

**Characterisation of Dof, an essential adaptor
molecule in fibroblast growth factor signalling in
*Drosophila melanogaster***

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1. Introduction

Fibroblast Growth Factor (FGF) signalling is a conserved mechanism from worms to humans. It regulates several cellular processes including cell growth, survival, differentiation and migration in developing and adult organisms. In *Drosophila melanogaster* the development of the mesoderm and the tracheal system are processes regulated by FGF signalling. In the search for additional mutations that affect the migration of tracheal and mesodermal cells or the differentiation of mesodermal derivatives, and thus, eventually interfere with the propagation of FGF signalling three groups found mutant alleles of the gene *dof/heartless/stumps* independently (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Homozygous mutant embryos showed similar defects in the mesoderm and the tracheae as mutants of the two known *Drosophila* FGF receptor genes. Genetic epistasis analysis showed that *dof* is an essential component of FGF signalling and placed it downstream of the FGF receptors and upstream or in parallel to Ras in the signalling cascade (therefore the name downstream of FGFR). The gene encodes a large molecule with no structural signs of known enzymatic domains characteristic for protein families with known functions. It shows weak homology to the B-cell receptor adaptors BCAP and BANK. The predicted domains of Dof (two ankyrin repeats and a coiled coil motif) are protein-protein interaction domains. In addition, several tyrosine residues of the sequence are located in consensus sites that could serve as binding sites for signalling molecules in their phosphorylated forms. Biochemical studies showed that Dof is tyrosine phosphorylated in an FGF receptor dependent manner and is able to form a complex with *Drosophila* FGF receptors (Wilson et al., 2004). Thus, its molecular characteristics, biochemical behaviour as well as genetic interactions indicated Dof to be an adaptor molecule in FGF signalling specific for *Drosophila*.

1.1 Adaptor proteins

The specific and appropriate response of cells to extracellular stimuli requires the integration of multiple signalling pathways. An emerging class of proteins that are major contributors in the regulation of cellular signalling networks are adaptor proteins. Adaptors are molecules of modular structure without enzymatic activity composed exclusively of multiple protein-protein or protein-lipid interacting domains. They link proteins to other proteins or to the plasma membrane, form large signalling complexes and specify subcellular localisation of other molecules thereby contributing to the specificity and efficiency of cellular responses in a spatio-temporal manner. Docking and scaffolding molecules are subgroups of the adaptor family with specialised functions. Docking proteins anchor interacting partners to subcellular structures, often membranes and so frequently contain protein-lipid interaction domains or sites for lipid modifications. Scaffolding proteins serve as interaction platforms for enzymatic cascades of multimeric signalling complexes. An alternative view also defines molecules as adaptors despite their enzymatic activity if they serve as a multi-modular platform for signalling complex assembly with a “built-in” enzymatic function or if the two functions are clearly distinguishable in different cellular responses.

1.1.1 Adaptors in the context of the specificity of cellular processes

Adaptors are described in studies of signalling and sorting processes. They have pivotal role in cell surface receptor signalling including receptor tyrosine kinase (RTK) signalling, G protein coupled receptor (GPCR) signalling, the Toll-like receptor (TLR) mediated inflammatory signalling

pathway as well as T- and B-cell receptor activation during lymphocyte development and immune response. Neurotransmission works with many structural and signalling adaptors on the side of the sensing (post-synaptic density) as well as on the side of the signal release. Adaptors have a general role in membrane and vesicle trafficking, e.g. in protein sorting in the Trans-Golgi network (TGN) and in endocytosis. The regulation of cell adhesion, cytoskeletal dynamics and active cell movements require whole sets of adaptors which act in integrin signalling, focal adhesion assembly and disassembly and actin polymerisation and crosslinking. Nuclear adaptors play role in transcriptional regulation. Even death needs the help of specific adaptors that form the “apoptosome”, a multiple protein complex essential to promote apoptosis.

Along the study of these different processes a growing body of evidence emerged for the existence of many adaptors which are involved in several signalling events. It is also known that the same canonical signalling cassettes are activated by many different signals. What are actually the tools in the hand of adaptors to increase specificity?

One way is that different combination of adaptors is utilised in different signalling events, called the combinatorial control. This can be emphasized and more specialised in a cell type dependent expression of adaptors. In general, interaction studies pointed out, that large protein complexes are the rule where simultaneous interaction of several components is required for signal transmission and the removal or exchange of a single component might modify the action of the complex. The assembly of signalling complexes might be influenced by mutually exclusive binding properties of the molecules and so factors might be displaced from a complex despite their ability to interact with components of the complex. These interfering interactions can be based on competitory or overlapping binding sites as well as on interaction surfaces which are dependent on conformational changes of the molecule and influenced by other interacting partners or modifications.

No less important is the subcellular compartmentalisation of active and inactive adaptors. Activation often means the recruitment to specific places of signalling at the cell membrane, the so called lipid rafts (membrane microdomains enriched in glyco-sphingolipids). Another example of specific recruitment is served by scaffolding molecules, binding platforms of enzymatic cascades. They synergise enzyme activity for one signalling and in parallel can keep away enzymes from other processes.

Even if same signalling cascades are activated by several signals, their amplitude and duration can be characteristic for the appropriate signalling event. This is achieved by the presence/absence of specific scaffolding molecules, by the capability of adaptors to oligomerise and thereby increase the local concentration of signalling molecules or by the fact that activation of cascades can happen in several alternative routes, which might be used with different redundancy in the one or the other signalling process.

Since Dof plays a role in FGF signalling - a subtype of RTK signalling – we focus in this work on adaptors which are involved in RTK signalling or linked directly to other protein tyrosine kinase (PTK) activity.

1.1.2 Modular assembly of adaptor molecules

A characteristic feature of adaptor proteins is the ability to bind at least two different molecules simultaneously. This capability derives from their multi-modular structure. They have protein domains with independent folding and binding capacity. Some of these domains are characteristic for the protein family of PTK-activity-linked adaptors and have defined binding sites whilst others are widely distributed modules often without preferring a defined binding domain.

