGAAGCC CTTGTGC	CGCGCGGTCGACTTAACTGGGCTT CATCCC
CD40	CGCGCGAGATCTAAAAAGGTGGCC AAGAAG	CGCGCGGTCGACTCACTGTCTCTC CTGCAC
EDAR	CGCGCGGGATCCTACATCCTGAAG ACAAAG	CGCGCGAAGCTTTCAGGATGCAGC ATGTGG
VeliB	CGCGCGCCATGGGTGCTGCGCTGGTG GAGCCG	CGCGCGGTCGACTCAACCTCGAGA CTCCAA

4.2 Generation of baculovirus and insect cells transfection/infection

Insect cell protein expression was carried out with a modified version of the Bac-to-Bac system (Invitrogen) as outlined below.

4.2.1 Production of recombinant bacmide

Grnd-ECD and Eiger-TNF (WT and carrying the Asn400Ala mutation) were cloned in a modified version of the pFast-Bac1 (pFB1) vector (ThermoFisher), containing the mellitin secretion signal, followed by a His-tag, and the PreScission cleavage site (Fig. 36).

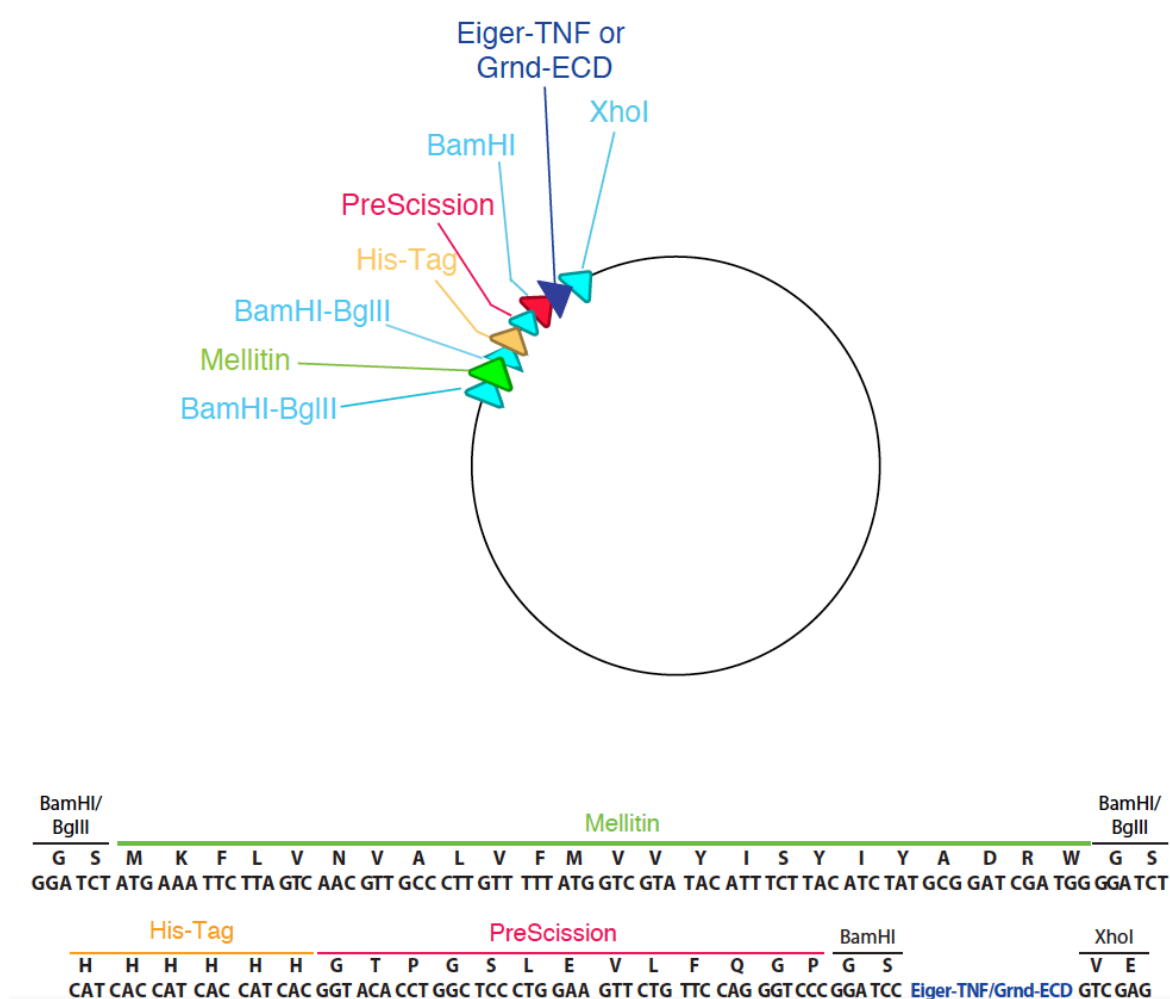


Fig. 36. Scheme of the engineered pFB1 vector. The pFB1 vector was modified to express extracellular proteins, by adding the mellitin secretion signal to the N-terminal region, and also a cleavable His-tag for affinity purification purposes. Cloning sites are indicated.

Transposition was performed using DH10MultiBac-YFP electrocompetent cells (Invitrogen), to which 1 μ L of DNA was added prior to electroporation. Bacteria were

selected on LB plates containing 50 µg/mL kanamycin, 7 µg/mL gentamycin, 10 µg/mL tetracycline, 200 µg/mL X-gal, and 40 µg/mL IPTG for at least 48 hours. DH10MultiBac-YFP cells hold a bacmide containing the LacZ gene, coding for β-galactosidase, which converts X-gal in a blue product. In recombinant bacteria, the insertion of the gene cloned in the pFastBac1 vector into the bacmide causes the disruption of the LacZ gene. As a consequence, recombinant bacteria are visualized as white colonies, from which the recombinant DNA is isolated using the Promega Wizard Plus SV Minipreps kit. Some modifications to the protocol was applied, *e.g.* the DNA precipitation with isopropanol instead of elution from the column kit.

4.2.2 Transfection of Sf21 cells with the recombinant bacmide

Sf21 cells (Invitrogen) were cultured at 27 °C in Sf-900II serum-free medium (SFM, Gibco) without addition of fetal calf serum (FCS) and antibiotics. Bacmide transection was performed using the Insectogene reagent (Biontex Laboratories) according to manufacturer's instructions. Cell transfection was monitored by looking at the appearance of YFP fluorescence. After 72 hours, the supernatant was collected, which contained the primary stock virus named "V0".

4.2.3 Infection of High Five cells

High Five cells grow in ExpressFive medium (Gibco) with addition of L-glutamine at 27 °C. Virus amplification was performed by infecting 25 ml of High Five cells at a density of 0.5×10^6 cells/ml using 2,5 ml of V0 virus. Infection was monitored by looking at YFP-positive cells and at the growth arrest caused by the viral production. After 72 hours from cell growth arrest, the medium was collected and harvested, and the virus ("V1") contained in the supernatant was used to perform a second infection. In this case, 5 ml of V1 were added to 400 mL of High Five cells at 0.5×10^6 cells/mL density. The presence of the mellitin secretion signal at the N-terminus of proteins cloned in the modified pFastBac1 vector

determines secretion of these proteins within the medium. After 48 hours from V1 infection of High Five cells, the medium-containing proteins were collected, and the proteins were then purified as described in paragraph 4.3.

4.3 Protein expression and purification

GST-Grnd intracellular fragments were cloned into a modified version of pGEX-6PI vector (GE Healthcare), and expressed in BL21 *E. coli* cells by induction with 1 mM IPTG at 37 °C for three hours. Cells were lysed in 0.1 M Tris-HCl pH 8, 0.3 M NaCl, 10 % glycerol, 0.5 mM EDTA, and 1 mM DTT, and centrifuged for 1 hour at 100000 g. Cleared lysates were affinity-purified by incubation with Glutathione (GSH) Sepharose-4 Fast-Flow beads (GE Healthcare). GST-Fas constructs were expressed by overnight induction with 0.5 mM IPTG at 18 °C and purified similarly.

Drosophila Veli full length (Veli1-195) and PDZ domain (Veli85-195) expression was induced in *E. coli* strain BL21 overnight at 20 °C with 0.5 mM IPTG as an amino terminal His₆- tagged proteins using a pET43 vector. Cells were lysed in 0.1 M Tris-HCl pH 8, 0.3 M NaCl, 10 % glycerol, 5 mM imidazole, and 2 mM β-mercaptoethanol, and cleared for 1 hour at 100000 g. Proteins were purified by affinity on Ni-NTA beads (Qiagen). The eluted proteins were dialyzed against 0.01 M Hepes pH 7.5, 0.06 M NaCl, 5 % glycerol, 0.5 mM EDTA and 1 mM DTT, concentrated and stored at -80 °C. Veli L27 domain (Veli1-81) was cloned with an N-terminal His₆-MBP-tag using a modified pET43 vector, and purified by a first affinity step on amylose beads (NEB). After washes, fusion proteins retained on beads were incubated with TEV protease (Invitrogen Life Technologies) for 1.5 hours at 20 °C to remove the His₆-MBP tag. The cleaved material was eluted from beads on a desalting buffer consisting of 0.01 M Tris pH 8, 0.1 M NaCl, 10% glycerol, 0.5 mM EDTA, and 1 mM DTT. After buffer dilution to reach a final salt concentration of 0.02 M NaCl, the protein was loaded on a Resource-Q anion-exchange column (GE Healthcare), eluted with a gradient of 0.02 -0.120 M NaCl in 20 column volumes, pooled, and stored at -80 °C.

The MATH domain of *Drosophila* Traf2 (Traf2-MATH, corresponding to residues 319-475) was similarly expressed as a His₆-tagged protein and purified by affinity on Ni-NTA beads. After elution with 200 mM imidazole, a dialysis overnight with 0.01 M Tris-HCl pH 6.8, 0.15 M NaCl, 5 % glycerol, 0.5 mM EDTA, and 1 mM DTT was performed, followed by ion-exchange chromatography on a Resource-S column (GE-Healthcare).

Drosophila GST-Baz-PDZ1 (amino acids 331-419) was affinity purified by incubation with GSH beads. Species retained on beads were cleaved overnight with GST-PreScission protease (GE Healthcare) overnight at 4 °C to remove the GST-tag and then purified by ion-exchange chromatography. Human VeliC-PDZ (85-196) and Dlg1-PDZ123, Dlg1-PDZ12, Dlg1-PDZ23, Dlg1-PDZ1, Dlg1-PDZ2, and Dlg1-PDZ3 (encompassing amino acids 221-546, 221-405, 314-546, 221-312, 314-405, 457-546, respectively) were produced as His₆-fusion proteins, purified by affinity on Ni-NTA beads, and further subjected to ion exchange chromatography. These proteins were expressed in *E. coli* BL21 strain upon induction with 0.5 mM IPTG at 18 °C.

Grnd extracellular domain, corresponding to residues 30-97, was cloned in a pETM14 vector with a cleavable N-terminal His₆-tag and expressed in SHuffle B cells (NEB) with 0.5 mM IPTG at 16 °C. The clear lysate was injected on a HiTrap chelating column (GE healthcare) loaded with Ni²⁺. The protein was eluted by applying a 5-200 mM imidazole gradient, and dialyzed overnight against 0.01 M Tris-HCl pH 8, 0.03 M NaCl and 5 % glycerol in the presence of PreScission protease to remove the His₆-tag prior to injection onto a Resource-Q anion exchange column. Grnd-ECD was eluted with a gradient of 0.03-0.120 M NaCl in 10 column volumes. Fractions containing pure protein were pooled and desalted in 0.01 M Hepes pH 7.5, 0.05 M NaCl. Grnd-ECD was also produced as a secreted protein by infection of High Five insect cells. The High five culture medium was collected, filtered through a Sartolab 0.22 µm PES membrane (Sartorius), and the protein purified by affinity on Ni Excel beads (GE Healthcare). The protein eluted from beads was further purified similarly to Grnd-ECD produced by bacteria SHuffle cells, as described above.

Eiger-ECD was cloned in a pET43 vector with an N-terminal His₆-tag and expressed in SHuffle cells similarly to Grnd-ECD. Clear cell lysates from three litres of culture were affinity-purified on a HiTrap column. After the elution from the HiTrap column, the peak fractions were pooled, concentrated to 1 mL and injected on an HiLoad 16/600 Superdex 200 column equilibrated in a buffer containing 0.02 M Tris pH 8, 0.3 M NaCl, 5 % glycerol. Eiger-TNF-WT and carrying the Asn400Ala mutation were cloned in the same baculovirus vector used for Grnd-ECD, and purified by affinity and gel filtration. All purification steps were conducted at 4 °C. Grnd and Eiger point mutations were generated by QuikChange strategy (Stratagene) according to manufacturer's instructions. All clones were sequence verified.

4.4. *In vitro* binding assays

4.4.1 Pull-down assays

For *in vitro* pull-down assay of Fig. 31B, 5 µM of GST-Grnd intracellular constructs immobilized on GSH-beads were incubated for 40 minutes at 4 °C with 10 µM of Veli full-length, L27 and PDZ domain in 0.01 M Hepes pH 7.4, 0.03 M NaCl, 5 % glycerol, 0.05 % Tween, 0.5 mM EDTA and 1 mM DTT. After washes, proteins retained on beads were separated by SDS-PAGE and analysed by Coomassie staining. The ability of Grnd-ICD to interact with Traf2-MATH was tested incubating 2 µM of Grnd, wild type or mutated on P200 and on P200/E202, with 10 µM Traf2-MATH in 0.01 mM Hepes pH 7.5, 0.15 M NaCl, 5 % glycerol, 0.05 % Tween, 0.5 mM EDTA, and 1 mM DTT for 1 hour at 4 °C. Species retained on beads were detected by SDS-PAGE.