Structural domains preferentially found in PTK-activity-linked adaptors

Src homology 2 (SH2) domains are protein modules of about 100 amino acids which bind phosphotyrosine containing peptide motifs of 5-10 amino acids length, generally C-terminal to a negatively charged region. They do not have affinity for unphosphorylated sequences and so are exclusive modules of tyrosine kinase signalling. The phosphopeptide binding site is bipartite. The pTyr binding pocket is highly conserved, whereas the second binding surface is more variable, and allows specific recognition of amino acids immediately C-terminal to the pTyr. These amino acids are decisive in binding specificity of different SH2 domains (Table 1) (Songyang et al., 1993; Songyang et al., 1994). Thus, tyrosine phosphorylation functions as an on-off switch for SH2 binding, while enabling the sequence context of the pTyr site to predict which SH2 domains, and so which signalling proteins are bound.

Table 1. Binding specificity of SH2, SH3 and PTB domains of various signalling molecules

SH2 domain binding specificities:	SH3 domain binding sites:
Src family: pY -[E/D/T]-[E/N/D]-[I/V/M/L]	general rule: x-P-p-x-P, x aliphatic
Abl: pY -[E/T/M]-[N/E/D]-[P/V/L]	Src: R -x-x-P-p-x-P
Crk: pY -[D/K/N]-[H/F/R]-[P/L]	Abl: T-x-x-P-p-x-P
Nck: pY - D-E -[P/D/V]	Grb2 C-term: P-x ₃ -R-x ₃ -P
Sem5: pY -[L/V/I/M]-N-[v/p]	Grb2 N-term: P-x-x-P
Csk: pY -[T/A/S]-[K/R/Q/N]-[M/I/V/R]	
3BP2: pY -[E/M/V]-[N/V/I]-x	
fps/fes: pY -E-x-[V/I]	
Drk: pY -[Y/I/V]-N-[F/L/I/V]	
Grb2: pY -[Q/Y/V]-N-[Y/Q/F]	
SHC: pY -[I/E/Y/L]-x-[I/L/M]	PTB domain binding sites:
Vav: pY -[M/L/E]-E-P	
ZAP-70: pY -x-x-I/L	Dok: Y/M-x-x-N-x-L-pY
p85 N-term: pY -[M/V/I/E]-x-M	minimal consensus: N-P-x-Y
C-term: pY -x-x-M	
PLCγ N-term: pY -[L/I/V]-[E/D]-[L/I/V]	
C-term: pY -[V/I]-[I/L]-[P/I/V]	
SHP2 N-term: pY -[I/V]-x-[VILP]	
Syk C-term: pY -[Q/T/E]-[E/Q]-[L/I]	
HCP N-term: pY -F-x-F	

Consensus sequence motifs were generated in a random peptide binding assay. Amino acids in the brackets separated by a slash were found without significant difference in that position. Bold letters remark outranking preference for that amino acid in that position. x stays for any amino acids. pY marks phosphorylated tyrosine residue. Modified and extended from (Songyang et al., 1993; Songyang et al., 1994).

Phospho-Tyrosine Binding (PTB) domains are modules of ~200 amino acid residues. Initially, the study of the adaptors Shc and IRS-1 showed that they recognise phospho-tyrosine containing peptide motifs that form a β turn. Binding to target sequences is determined by residues N-terminal to the pTyr (Table 1). Later findings demonstrated that PTB domains are able to bind unphosphorylated tyrosine-containing peptides (Margolis et al., 1999) or even do not require tyrosine for their binding to targets (Ong et al., 2000). Thus, sequence binding preference of PTB domains is less defined than of SH2 domains, which makes target prediction difficult for this domain.

Src homology 3 (SH3) domains bind proline rich peptides of approximately 10 amino acids. The core binding sequence contains the consensus x-P-x/p-x-P, where prolines at defined positions are of structural need and ensure high affinity binding. Each SH3 domain has a distinct binding preference and the nature of the non-proline residues in and around the core sequence serves for specificity (Table 1) (Pawson, 1995). SH3-binding does not depend on modification like phosphorylation enabling theoretically a constitutive protein-protein interaction. However, serine and threonine phosphorylation adjacent to the proline-rich motif may influence SH3 domain interactions and result in uncoupling of proteins.

Pleckstrin homology (PH) domains are lipid interacting modules of approximately 100 amino acids length. They bind the charged headgroups of specific poly-phosphoinositides and may thereby regulate the subcellular targeting of proteins to specific regions of the plasma membrane, where their ligand is enriched.

Classification of PTK-activity-linked adaptors based on their structural domains

Most of the PTK-activity-linked adaptors contain at least one of the preferentially used domains. Adaptors contain either exclusively the characteristic domains in single or multiple copies or combine these modules with diverse other domains. Some of them lack domains typical for PTK-activity-linked adaptors. Another characteristic feature is that they often harbour proline rich regions and tyrosine motifs – binding sites for SH3 and SH2 domains respectively. Figure 1 gives an overview of the different classes of PTK-activity-linked adaptors based on their structural domains.

Without going into details with each group we can make several general remarks. First, beside the preferential domains several other domains are represented in the different adaptors. Some of them appear in more than one protein, like PDZ domains, ankyrin repeats or coiled coil motifs whereas others are unique for a particular adaptor molecule, e.g. sorbitol homology (SoHo) domains in the Vinnexin family. Second, if the phospho-tyrosine binding motif SH2 is present in an adaptor then only in a single copy whilst conserved tyrosine motifs, potential binding sites for different SH2 domains are abundant. Thus, an SH2 domain-containing adaptor can bind only to a single phosphorylated partner which might cause a competition of molecules that contain binding sites for the particular SH2 domain. On the other hand tyrosine phosphorylated adaptors are able to recruit many SH2 containing adaptors at the same time. Third, the number of SH3 domains in an adaptor is less well defined and can vary from zero up to three, albeit often with different binding specificities.

The same is true for their binding sites, the proline rich regions. Finally, the presence and order of the different domains is unique for the different adaptor families. In other words, the order of moduls, rather than only the presence of the identical moduls determines homology.