Similarly, 5 µM of GST-Fas fragments adsorbed on beads were incubated for 1 hour with 10 µM VeliC-PDZ or Dlg1-PDZ constructs in 0.01 mM Hepes pH 7.5, 0.1 mM NaCl, 5 % glycerol, 0.05 % Tween, 0.5 mM EDTA, and 1 mM DTT. Bound species were visualized as described above. For the *in vitro* competition pull-down assay of Fig. 34, 3 µM of Fas-319-335 were incubated with 7.5 µM VeliC-PDZ in 0.01 M Hepes pH 7.5, 0.05 M NaCl, 5 %

glycerol, 0.05 % Tween, 0.5 mM EDTA, and 1 mM DTT. After one hour, Dlg1-PDZ123 was added at 0 μ M, 5 μ M, 10 μ M, 30 μ M, and 50 μ M, and incubated for additional 60 minutes. Species retained on beads were separated on SDS-PAGE and visualized by Coomassie blue.

To analyse the molecular determinants of the interaction of Grnd-ECD with Eiger-ECD in Fig. 26A and Fig.26E, 2 μ M of Eiger-ECD fused with a strep-tag were adsorbed on Strep-tactin beads and incubated with 10 μ M of Grnd-ECD either wild-type or carrying specific mutations, in 0.01 M Hepes pH 7.5, 0.1 M NaCl, 5 % glycerol, 0.05 % Tween, 0.5 mM EDTA.

For pull-experiments against cell lysates (Fig. 33A), 5 μ M GST-Fas constructs immobilized on GSH beads were incubated with 2 mg of HEK293T lysates for 2 hours at 4 °C in JS buffer (0.05 M Hepes pH 7.5, 0.15 M NaCl, 10 % glycerol, 1 % tryton, 1.5 mM MgCl₂, 5 mM EDTA) supplemented with proteases and phosphatases inhibitors (PhosSTOP, Roche). After three washing steps, detection of Dlg1 bound to Fas was performed by immunoblotting using a monoclonal antibody generated in-house. The Ponceau-stained membrane was used as a loading control for GST-fusion proteins.

4.4.2 Analytical Size Exclusion Chromatography (SEC)

For analytical SEC analyses of Fig. 16A, Fig. 28C, Fig. 29B-C and Fig.30, 30 μ M of Eiger-ECD and Eiger-TNF-WT or Asn400Ala or unmodified were loaded singularly or in combination with Grnd-ECD in a 1:1.1 molar ratio on a Superdex 5/150 (GE Healthcare) equilibrated in 0.01 M Hepes pH 7.5, 0.3 M NaCl, 5% glycerol, and eluted in 50 μ l fractions. The presence of the proteins in the elution volume was monitored by absorbance at 280 nm (expressed as mAu), and subsequently checked by SDS-PAGE followed by Coomassie staining.

4.4.3 Isothermal Titration Calorimetry (ITC)

Purified Eiger-ECD and Grnd-ECD (wild-type and mutated species) were dialyzed overnight against 0.02 M Tris pH 8 and 0.3 M NaCl. The thermodynamical parameters of the association between Eiger and Grnd were measured using a MicroCal PEAQ-ITC (Malvern, UK). At 25 °C 8 µM of Eiger-ECD were titrated with 19 µl injections in 100 µM of Grnd-ECD. Heats of ligand binding were fitted to a single-site binding curve with the MicroCal PEAQ-ITC software.

4.5 Analysis of protein glycosylation state

Deglycosylation of Eiger-TNF secreted from High Five cells (Fig. 28B) was performed incubating 30 µg of the sample for 10 minutes at 100 °C in the presence of SDS to denature the protein. The sample was then incubated with GlycoBuffer2 1X (NEB) and 2 µl of PNGase-F (NEB) for one hour at 37 °C. Deglycosylation of the sample was assessed by SDS-PAGE and Coomassie blue staining.

4.6 Immunoblotting

To assess the levels of Dlg1 bound to Fas (Fig. 33A), species retained on beads as described before were separated by SDS-PAGE and transferred onto a nitrocellulose 0.45 µm membrane (GE Healthcare). 50 µM of extracts per lane were loaded. Blocking and antibody incubations were performed at room temperature in TBS containing 0.1 % Tween-20 and 1% low-fat milk. The antibody used was mouse anti-Dlg1 (Mapelli's lab, at 1:1000 dilution). To study the pattern of degradation of Eiger-ECD (Fig. 21B), crystals of the Eiger-ECD:Grnd-ECD complex were collected, dissolved in water, and loaded on a SDS-PAGE that was transferred to a nitrocellulose membrane. Immunoblot analysis was done incubating the membrane with a mouse anti-His antibody (Santa Cruz Biotechnology, at 1:200 dilution) at room temperature in TBS additioned with 0.1% Tween 20 and 1 % low-fat milk.

4.7 Sequence alignment

The Grnd sequence from *Drosophila melanogaster* (Uniprot entry Q9VJ83) was aligned to human TNFR BCMA (Uniprot entry Q02223), TACI (Uniprot entry O14836), Fas (Uniprot entry P25445), DcR1 (Uniprot entry O14798), DcR2 (Uniprot entry Q9UBN6), DR4 (Uniprot entry O00220), DR5 (Uniprot entry O14763), Fn14 (Uniprot entry Q9NP84), BAFFR (Uniprot entry Q96RJ3), TNFRI (Uniprot entry P19438), and to *Xenopus laevis* Fn14 (Q6SIX7) via Clustal Omega (185), and coloured by percentage of identity with Jalview (186) (Fig. 14A). Fly Veli (Uniprot entry Q8IMT8), human VeliA (Uniprot entry O14910), VeliB (Uniprot entry Q9HAP6), and Veli C (Uniprot entry Q9NUP9) were analysed with the same procedure described above (Fig. 32A).

4.8 Static Light Scattering

Static Light Scattering (SLS) analyses of Grnd-ECD were performed on a Viscotek GPCmax/TDA (Malvern, UK) instrument, connected in tandem with two TSKgel G3000PWxl size-exclusion chromatography columns (Tosoh bioscience) in series. The system was equilibrated in a buffer containing 0.01 M Hepes pH 7.5 and 0.05 M NaCl, and calibrated with BSA. Eiger-ECD and the Eiger-ECD:Grnd-ECD complex was loaded on a Superdex200 10/300 increase column (GE Healthcare) coupled to the same Viscotek GPCmax instrument equilibrated in a buffer containing 0.02 M Tris-HCl pH 8, 0.3 M NaCl, 5 % glycerol. About 75 μ L of the samples concentrated at about 2 mg/mL for the Eiger-ECD:Grnd-ECD complex were loaded on the columns, and eluted isocratically.

4.9 Sample preparation for N-terminal sequencing and intact MW determination

In order to define the sequence of the fragment of Eiger-ECD obtained by proteolysis (Fig. 21C), 150 μ g of the Eiger-ECD:Grnd-ECD purified complex were incubated with trypsin

(Sigma Aldrich) with a 1:20 weight:weight ratio for 30 minutes at 4 °C. Proteins were further purified on a Superdex 75 15/150 column equilibrated in a buffer containing 0.01 M Tris-HCl pH 8 and 0.3 M NaCl. The fractions containing the trypsinized proteins were collected, and sent in solution to IFOM proteomics facility (Milan, Italy) for intact MW determination by MALDI-TOF MS. The same fractions were separated on SDS-PAGE and transferred onto a PVDF membrane. After visualization by Ponceau staining, the band containing the proteolyzed Eiger-ECD fragment was excised, and sent to AltaBioscience (Redditch, UK) for N-terminal sequencing.