What can one conclude from these structural characteristics for the function of the different adaptors? The conserved and unique domain structure of the different adaptor families indicates their important and specific role in signalling events. However, the diversity in the presence and combination of SH2 and SH3 domains and tyrosine and proline rich motifs alone gives already such high level of networking potential for these adaptors, that it makes their cellular role in signalling unpredictable. Indeed recent proteomics data showed the tyrosine phosphorylation of plenty of cytosolic proteins in the cell upon a single stimulation indicating the wide range of possibilities for phosphorylation based interactions. The presence of specific domains might be helpful revealing the function of adaptors, like the actin binding domain in Cortactin and Paxillin or the receptor binding PTB domain of FRS2.

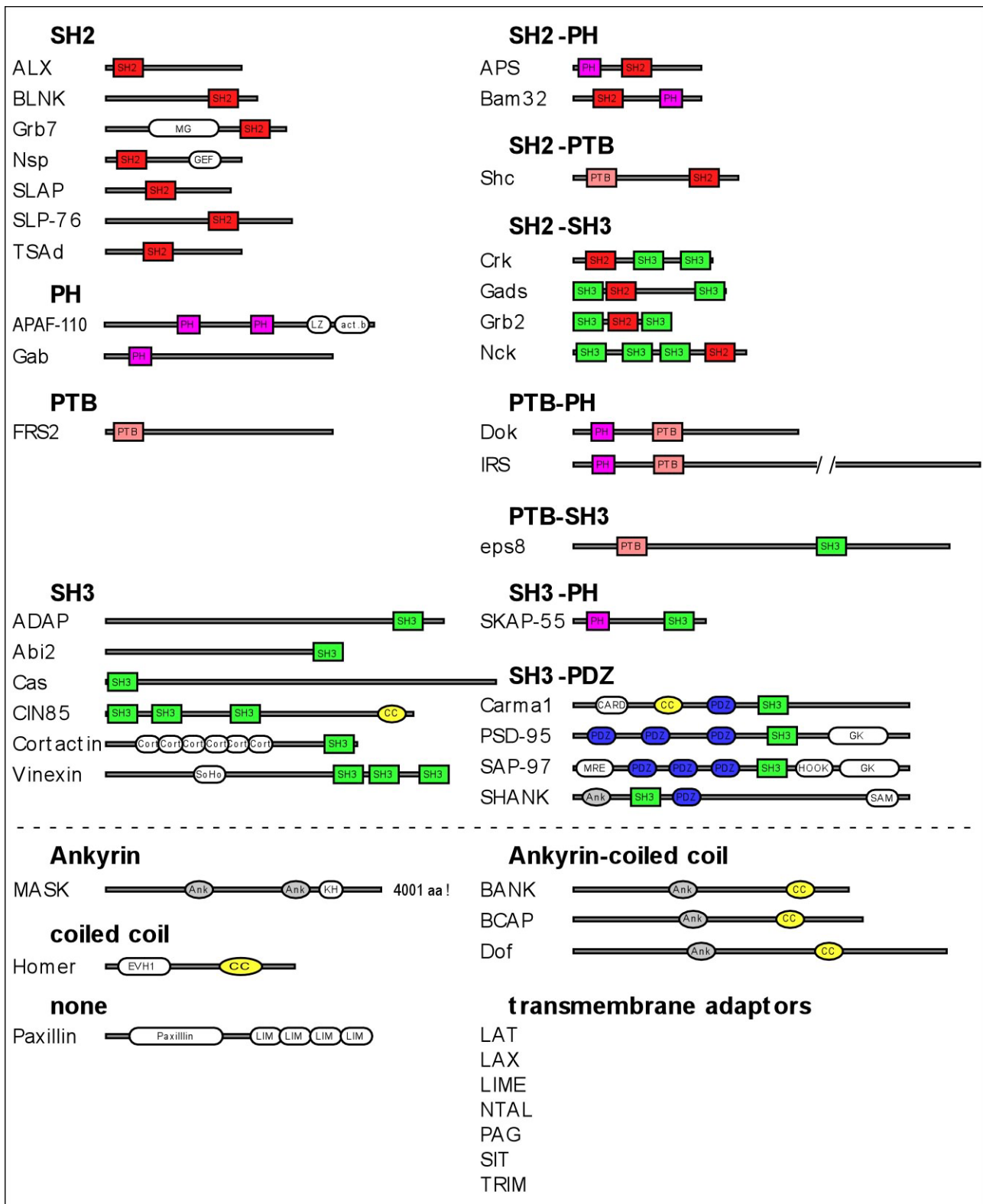


Figure 1. Classes of PTK-activity-linked adaptors based on their structural domains. Gray bars represent the relative length of the proteins with the exception of IRS and MASK. Colour coded boxes show the domains characteristic for PTK-activity-linked adaptors, colour coded ovals represent domains that are shared by several members of PTK-activity-linked adaptors but do not belong to the characteristic ones and white ovals stand for domains which are unique among these adaptors. Dashed line separates adaptors with characteristic domains (top) from adaptors with non-characteristic domains (bottom). Since transmembrane adaptors contain no recognisable domain in their amino acid sequences beside the transmembrane region they are listed only by name as a separate group. The domain structure of the proteins was constructed

1.1.3 Functional dissection – adaptors as molecular interneurons of the cells

Adaptors are linkers that couple sensing processes of the cell to effector molecules. Their regulation enables to modulate signalling outputs. Thus, the number of adaptors involved in a signalling pathway might reflect the fine-tuning capacity of the appropriate system. Most extreme examples are T- and B-cell receptor signalling events, where up to eight components are utilised to activate the Ras-MAPK cascade (Simeoni, 2004). In other systems e.g. in case of several RTKs a single adaptor is sufficient to activate the same cascade.

It raises an interesting option to distinguish adaptors based on the criterion if they can directly bridge activator and effector, bind activator or effector or function as intermedator by linking adaptors together. It could give a kind of hierarchy of adaptors in the signalling cascades. However the position of an adaptor might be different in the activation of the one or the other signalling cascade. Taken the example of the adaptor Grb2: it can directly bridge EGF receptors to the Ras activator Sos. In B-cell activation it links the adaptor LAT to the effector Sos, whereas in FGF signalling it links the sensor/receptor binding FRS2 adaptor to Sos. It acts also as an intermedator adaptor in FGF signalling by binding to FRS2 and recruiting the adaptor Gab1, which then activates the effector PI3K. The validity of such a positioning relays on the selective specificity of the interactions; it is not an intrinsic feature of the adaptors. The identification of domains in the adaptors with binding specificity for receptors/activators or effectors might help for positioning but a particular position should not be generalised for all signalling events.

An alternative way to classify adaptors might be their specificity to different signalling pathways. Several adaptors are involved only in one signalling cascade – e.g. scaffolding proteins of enzymatic cascades, many B- and T-cell adaptors or the adaptor FRS2 in mammalian FGF signalling – whilst others are utilised by many signalling events, e.g. members of the Grb2, Crk, Gab or Nck adaptor families. The first group might provide specificity to the different signalling events whereas the second group might enable crosstalk and balancing between signalling events in the cell.

1.1.4 Tools to regulate adaptor protein activity

Adaptor proteins function as molecular switches in signal transduction. Their activity requires a tight regulation to ensure signal propagation or inhibition. Diverse strategies are used for both positive as well as negative regulation including phosphorylation, proteolytic cleavage, proteosomal degradation and changes in the conformation or in the oligomerisation capacity of the molecules. Regulatory changes can have temporary/reversible effects like phosphorylation and conformational changes or definitive/irreversible consequences like cleavage or degradation.

Posttranslational modifications

Tyrosine phosphorylation

Tyrosine phosphorylation acts as a general switch for activation in signalling cascades. Most PTK-activity-linked adaptors are tyrosine phosphorylated in a stimulation dependent manner and a

using PFAM (<http://www.sanger.uk/Pfam>). act.b, actin binding domain; Ank, ankyrin repeat; CARD, caspase-recruitment domain; CC, coiled coil; Cort, cortactin repeat; GK, guanylate kinase-like; GM, Grb7/Mig-10 homology region; KH, Kash homology; LIM, lin-11/isl-1/mec-3 domain; LZ, leucine zipper; MRE, MAGUK recruitment domain; PDZ, PSD-95/Disks-large/ZO-1 domain; PH, Pleckstrin homology; PTB, phosphotyrosine binding domain; SAM, sterile α motif; SH2, Src-homology 2; SH3, Src-homology 3; SoHo, Sorbitol homology.

cooperative action of kinases and phosphatases balances the actual tyrosine phosphorylation state of these adaptors during signalling. Only very few of them do not show detectable tyrosine phosphorylation, like the adaptor E3B1/Abi2 (Ichigotani et al., 2002).

The primary role of phosphorylated tyrosine residues is to provide signalling-dependent binding surface for the highly specialised SH2 domains thereby recruiting downstream transducers which propagate signalling and activate effectors.

However, we also find inhibitory effects of tyrosine phosphorylation. Cortactin, an actin binding adaptor protein is phosphorylated on tyrosine residues adjacent to its SH3 domain. This blocks the binding capacity of the SH3 domain (Martinez-Quiles et al., 2004). Crk function is regulated by tyrosine phosphorylation-dependent auto-inhibition. Its SH2 domain interacts with a phosphorylated tyrosine residue of the own molecule (Feller, 2001). This has dual consequences: SH2 domain is occupied for other interactors in a competitive manner and interactors of the SH3 domain are lost due to the conformational changes caused by the intramolecular interaction.

Serine/Threonine phosphorylation

If tyrosine phosphorylation is the general switch then serine/threonine phosphorylation is the modifier in signalling events. It serves as fine tuner in the regulation of adaptor proteins. A broad range of Ser/Thr kinases and phosphatases can act on adaptors. They are either activated by the same signalling the respective adaptor involved in (often as feedback regulators) or by independent signalling cascades thereby providing cross talk between different signalling processes in the cell. We find examples of constitutive as well as signalling dependent phosphorylation of adaptors on Ser/Thr residues with positive or negative regulatory role. Ser/Thr phosphorylation acts through its allosteric effect on the molecule and can cause diverse functional changes. It can sensitise or desensitise adaptors for specific interactions by inducing changes in the conformation of the molecule (e.g. IRS, FRS2 and Grb7/10/14). A local allosteric effect of Ser/Thr phosphorylation is the disruption of the phospho-tyrosine binding capacity of SH2 domains (Gab1 and IRS). The local effect of Ser/Thr phosphorylation in binding site disruption can cause global conformational changes through the inhibition of intramolecular interaction (Cortactin) or interfering with the auto-inhibitory binding of adaptors (AFAP-110).

IRS proteins are examples for complex roles of S/T phosphorylation. In quiescent cells a basal level of S/T phosphorylation on particular residues is necessary for an effective subsequent tyrosine phosphorylation by the insuline receptor. On the other hand, elevated S/T phosphorylation on different residues inhibits the binding of IRS-1 and -2 to the juxtamembrane region of the insuline receptor impairing their ability to undergo tyrosine phosphorylation. Thus, position of S/T phosphorylation in IRS determines the positive or negative influence on insuline receptor binding affinity. Further negative regulatory mode is the specific phosphorylation of serine and threonine residues adjacent to SH2 binding motifs thereby decreasing the binding affinity of SH2 domain based interactors to IRS. Additional observation is that S/T phosphorylation commits IRS to proteosomal degradation (reviewed in (Johnston et al., 2003)).

The activity of the adaptor FRS2, key mediator of FGF signalling stands also under the dual control of tyrosine and threonine phosphorylation. FRS2 binds constitutively to FGF receptors in cells via its PTB domain (Ong et al., 2000). Upon FGF stimulation FRS2 is phosphorylated by the FGF receptor on several tyrosine residues that serve as docking sites for the MAPK cascade-activating molecules Grb2 and SHP2 (Ong et al., 1997). Activated MAPK has a negative regulatory feedback on FRS2 by phosphorylating it on several threonine residues. Threonine phosphorylation inhibits further tyrosine phosphorylation of FRS2, leading to the downregulation of the signalling cascade (Lax et al., 2002). Similarly, threonine phosphorylation of FRS2 is induced in response to other growth factors (e.g. PDGF, EGF). FRS2 is able to bind to the EGF receptor in a ligand-stimulated manner and undergo threonine and weak tyrosine phosphorylation (Wu et al., 2003).