4.10 Protein crystallization and structure determination

4.10.1 Grnd-ECD sample preparation and crystallization conditions

Grnd-ECD was incubated with trypsin in a 1:50 ratio for 16 hours at 4 °C, and subsequently purified on a HiLoad 16/600 Superdex 200 in 0.02 M Tris-HCl pH 8, and 0.05 M NaCl. Initial crystallization trials of the Grnd-ECD sample were performed with the commercial screen Index HT and Hampton 12 (Hampton Research) at 82 mg/ml, and conducted in sitting-drop vapor-diffusion format using a Mosquito nanodispenser (TTP Labtech) in MRC 2 well crystallization plates (Swissci, Hampton Research). 100 nl protein solution were mixed with an equal volume of reservoir solution at 20 °C. Crystals appeared after 2 days in about 30 % of conditions containing PEG and MPD. Crystals were flash-cooled in liquid nitrogen without additional cryo-protection.

4.10.2 Data collection, processing, and structure determination of Grnd-ECD

The best crystals of Grnd-ECD grew in condition E4 of Index screen, composed by 0.1 M Hepes pH 7.5, 45% MPD, 0.2 M ammonium acetate and diffracted at 0.9256 Å. A 0.9256 Å native dataset of these crystals was collected at a wavelength of 0.978 Å at beamline PXIII of the Swiss Light Source (SLS), Villigen, Switzerland. Crystals belong to the space group P4₁2₁2, with a single molecule per asymmetric unit. Due to the lack of homologous

structures, and thanks to the high sulphur content of Grnd-ECD (more than 10% of the protein sequence were Cys or Met), experimental phases were obtained by Sulphur Single-wavelength Anomalous Diffraction (S-SAD) data collection with X-ray at 2.075 Å wavelength. In this experiment, crystals diffracted to 2.5 Å resolution. Anomalous signal coming from native sulphurs allowed the localization of these elements and the calculation of initial maps. The model built from the S-SAD dataset was used for molecular replacement in the 0.9256 Å native dataset, and refined in Phenix (165) performing molecular replacement of the initial density map against the 0.9256 Å resolution data to a final R_{free} of 15.6 % And an R_{work} of 13 %. Data collection statistics are shown in Table 2. The structure was illustrated with PyMol (DeLano Scientific LLC).

4.10.3 Crystallization of Eiger-ECD:Grnd-ECD

Purified Eiger-ECD and Grnd-ECD were incubated in a 1:1.3 molar ratio for 45 minutes at 4 °C, then trypsin was added at a 1:50 weight:weight ratio for 2 hours at 4 °C. The complex was further purified by gel filtration on a Superdex 200 10/300 column (GE Healthcare) and concentrated to 28 mg/ml. An initial manual screening was performed by mixing 1 µl protein with 1 µl of reservoirs with the PCT reagents (Pre-Crystallization Test, Hampton Research). Crystals grew up after few hours in condition B2 of the PCT, consisting of 0.1 M Tris-HCl pH 8.5, 0.2 M MgCl_2 , 15 % PEG 4000. In order to obtain larger crystals and to slow down their growth, we performed a series of grid screens around the initial condition. In particular, we varied the concentration of PEG4000 and of the protein, the pH values, the temperature, and the seeding time, as detailed in paragraph 2.4.2. Crystals were cryo-protected by adding 20 % ethylene glycol to the reservoir condition, and flash-cooled in liquid nitrogen.

4.10.4 Eiger-ECD:Grnd-ECD: data collection, processing, and structure determination

X-ray diffraction data of Eiger-ECD:Grnd-ECD crystals were collected at the beamline ID23-2 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The best

crystals grew in a buffer consisting of 0.1 M Tris-HCl pH 8.5, 14 % PEG 4000, 0.2 M MgCl₂, and diffracted at 2.02 Å resolution. They belong to the spacegroup P321. A copy of Grnd-ECD and Eiger-ECD was present in the asymmetric unit, consistent with a Matthews coefficient of 2.24 Å³ Da⁻¹ and a solvent content of 45.12 %. The structure was solved by molecular replacement with *Phaser* (168), using as a search model the coordinates of human EDA-A2 (PDB ID: 1RJ8), after threading of the Eiger sequence in Phyre2 (169) (see main text for more details). The model was refined to a final R_{free} of 21.4 % and R_{work} of 19.2 %. Data collection statistics are given in Table 3. The structure was shown with PyMol.

4.11 Protocols for *in vivo* assays in *Drosophila melanogaster*

4.11.1 Cell culture

Drosophila S2 and S2R⁺ cells were grown in Complete Schneiders Medium (CSM, Invitrogen) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, BioWhittaker), 50 U/ml penicillin, and 50 mg/ml streptomycin (Invitrogen) at 25 °C. Transfections were performed using Effectene reagent (Qiagen) according to manufacturer's instructions.

4.11.2 Immunoprecipitation

IPs were performed from about 1 x 10⁷ S2 cells. Cells were lysed in 200 µl of a buffer composed by 0.05 M Tris-HCl pH 8, 0.15 M NaCl, 1% NP-50, 1 mM EGTA, 0.5 M NaF, phosphatase inhibitor cocktail 2 (Sigma), Complete protease inhibitor cocktail (Roche), and cell extracts were cleared by centrifugation at 10,000 rpm for 15 min. The cleared extracts were incubated with protein G-SepharoseTM beads (Sigma) for 1 hour to reduce unspecific binding of proteins to the beads. The precleared extracts were then incubated with 80 µl of protein G-Sepharose beads and 1 µl of the specific antibody for 3 hrs. Subsequently, beads were washed 3 times in the lysis buffer, boiled in sample and reducing buffers (Invitrogen).

For further details on protocols for *in vivo* assay in *Drosophila melanogaster*, refer to Andersen *et al.*, 2015 (153).