Thus, MAPK dependent threonine phosphorylation of FRS2 might ensure a controlled dosage of signalling via FGF receptors balanced also by other growth factor signalings. Since FRS2 is constitutively associated with the FGF receptor, threonine phosphorylation might provide an additional tight control to prevent activation of FGF-dependent signalling by random receptor autoactivation.

AFAP-110 and Cortactin are good examples how S/T phosphorylation can lead to conformational changes by altering intramolecular interactions and thereby propagate signalling. The phosphorylation of AFAP-110 alters its multimerisation behaviour and enables novel downstream interactions (Baisden et al., 2001). Cortactin SH3 C-terminal domain forms an auto-inhibitory loop by binding to proline rich motifs of the same molecule. Upon Erk activation Erk phosphorylates serine residues within the proline rich motif and thereby liberates cortactin SH3 domains to interact with proline rich regions of other molecules (Campbell et al., 1999). As mentioned above phosphorylation of adjacent tyrosine residues block the ability of the SH3 domain for effective binding (Martinez-Quiles et al., 2004). Thus, tyrosine and serine phosphorylations act on the interaction behaviour of the same domain with opposing effects.

Gab1 adaptor function on PI3K activation is negatively regulated by serine/threonine phosphorylation (Lehr et al., 2004). Phosphorylation of serine/threonine residues adjacent to tyrosine motifs which are phosphorylated upon RTK signalling and serve as binding sites for the SH2 domain of PI3K disrupts this interaction.

Much less is known about the role of serine/threonine phosphorylation of paxillin, E3B1/Abi2 and the Grb7/10/14 family adaptors. Phosphorylation of particular serine residues play a key role in cell migration and in the regulation of focal adhesion organisation (Huang et al., 2004). However, the mechanism of the modulatory effect of these phosphorylations is not uncovered as well as the biological relevance of other phospho-serine/threonine residues in the molecule remains to be elucidated. E3B1/Abi2 becomes hyperphosphorylated on serine residues following mitogenic stimulation (Ichigotani et al., 2002). However little is known about the clear cause and consequence of the phosphorylation. Grb7/10/14 family adaptors are phosphorylated on serine and threonine residues at basal level in quiescent cells which is increased in case of Grb10 and Grb14 upon growth factor stimulation (Han et al., 2001). Grb14 interacts directly with activated insulin receptor and its serine/threonine phosphorylation inhibits insulin signalling probably by maintaining the bound receptor in an inactive conformation (Cariou et al., 2004). Functional significance of serine/threonine phosphorylation of other Grb7 family members is not yet understood.

Caspase cleavage

Signalling can serve as survival factor in cells. Effective shut down of signalling events is therefore a prerequisite of apoptosis. As a consequence many adaptor proteins are targets of caspases which are the major initiator and executor proteases activated in programmed cell death. Interestingly, caspase activity is observed and required in the regulation of some adaptors not exclusively under apoptotic conditions.

Cas family adaptor proteins are involved in the assembly of multiple protein interactions at focal adhesion sites (Bouton et al., 2001). Human enhancer of filamentation 1, HEF1, a member of this family shows a cell cycle dependent regulation. In cells in G1-S-G2 phases full length forms of the molecule accumulate at focal adhesions, while at G2/M transition HEF1 is cleaved at a caspase consensus site to generate an N-terminal HEF1 form, p55HEF1, which localises to the mitotic spindle. C-terminal cleavage products are apparently degraded (Law et al., 1998). Additional finding was that proapoptotic stimuli or HEF1 overexpression lead also to the cleavage of HEF1 by caspases. The same caspase cleavage site is used as during mitosis to generate the N-terminal 55 kDa and a C-terminal 65 kDa fragment, while a novel caspase cleavage site was identified to be responsible for the generation of a carboxy-terminal small 28 kDa fragment (Law et al., 2000). This

second caspase site is also present in p130Cas, an other member of the Cas family, which is similarly cleaved at this site to produce a 28 kDa protein species following proapoptotic stimulus (Bannerman et al., 1998). The proapoptotic activity of HEF1 was found to depend on the expression of the C-terminal 28 kDa region of the protein. These findings together suggest a model in which separate parts of HEF1 have functions distinct from the full length molecule. Regulation of the cleavage and abundance of HEF1 (and p130Cas) might propagate the destruction of focal adhesion sites and regulate onset of mitosis or apoptosis (Law et al., 2000). Thus, modulated caspase activity towards Cas adaptors may be important for cytoskeletal organisation and cell cycle progression, while a dramatic increase or dysregulation of caspase activity is necessary to promote apoptosis.

Another example, how caspase cleavage can regulate signalling events, is served by the adaptor molecule Gads. Gads functions specifically in T-cell receptor signalling by linking the adaptor SLP-76 to tyrosine phosphorylated LAT. This linker function of Gads is essential to recruit SLP-76 to the membrane which then upon phosphorylation activates downstream signalling events (Liu et al., 2001). Apoptosis induction results in detectable cleavage of Gads at a predicted caspase cleavage site. Cleavage products retain their binding specificity for LAT and SLP-76 respectively. Expression of Gads cleavage products showed a dominant inhibitory effect on signal propagation. Hence, the functional relevance of caspase cleavage of Gads is likely to uncouple LAT and SLP-76 interaction (Berry et al., 2001). The surprising finding, that cell death receptor mediated signalling is required for activation induced proliferation of T-cells (Kennedy et al., 1999) rises the hypothesis that cleavage of signalling molecules downstream of the T-cell receptor could modulate the spectrum of activated pathways in a way that propagates proliferation.