14.12 Genotypes

Figure 14:

D: w^- ; +; *rn-Gal4/puc-lacZ*

E: w^- ; *elav-Gal80/+*; *rn-Gal4, UAS avl RNAi GD/puc-lacZ*

F: w^- ; *elav-Gal80/grnd RNAi KK*; *rn-Gal4, UAS avl RNAi GD/puc-lacZ*

G: w^- ; *elav-Gal80/tak1 RNAi KK*; *rn-Gal4, UAS avl RNAi GD/puc-lacZ*

Figure 31:

E: w^- ; *UAS crb-intra/+* ; *rn-Gal4 /+*

F: w^- ; *UAS crb-intra/ UAS puc^{2A}*; *rn-Gal4 /+*

G: w^- ; *UAS crb-intra/grnd RNAi KK*; *rn-Gal4 /+*

H: w^- ; *UAS crb-intra/veli RNAi* ; *rn-Gal4/veli RNAi*

5 REFERENCES

1. Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3(9):745-756.
2. Bodmer JL, Schneider P, & Tschopp J (2002) The molecular architecture of the TNF superfamily. *Trends Biochem Sci* 27(1):19-26.
3. Vanamee ES & Faustman DL (2018) Structural principles of tumor necrosis factor superfamily signaling. *Sci Signal* 11(511).
4. Black RA, *et al.* (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 385(6618):729-733.
5. Powell WC, Fingleton B, Wilson CL, Boothby M, & Matrisian LM (1999) The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr Biol* 9(24):1441-1447.
6. Chen Y, *et al.* (2001) Mutations within a furin consensus sequence block proteolytic release of ectodysplasin-A and cause X-linked hypohidrotic ectodermal dysplasia. *Proc Natl Acad Sci U S A* 98(13):7218-7223.
7. Audo R, *et al.* (2014) Distinct effects of soluble and membrane-bound fas ligand on fibroblast-like synoviocytes from rheumatoid arthritis patients. *Arthritis Rheumatol* 66(12):3289-3299.
8. Matsumoto H, *et al.* (2015) Membrane-bound and soluble Fas ligands have opposite functions in photoreceptor cell death following separation from the retinal pigment epithelium. *Cell Death Dis* 6:e1986.
9. Locksley RM, Killeen N, & Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104(4):487-501.
10. Degli-Esposti MA, *et al.* (1997) Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* 186(7):1165-1170.
11. Durkop H, *et al.* (1992) Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. *Cell* 68(3):421-427.
12. Liu Y, *et al.* (2003) Ligand-receptor binding revealed by the TNF family member TALL-1. *Nature* 423(6935):49-56.
13. Naismith JH & Sprang SR (1998) Modularity in the TNF-receptor family. *Trends Biochem Sci* 23(2):74-79.
14. Gordon NC, *et al.* (2003) BAFF/BLyS receptor 3 comprises a minimal TNF receptor-like module that encodes a highly focused ligand-binding site. *Biochemistry* 42(20):5977-5983.
15. Kim HM, *et al.* (2003) Crystal structure of the BAFF-BAFF-R complex and its implications for receptor activation. *Nat Struct Biol* 10(5):342-348.
16. Naismith JH, Devine TQ, Kohno T, & Sprang SR (1996) Structures of the extracellular domain of the type I tumor necrosis factor receptor. *Structure* 4(11):1251-1262.
17. He F, *et al.* (2009) Solution structure of the cysteine-rich domain in Fn14, a member of the tumor necrosis factor receptor superfamily. *Protein Sci* 18(3):650-656.
18. Pellegrini M, *et al.* (2013) Structure of the extracellular domains of human and *Xenopus* Fn14: implications in the evolution of TWEAK and Fn14 interactions. *FEBS J* 280(8):1818-1829.
19. Hymowitz SG, *et al.* (2005) Structures of APRIL-receptor complexes: like BCMA, TACI employs only a single cysteine-rich domain for high affinity ligand binding. *J Biol Chem* 280(8):7218-7227.
20. Zhan C, *et al.* (2011) Decoy strategies: the structure of TL1A:DcR3 complex. *Structure* 19(2):162-171.
21. An HJ, *et al.* (2011) Crystallographic and mutational analysis of the CD40-CD154 complex and its implications for receptor activation. *J Biol Chem* 286(13):11226-11235.
22. Ramamurthy V, *et al.* (2015) The structure of the death receptor 4-TNF-related apoptosis-inducing ligand (DR4-TRAIL) complex. *Acta Crystallogr F Struct Biol Commun* 71(Pt 10):1273-1281.
23. Naismith JH, Devine TQ, Brandhuber BJ, & Sprang SR (1995) Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor. *J Biol Chem* 270(22):13303-13307.
24. Papoff G, *et al.* (1999) Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. *J Biol Chem* 274(53):38241-38250.
25. Siegel RM, *et al.* (2000) Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* 288(5475):2354-2357.
26. Chan FK, *et al.* (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* 288(5475):2351-2354.

27. Clancy L, *et al.* (2005) Preligand assembly domain-mediated ligand-independent association between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis. *Proc Natl Acad Sci U S A* 102(50):18099-18104.
28. Smulski CR, *et al.* (2013) Cysteine-rich domain 1 of CD40 mediates receptor self-assembly. *J Biol Chem* 288(15):10914-10922.
29. Edmond V, *et al.* (2012) Precise mapping of the CD95 pre-ligand assembly domain. *PLoS One* 7(9):e46236.
30. Chan FK (2007) Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. *Cytokine* 37(2):101-107.
31. Brown SA, Hanscom HN, Vu H, Brew SA, & Winkles JA (2006) TWEAK binding to the Fn14 cysteine-rich domain depends on charged residues located in both the A1 and D2 modules. *Biochem J* 397(2):297-304.
32. Liu W, *et al.* (2014) Mechanistic basis for functional promiscuity in the TNF and TNF receptor superfamilies: structure of the LIGHT:DcR3 assembly. *Structure* 22(9):1252-1262.
33. Hehlhans T & Pfeffer K (2005) The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* 115(1):1-20.
34. Huang B, Eberstadt M, Olejniczak ET, Meadows RP, & Fesik SW (1996) NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. *Nature* 384(6610):638-641.
35. Scott FL, *et al.* (2009) The Fas-FADD death domain complex structure unravels signalling by receptor clustering. *Nature* 457(7232):1019-1022.
36. Park YH, Jeong MS, & Jang SB (2014) Death domain complex of the TNFR-1, TRADD, and RIP1 proteins for death-inducing signaling. *Biochem Biophys Res Commun* 443(4):1155-1161.
37. Yurchenko M, Shlapatska LM, & Sidorenko SP (2012) The multilevel regulation of CD95 signaling outcome. *Exp Oncol* 34(3):153-159.
38. Andera L (2009) Signaling activated by the death receptors of the TNFR family. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 153(3):173-180.
39. Ye H, Park YC, Kreishman M, Kieff E, & Wu H (1999) The structural basis for the recognition of diverse receptor sequences by TRAF2. *Mol Cell* 4(3):321-330.
40. Wu H, Park YC, Ye H, & Tong L (1999) Structural studies of human TRAF2. *Cold Spring Harb Symp Quant Biol* 64:541-549.
41. Moreno E, Yan M, & Basler K (2002) Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. *Curr Biol* 12(14):1263-1268.
42. Igaki T, *et al.* (2002) Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. *Embo J* 21(12):3009-3018.
43. Shklover J, Levy-Adam F, & Kuran E (2015) The role of Drosophila TNF Eiger in developmental and damage-induced neuronal apoptosis. *FEBS Lett* 589(8):871-879.
44. Kauppila S, *et al.* (2003) Eiger and its receptor, Wengen, comprise a TNF-like system in Drosophila. *Oncogene* 22(31):4860-4867.
45. Agrawal N, *et al.* (2016) The Drosophila TNF Eiger Is an Adipokine that Acts on Insulin-Producing Cells to Mediate Nutrient Response. *Cell Metab* 23(4):675-684.
46. Perez-Garijo A, Fuchs Y, & Steller H (2013) Apoptotic cells can induce non-autonomous apoptosis through the TNF pathway. *Elife* 2:e01004.
47. Wang SL, Hawkins CJ, Yoo SJ, Muller HA, & Hay BA (1999) The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* 98(4):453-463.
48. Goyal L, McCall K, Agapite J, Hartwig E, & Steller H (2000) Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function. *Embo J* 19(4):589-597.
49. Igaki T, Pastor-Pareja JC, Aonuma H, Miura M, & Xu T (2009) Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. *Dev Cell* 16(3):458-465.
50. Ruan W, Srinivasan A, Lin S, Kara k I, & Barker PA (2016) Eiger-induced cell death relies on Rac1-dependent endocytosis. *Cell Death Dis* 7:e2181.
51. Vidal M (2010) The dark side of fly TNF: an ancient developmental proof reading mechanism turned into tumor promoter. *Cell Cycle* 9(19):3851-3856.
52. Uhlirova M & Bohmann D (2006) JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. *Embo J* 25(22):5294-5304.
53. Igaki T, Pagliarini RA, & Xu T (2006) Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. *Curr Biol* 16(11):1139-1146.
54. Cordero JB, *et al.* (2010) Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. *Dev Cell* 18(6):999-1011.
55. Kanda H, Igaki T, Kanuka H, Yagi T, & Miura M (2002) Wengen, a member of the Drosophila tumor necrosis factor receptor superfamily, is required for Eiger signaling. *J Biol Chem* 277(32):28372-28375.

56. Ruan W, Unsain N, Desbarats J, Fon EA, & Barker PA (2013) Wengen, the sole tumour necrosis factor receptor in *Drosophila*, collaborates with moesin to control photoreceptor axon targeting during development. *PLoS One* 8(3):e60091.
57. Yonehara S, Ishii A, & Yonehara M (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J Exp Med* 169(5):1747-1756.
58. Trauth BC, *et al.* (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 245(4915):301-305.
59. Itoh N, *et al.* (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66(2):233-243.
60. Eramo A, *et al.* (2004) CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells. *Eur J Immunol* 34(7):1930-1940.
61. Medema JP, *et al.* (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J* 16(10):2794-2804.
62. Salvesen GS & Dixit VM (1999) Caspase activation: the induced-proximity model. *Proc Natl Acad Sci U S A* 96(20):10964-10967.
63. Peter ME & Krammer PH (2003) The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 10(1):26-35.
64. Scaffidi C, *et al.* (1998) Two CD95 (APO-1/Fas) signaling pathways. *Embo J* 17(6):1675-1687.
65. Wei MC, *et al.* (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 14(16):2060-2071.
66. Adrain C, Creagh EM, & Martin SJ (2001) Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *Embo J* 20(23):6627-6636.
67. Shinohara H, Yagita H, Ikawa Y, & Oyaizu N (2000) Fas drives cell cycle progression in glioma cells via extracellular signal-regulated kinase activation. *Cancer Res* 60(6):1766-1772.
68. Barnhart BC, *et al.* (2004) CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. *Embo J* 23(15):3175-3185.
69. Chen L, *et al.* (2010) CD95 promotes tumour growth. *Nature* 465(7297):492-496.
70. Lee SM, Kim EJ, Suk K, & Lee WH (2011) Stimulation of Fas (CD95) induces production of pro-inflammatory mediators through ERK/JNK-dependent activation of NF-kappaB in THP-1 cells. *Cell Immunol* 271(1):157-162.
71. Stronach B (2005) Dissecting JNK signaling, one KKKinase at a time. *Dev Dyn* 232(3):575-584.
72. Hibi M, Lin A, Smeal T, Minden A, & Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* 7(11):2135-2148.
73. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* 103(2):239-252.
74. Takatsu Y, *et al.* (2000) TAK1 participates in c-Jun N-terminal kinase signaling during *Drosophila* development. *Mol Cell Biol* 20(9):3015-3026.
75. Glise B, Bourbon H, & Noselli S (1995) hemipterous encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. *Cell* 83(3):451-461.
76. Perkins KK, Admon A, Patel N, & Tjian R (1990) The *Drosophila* Fos-related AP-1 protein is a developmentally regulated transcription factor. *Genes Dev* 4(5):822-834.
77. Xue L, *et al.* (2007) Tumor suppressor CYLD regulates JNK-induced cell death in *Drosophila*. *Dev Cell* 13(3):446-454.
78. Ma X, *et al.* (2013) dUev1a modulates TNF-JNK mediated tumor progression and cell death in *Drosophila*. *Dev Biol* 380(2):211-221.
79. Ma X, *et al.* (2014) Bendless modulates JNK-mediated cell death and migration in *Drosophila*. *Cell Death Differ* 21(3):407-415.
80. Adhikari A & Chen ZJ (2009) Diversity of polyubiquitin chains. *Dev Cell* 16(4):485-486.
81. Shi CS & Kehrl JH (2003) Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). *J Biol Chem* 278(17):15429-15434.
82. Habelhah H, *et al.* (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. *Embo J* 23(2):322-332.
83. Martin-Blanco E, *et al.* (1998) puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev* 12(4):557-570.
84. Dobens LL, Martin-Blanco E, Martinez-Arias A, Kafatos FC, & Raftery LA (2001) *Drosophila* puckered regulates Fos/Jun levels during follicle cell morphogenesis. *Development* 128(10):1845-1856.
85. McEwen DG & Peifer M (2005) Puckered, a *Drosophila* MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. *Development* 132(17):3935-3946.
86. Kyriakis JM & Avruch J (1990) pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem* 265(28):17355-17363.