Dimerisation/oligomerisation

Oligomerisation of adaptors can have both positive as well as negative effects on their activity. It is mediated either by homophilic interaction of the same domains or by heterophilic interaction between two different regions of the molecules. However, based on the experimental approaches it is not distinguishable in each case, whether these domains are utilised for intra- or for intermolecular interactions.

Preferential domains of homophilic interactions are leucine zipper domains, coiled coil motifs and helix-loop-helix structures. Less defined are the domains for heterophilic interactions: the one part is often determined only as a molecular region without known domain structure. Examples below show that heterophilic interaction of adaptors is usually regulated by tyrosine phosphorylation.

CIN85/CMS family adaptor proteins are involved in the regulation of RTK downregulation via endocytosis (reviewed in (Dikic, 2002)). They are able to homodimerise via their coiled-coil domains. Overexpression of CIN85 or CMS leads to the formation of multiple endosomal-like vesicles in the cells, the existence of which depends on the presence of the coiled-coil domain. One possible explanation is that homo-oligomerisation of CIN85/CMS increases the local concentration of recruited signalling complexes in the proximity of the receptor (cargo) and thereby positively regulates endocytosis.

Members of the Dok adaptor family inhibit the Ras pathway by recruiting RasGAP in a tyrosine phosphorylation-dependent manner. Mutations in Dok that prevent oligomerisation also abrogate inhibitory activity of Dok1 without affecting the overall phosphorylation state of the molecule, indicating that domains involved in oligomerisation play a crucial role in downstream interactions without influencing Dok1 activation itself (Songyang, 2001). It is not understood at present if the dual requirement of PTB domain and phosphorylated Tyr146 in Dok1 function is primarily due to their capability to induce oligomerisation.

The regulation of the oligomerisation capacity of the adaptor AFAP-110 is also phosphorylation dependent and involves both inhibitory and activation steps. AFAP-110 has the

intrinsic capability to form multimers via its C-terminal leucine zipper motif. This domain has an auto-inhibitory binding to N-terminal regions of the molecule and this interaction keeps AFAP-110 in a tetrameric complex (Qian et al., 2004). Upon phosphorylation auto-inhibition is released and conformational changes lead to a reduction of the multimeric status of AFAP-110. Dimers are formed probably via new interacting surfaces in the molecule. The new conformation allows the binding and activation of Src kinase and increases the actin crosslinking activity of AFAP-110 (Qian et al., 2002).

Oligomerisation of adaptor molecules can have also inhibitory effect on interactions of signal transduction events. The adaptor Grb10 forms oligomers in a way, that N-terminal amino acid sequences of the one molecule interact with the C-terminal BPS-SH2 domains of the other molecule (Dong et al., 1998). BPS-SH2 domains are important functional modules, which can directly bind activated insulin receptor. Oligomerisation therefore may provide a reservoir of Grb10 that is prevented to bind phospho-tyrosine containing molecules and stimulation may release Grb10 molecules from this pool. Alternatively Grb10 oligomers may have a more active function serving as docking complex to recruit multiple signalling molecules. Another piece to the complexity is that Grb7, member of the same adaptor family was also able to interact with the C-terminal PBS/SH2 domains of Grb10 in yeast two hybrid assay (Liu and Roth, 1998). This potentiates the homo-/hetero-oligomerisation capacity of Grb7 family adaptors as a broader regulatory tool in signal transduction. However, no physiological relevance of these interaction has been shown so far.

The Cas family adaptor protein HEF1 is also able to dimerise via its C-terminal helix-loop-helix (HLH) domain. This motif mediates not only HEF1 homodimerisation, but also heterodimerisation with the HLH domain of the other Cas family member p130cas as well as with the transcriptional regulatory HLH proteins Id2, E12 and E47 (Law et al., 1999). Dimerisation capacity was analysed only for the C-terminal region of HEF1 but not for the full length molecule. HEF1 is processed in T cells (see above). Thus, it might be possible, that dimerisation is an intrinsic feature of a sole C-terminal fragment of HEF1 but not of the full length molecule. Functional relevance of dimerisation is not understood.

Intramolecular interactions, conformational changes

Conformational changes are stimulation-dependent temporary alterations that affect the activity of adaptors. They are induced upon phosphorylation of the adaptors which causes allosteric distortion or frequently alters intramolecular interactions of the molecule. As discussed earlier with examples phosphorylation can disrupt intramolecular interactions and result an “open” molecular structure where new protein interaction domains become available (e.g. Cortactin). Phosphorylation can also generate new binding sites for own phospho-tyrosine binding domains propagating a “closed” form of the molecule which will mask interaction surfaces for other binding partners (e.g. Crk). Thus, conformational change is a potent tool to alter interaction surfaces of adaptors and therefore mutually exclude interacting partners in a signalling dependent manner.

Protein degradation

Protein degradation is an irreversible way to downregulate adaptor activity. The ubiquitin E3 ligase Cbl plays important role in this negative signalling sending not only adaptors but several receptors and signalling effectors for degradation (Dikic and Giordano, 2003). It contributes in the poly-ubiquitination of FRS2 which leads to the proteosomal degradation of this adaptor (Wong et al., 2002). The adaptor Cin85, involved in the ligand-induced endocytosis of several receptors undergoes Cbl dependent mono-ubiquitination during this process which provides the sorting signal for lysosomal degradation of the whole receptor-Cbl-Cin85 complex (Dikic, 2002). SOCS proteins play a role in proteosomal targeting of associated proteins (Kamura et al., 1998). It has been shown

that they contribute to the proteosomal degradation of IRS adaptors (Johnston et al., 2003). Studies on IRS also showed that S/T phosphorylation promoted its degradation through an unknown mechanism.

The discussed examples show that adaptor proteins are regulated in a complex way. Regulatory modifications can have opposing effects and can be a prerequisite or inducer of further regulatory changes. Hence, regulatory tools act in concert to achieve an appropriate activity level of adaptors.

1.2 FGF signalling-dependent morphogenetic processes in flies

Initially *dof* mutants were found in genetic screens affecting mesodermal and tracheal development in the embryo (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Both processes are controlled by FGF (Ghabrial et al., 2003; Wilson and Leptin, 2000). Later analysis showed that *dof* is generally involved in developmental processes where FGF signalling plays a role, e.g. also in the migration of midline glia cells (Vincent et al., 1998) and in the formation of air sacs in the pupa (Sato and Kornberg, 2002). Here I will focus on the embryonic development of the mesoderm and the tracheae since the *in vivo* function of *dof* and other components of the FGF signalling cascade were studied in these developmental processes to the greatest detail including experimental results presented in this work. Thus, mesoderm and tracheal development are the two FGF-dependent processes in *Drosophila* about the regulation of which we understand the most at present.

1.2.1 Regulation of tracheal morphogenesis

The *Drosophila* embryonic trachea is a tubular network and serves as the respiratory organ throughout larval life (reviewed in (Affolter et al., 2003; Ghabrial et al., 2003)). It derives from ten clusters of dorsal ectodermal cells from both side of the embryo, the so called tracheal placodes (Figure 2a). They invaginate into the mesoderm to form the tracheal pits, each containing approximately 80 cells. Without further cell division the 20 sacs establish independent tracheal metamers with highly similar branching patterns through stereotypic, directed migration and cell shape changes (Figure 2b,c). Six primary branches are formed into different directions and each branch contains a well defined, invariable number of tracheal cells. The metamers become interconnected by subsequent fusion events through specialised fusion cells of particular tracheal branches (Figure 2d). As last step fine subcellular extensions, the terminal branches grow out to deliver oxygen to target tissues guided directly by different oxygen requirement of the cells (Figure 2g). Tracheal branches migrate into different parts of the embryo invading different tissues. The surrounding tissue provides additional specific interactions for the corresponding branches and thereby contributes to the final pattern of the tracheal network (reviewed in (Uv et al., 2003)).

At molecular level the JAK/STAT pathway is responsible to initiate tracheal cell fate. It induces the expression of the transcription factor Trachealless in the tracheal placodes. Trachealless is responsible to make tracheal cells competent for migration by inducing the expression of *Dof* and the FGF receptor *Breathless* (*Btl*). The *Btl* specific FGF ligand *Branchless* (*Bnl*) is expressed in dynamic pattern in surrounding cells of the tracheal placodes and predefines the directional outgrowth of the six primary branches (Figure 2e) (Sutherland et al., 1996). *Bnl* is acting as a chemoattractant for tracheal cells that is nicely demonstrated by its ability to reroute tracheal cells if ectopically expressed or to attract fine terminal branches in larvae towards oxygen-starved cells that