87. Botella JA, *et al.* (2001) The Drosophila cell shape regulator c-Jun N-terminal kinase also functions as a stress-activated protein kinase. *Insect Biochem Mol Biol* 31(9):839-847.
88. Ohsawa S, *et al.* (2011) Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila. *Dev Cell* 20(3):315-328.
89. Bilder D (2004) Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* 18(16):1909-1925.
90. Pagliarini RA & Xu T (2003) A genetic screen in Drosophila for metastatic behavior. *Science* 302(5648):1227-1231.
91. Bergmann A, Agapite J, McCall K, & Steller H (1998) The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. *Cell* 95(3):331-341.
92. Kurada P & White K (1998) Ras promotes cell survival in Drosophila by downregulating hid expression. *Cell* 95(3):319-329.
93. Kulshammer E, *et al.* (2015) Interplay among Drosophila transcription factors Ets21c, Fos and Ftz-F1 drives JNK-mediated tumor malignancy. *Dis Model Mech* 8(10):1279-1293.
94. Pastor-Pareja JC, Wu M, & Xu T (2008) An innate immune response of blood cells to tumors and tissue damage in Drosophila. *Dis Model Mech* 1(2-3):144-154; discussion 153.
95. La Fortezza M, *et al.* (2016) JAK/STAT signalling mediates cell survival in response to tissue stress. *Development* 143(16):2907-2919.
96. Laprise P & Tepass U (2011) Novel insights into epithelial polarity proteins in Drosophila. *Trends Cell Biol* 21(7):401-408.
97. Martin-Belmonte F & Perez-Moreno M (2012) Epithelial cell polarity, stem cells and cancer. *Nat Rev Cancer* 12(1):23-38.
98. Ellenbroek SI, Iden S, & Collard JG (2012) Cell polarity proteins and cancer. *Semin Cancer Biol* 22(3):208-215.
99. Tepass U & Knust E (1990) Phenotypic and developmental analysis of mutations at the crumbs locus, a gene required for the development of epithelia in Drosophila melanogaster. *Roux Arch Dev Biol* 199(4):189-206.
100. Tepass U, Theres C, & Knust E (1990) crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. *Cell* 61(5):787-799.
101. Bachmann A, Schneider M, Theilenberg E, Grawe F, & Knust E (2001) Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. *Nature* 414(6864):638-643.
102. Roh MH, *et al.* (2002) The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J Cell Biol* 157(1):161-172.
103. Bulgakova NA, Kempkens O, & Knust E (2008) Multiple domains of Stardust differentially mediate localisation of the Crumbs-Stardust complex during photoreceptor development in Drosophila. *J Cell Sci* 121(Pt 12):2018-2026.
104. Zhang J, *et al.* (2012) Structure of an L27 domain heterotrimer from cell polarity complex Patj/Pals1/Mals2 reveals mutually independent L27 domain assembly mode. *J Biol Chem* 287(14):11132-11140.
105. Grawe F, Wodarz A, Lee B, Knust E, & Skaer H (1996) The Drosophila genes crumbs and stardust are involved in the biogenesis of adherens junctions. *Development* 122(3):951-959.
106. Wodarz A, Hinz U, Engelbert M, & Knust E (1995) Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* 82(1):67-76.
107. Sotillos S, Diaz-Meco MT, Caminero E, Moscat J, & Campuzano S (2004) DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. *J Cell Biol* 166(4):549-557.
108. Straight SW, *et al.* (2004) Loss of PALS1 expression leads to tight junction and polarity defects. *Mol Biol Cell* 15(4):1981-1990.
109. Michel D, *et al.* (2005) PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells. *J Cell Sci* 118(Pt 17):4049-4057.
110. Olsen O, *et al.* (2007) Renal defects associated with improper polarization of the CRB and DLG polarity complexes in MALS-3 knockout mice. *J Cell Biol* 179(1):151-164.
111. Bohl J, Brimer N, Lyons C, & Vande Pol SB (2007) The stardust family protein MPP7 forms a tripartite complex with LIN7 and DLG1 that regulates the stability and localization of DLG1 to cell junctions. *J Biol Chem* 282(13):9392-9400.
112. Penkert RR, DiVittorio HM, & Prehoda KE (2004) Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. *Nat Struct Mol Biol* 11(11):1122-1127.
113. Wang Q, Hurd TW, & Margolis B (2004) Tight junction protein Par6 interacts with an evolutionarily conserved region in the amino terminus of PALS1/stardust. *J Biol Chem* 279(29):30715-30721.
114. Kemphues KJ, Priess JR, Morton DG, & Cheng NS (1988) Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell* 52(3):311-320.
115. Tabuse Y, *et al.* (1998) Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. *Development* 125(18):3607-3614.

116. Kuchinke U, Grawe F, & Knust E (1998) Control of spindle orientation in *Drosophila* by the Par-3-related PDZ-domain protein Bazooka. *Curr Biol* 8(25):1357-1365.
117. Betschinger J, Mechtler K, & Knoblich JA (2003) The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* 422(6929):326-330.
118. Yamanaka T, *et al.* (2003) Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr Biol* 13(9):734-743.
119. Plant PJ, *et al.* (2003) A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat Cell Biol* 5(4):301-308.
120. Horikoshi Y, *et al.* (2009) Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. *J Cell Sci* 122(Pt 10):1595-1606.
121. Morais-de-Sa E, Mirouse V, & St Johnston D (2010) aPKC phosphorylation of Bazooka defines the apical/lateral border in *Drosophila* epithelial cells. *Cell* 141(3):509-523.
122. Nagai-Tamai Y, Mizuno K, Hirose T, Suzuki A, & Ohno S (2002) Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells* 7(11):1161-1171.
123. Benton R & St Johnston D (2003) A conserved oligomerization domain in *Drosophila* Bazooka/PAR-3 is important for apical localization and epithelial polarity. *Curr Biol* 13(15):1330-1334.
124. Mizuno K, *et al.* (2003) Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. *J Biol Chem* 278(33):31240-31250.
125. Bilder D & Perrimon N (2000) Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* 403(6770):676-680.
126. Woods DF & Bryant PJ (1991) The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* 66(3):451-464.
127. Navarro C, *et al.* (2005) Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene* 24(27):4330-4339.
128. Kallay LM, McNickle A, Brennwald PJ, Hubbard AL, & Braiterman LT (2006) Scribble associates with two polarity proteins, Lgl2 and Vangl2, via distinct molecular domains. *J Cell Biochem* 99(2):647-664.
129. Mathew D, *et al.* (2002) Recruitment of scribble to the synaptic scaffolding complex requires GUK-holder, a novel DLG binding protein. *Curr Biol* 12(7):531-539.
130. Laprise P, Viel A, & Rivard N (2004) Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. *J Biol Chem* 279(11):10157-10166.
131. Lee S, Fan S, Makarova O, Straight S, & Margolis B (2002) A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. *Mol Cell Biol* 22(6):1778-1791.
132. Laprise P, *et al.* (2009) Yurt, Coracle, Neurexin IV and the Na(+),K(+)-ATPase form a novel group of epithelial polarity proteins. *Nature* 459(7250):1141-1145.
133. Laprise P, *et al.* (2006) The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. *Dev Cell* 11(3):363-374.
134. Gamblin CL, *et al.* (2018) Oligomerization of the FERM-FA protein Yurt controls epithelial cell polarity. *J Cell Biol* 217(11):3853-3862.
135. Gamblin CL, Hardy EJ, Chartier FJ, Bisson N, & Laprise P (2014) A bidirectional antagonism between aPKC and Yurt regulates epithelial cell polarity. *J Cell Biol* 204(4):487-495.
136. Lamb RS, Ward RE, Schweizer L, & Fehon RG (1998) *Drosophila* coracle, a member of the protein 4.1 superfamily, has essential structural functions in the septate junctions and developmental functions in embryonic and adult epithelial cells. *Mol Biol Cell* 9(12):3505-3519.
137. Ward RE, Lamb RS, & Fehon RG (1998) A conserved functional domain of *Drosophila* coracle is required for localization at the septate junction and has membrane-organizing activity. *J Cell Biol* 140(6):1463-1473.
138. Royer C & Lu X (2011) Epithelial cell polarity: a major gatekeeper against cancer? *Cell Death Differ* 18(9):1470-1477.
139. Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646-674.
140. Khursheed M & Bashyam MD (2014) Apico-basal polarity complex and cancer. *J Biosci* 39(1):145-155.
141. Feigin ME, *et al.* (2014) Mislocalization of the cell polarity protein scribble promotes mammary tumorigenesis and is associated with basal breast cancer. *Cancer Res* 74(11):3180-3194.
142. Kapil S, *et al.* (2017) The cell polarity protein Scrib functions as a tumor suppressor in liver cancer. *Oncotarget* 8(16):26515-26531.
143. Gardiol D, Zacchi A, Petrera F, Stanta G, & Banks L (2006) Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *Int J Cancer* 119(6):1285-1290.
144. Kuphal S, *et al.* (2006) Expression of Hugl-1 is strongly reduced in malignant melanoma. *Oncogene* 25(1):103-110.

145. Storrs CH & Silverstein SJ (2007) PATJ, a tight junction-associated PDZ protein, is a novel degradation target of high-risk human papillomavirus E6 and the alternatively spliced isoform 18 E6. *J Virol* 81(8):4080-4090.
146. Knoblich JA (2008) Mechanisms of asymmetric stem cell division. *Cell* 132(4):583-597.
147. Raman R, Pinto CS, & Sonawane M (2018) Polarized Organization of the Cytoskeleton: Regulation by Cell Polarity Proteins. *J Mol Biol* 430(19):3565-3584.
148. Shelly M, *et al.* (2003) Polar expression of ErbB-2/HER2 in epithelia. Bimodal regulation by Lin-7. *Dev Cell* 5(3):475-486.
149. Straight SW, Chen L, Karnak D, & Margolis B (2001) Interaction with mLin-7 alters the targeting of endocytosed transmembrane proteins in mammalian epithelial cells. *Mol Biol Cell* 12(5):1329-1340.
150. Olsen O, Liu H, Wade JB, Merot J, & Welling PA (2002) Basolateral membrane expression of the Kir 2.3 channel is coordinated by PDZ interaction with Lin-7/CASK complex. *Am J Physiol Cell Physiol* 282(1):C183-195.
151. Hong X, Avetisyan M, Ronilo M, & Standley S (2015) SAP97 blocks the RXR ER retention signal of NMDA receptor subunit GluN1-3 through its SH3 domain. *Biochim Biophys Acta* 1853(2):489-499.
152. Nemetschke L & Knust E (2016) Drosophila Crumbs prevents ectopic Notch activation in developing wings by inhibiting ligand-independent endocytosis. *Development* 143(23):4543-4553.
153. Andersen DS, *et al.* (2015) The Drosophila TNF receptor Grindelwald couples loss of cell polarity and neoplastic growth. *Nature*.
154. Sun G & Irvine KD (2011) Regulation of Hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors. *Dev Biol* 350(1):139-151.
155. Muzzopappa M, Murcia L, & Milan M (2017) Feedback amplification loop drives malignant growth in epithelial tissues. *Proc Natl Acad Sci U S A* 114(35):E7291-E7300.
156. Miles WO, Dyson NJ, & Walker JA (2011) Modeling tumor invasion and metastasis in Drosophila. *Dis Model Mech* 4(6):753-761.
157. Gonzalez C (2013) Drosophila melanogaster: a model and a tool to investigate malignancy and identify new therapeutics. *Nat Rev Cancer* 13(3):172-183.
158. Ye H, *et al.* (2002) Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* 418(6896):443-447.
159. Bradley JR & Pober JS (2001) Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* 20(44):6482-6491.
160. Lobstein J, *et al.* (2012) SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microb Cell Fact* 11:56.
161. de Vreede G, *et al.* (2018) A Drosophila Tumor Suppressor Gene Prevents Tonic TNF Signaling through Receptor N-Glycosylation. *Dev Cell* 45(5):595-605 e594.
162. Hymowitz SG, *et al.* (2003) The crystal structures of EDA-A1 and EDA-A2: splice variants with distinct receptor specificity. *Structure* 11(12):1513-1520.
163. Liu Y, *et al.* (2002) Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. *Cell* 108(3):383-394.
164. Wallweber HJ, Compaa DM, Starovasnik MA, & Hymowitz SG (2004) The crystal structure of a proliferation-inducing ligand, APRIL. *J Mol Biol* 343(2):283-290.
165. Adams PD, *et al.* (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):213-221.
166. Patel DR, *et al.* (2004) Engineering an APRIL-specific B cell maturation antigen. *J Biol Chem* 279(16):16727-16735.
167. Aricescu AR, Lu W, & Jones EY (2006) A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr D Biol Crystallogr* 62(Pt 10):1243-1250.
168. McCoy AJ, *et al.* (2007) Phaser crystallographic software. *J Appl Crystallogr* 40(Pt 4):658-674.
169. Kelley LA, Mezulis S, Yates CM, Wass MN, & Sternberg MJ (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10(6):845-858.
170. Giot L, *et al.* (2003) A protein interaction map of Drosophila melanogaster. *Science* 302(5651):1727-1736.
171. Lee HJ & Zheng JJ (2010) PDZ domains and their binding partners: structure, specificity, and modification. *Cell Commun Signal* 8:8.
172. Lu H & Bilder D (2005) Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat Cell Biol* 7(12):1232-1239.
173. Moller P, *et al.* (1994) Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. *Int J Cancer* 57(3):371-377.
174. Peduto Eberl L, *et al.* (1999) Fas and Fas ligand expression in tumor cells and in vascular smooth-muscle cells of colonic and renal carcinomas. *Int J Cancer* 81(5):772-778.
175. Gagnoux-Palacios L, *et al.* (2018) Cell polarity and adherens junction formation inhibit epithelial Fas cell death receptor signaling. *J Cell Biol*.

176. Lozovatsky L, Abayasekara N, Piawah S, & Walther Z (2009) CASK deletion in intestinal epithelia causes mislocalization of LIN7C and the DLG1/Scrib polarity complex without affecting cell polarity. *Mol Biol Cell* 20(21):4489-4499.
177. Day ES, Cote SM, & Whitty A (2012) Binding efficiency of protein-protein complexes. *Biochemistry* 51(45):9124-9136.
178. Al-Shamkhani A, Mallett S, Brown MH, James W, & Barclay AN (1997) Affinity and kinetics of the interaction between soluble trimeric OX40 ligand, a member of the tumor necrosis factor superfamily, and its receptor OX40 on activated T cells. *J Biol Chem* 272(8):5275-5282.
179. Nelson CA, Warren JT, Wang MW, Teitelbaum SL, & Fremont DH (2012) RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor. *Structure* 20(11):1971-1982.
180. Dufour F, *et al.* (2017) N-glycosylation of mouse TRAIL-R and human TRAIL-R1 enhances TRAIL-induced death. *Cell Death Differ* 24(3):500-510.
181. Wagner KW, *et al.* (2007) Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med* 13(9):1070-1077.
182. Karnak D, Lee S, & Margolis B (2002) Identification of multiple binding partners for the amino-terminal domain of synapse-associated protein 97. *J Biol Chem* 277(48):46730-46735.
183. von Ossowski I, *et al.* (2006) Crystal structure of the second PDZ domain of SAP97 in complex with a GluR-A C-terminal peptide. *FEBS J* 273(22):5219-5229.
184. Lee KH, *et al.* (2006) The role of receptor internalization in CD95 signaling. *Embo J* 25(5):1009-1023.
185. Sievers F & Higgins DG (2014) Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol* 1079:105-116.
186. Waterhouse AM, Procter JB, Martin DM, Clamp M, & Barton GJ (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9):1189-1191.

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