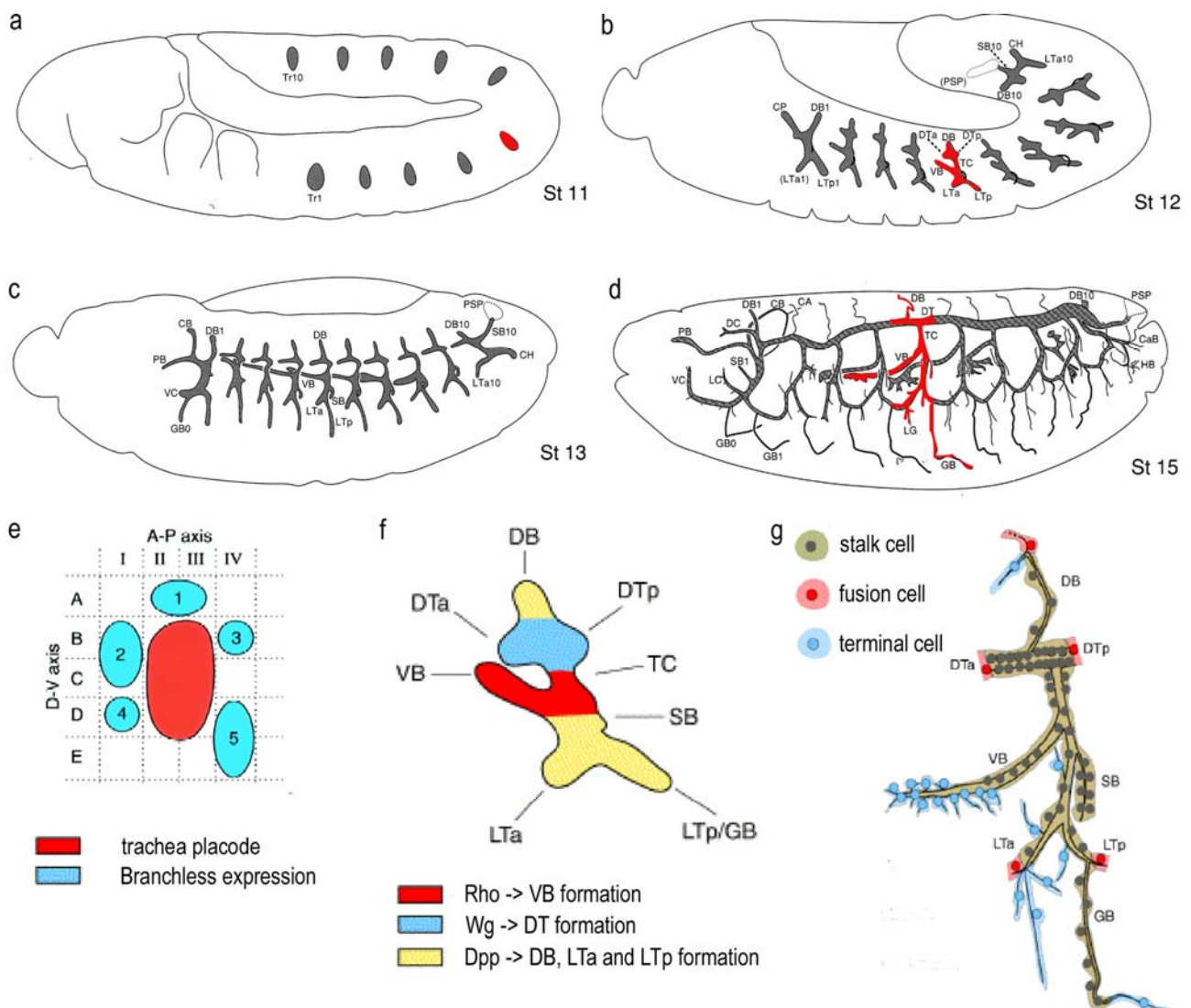


Figure 2. Schematic representation of the developmental stages of tracheal morphogenesis during embryogenesis. (a-d) Schematic drawing of *Drosophila* embryos from lateral view at developmental stages 11, 12, 13 and 15 respectively. Anterior to the left. Gray structures show the morphology of tracheal anlage/tissue at different stages. (a) Invaginated tracheal palcodes prior branching, (b) Initial branching of the six primary branches, (c) trachea metamers with branched out primary branches prior fusion and (d) tracheal network after fusion occurred and secondary branches are formed. (e-g) blow ups of regions in a, b and d highlighted with red. (e) shows the arrangement of Branchless expression clusters (blue) surrounding a trachea placode (red). (f) Regional differentiation of tracheal cells at early primary branching stage. Colour code shows the regionally acting signalling pathways which determine different cellular fates of the developing primary branches according their anterior-posterior, dorsal-ventral position in the placode. (g) tracheal unit of one hemisegment showing the positions of specified tracheal cells for fusion and terminal branching. CA, cerebral anastomosis; CaB, caudal branch; CB, cerebral branch; DB, dorsal branch; DC, dorsal cephalic branch; DTa, dorsal trunk anterior; DTp, dorsal trunk posterior; GB, ganglionic branch; HB, hindgut branch; LC, lateral cephalic branch; LG, lateral group branch G; LTA, lateral trunk anterior; LTP, lateral trunk posterior; PB, pharyngeal branch; SB, spiracular branch; TC, transverse connective; VB, visceral branch. Figures were taken and modified from (Bate, 1993) (a-d), (Metzger and Krasnow, 1999) (e) and (Uv et al., 2003) (f-g).

express it at high levels. In the absence of Bnl, Btl or Dof cell migration and subsequent development of the tracheae fails to occur (reviewed in (Ribeiro et al., 2003)).

Tracheal cells show a regional differentiation at placode stage as a consequence of the differentially localised activity of Decapentaplegic (Dpp), Rhomboid (Rho) and Wingless (Wg) (Figure 2f) (reviewed in (Uv et al., 2003)). Their activity is manifested by a selective expression of

transcription factors that act as sorting markers of tracheal cells for different primary branches. FGF signalling controls motility of tracheal cells by inducing filopodia formation in a spatially specific manner but is not sufficient for successful outgrowth of tracheal branches (Ribeiro et al., 2002). Regional differentiation might prepare and specialise cells of different branches for oriented migration towards the sources of Bnl along distinct substrates. Signalling systems provided by the regional differentiation integrate cell motility and thereby allowing the correct morphogenesis of individual branches.

1.2.2 Regulation of mesoderm development

The mesoderm invaginates as an epithelial layer on the ventral side of the embryo driven by cell shape changes (Figure 3a). After the central part of the mesoderm is fully internalised and the ventral furrow is closed, the epithelial tube initiates contact with the ectoderm (Figure 3b), followed by epithelial mesenchymal transition (Figure 3c). Mesodermal cells dissociate, resume mitosis and spread out on the ectoderm to form a single-cell layer (Figure 3d). Based on dorso-ventral inductive signals from the underlying ectoderm mesodermal cells differentiate into specific tissues along the dorso-ventral axis (Figure 3d). Thus, the formation of mesodermal derivatives requires a sequential interplay of cell migration and cell differentiation (reviewed in (Michelson et al., 1998).

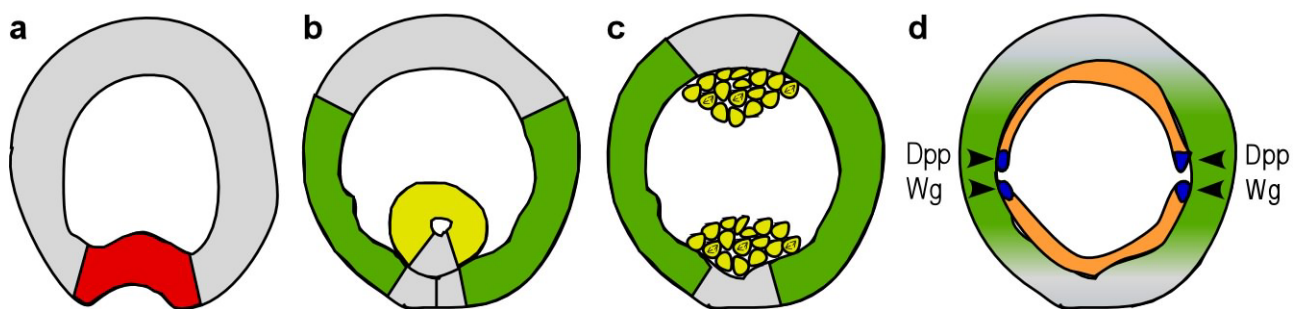


Figure 3. Schematic representation of mesoderm morphogenesis. (a-d) Schematic drawings of cross-sections of embryos at four successive stages of mesoderm development. (a) Mesoderm invagination begins with the formation of the ventral furrow by the most ventral cells. Presumptive mesoderm expresses Twist (red). (b) Invaginated mesoderm forms an epithelial tube and begins to make contact with the ectoderm. Heartless and Dof are expressed in the mesoderm (yellow) and the two FGF ligands in the complementary parts of the ectoderm (green). (c) Mesodermal tube flatten against the ectoderm, cells lose their epithelial integrity and begin to divide. Note that at this time germ band extension is in a progressive stage. (d) Mesodermal cells (orange) migrated out dorsally on the surface of the underlying ectoderm and start to differentiate. The dorsal-most cells of the mesodermal sheet form Eve-positive cell clusters (blue) upon inductive signals of the ectoderm (Dpp, Wg). FGF signalling is also required for this process; expression of the two FGF ligands becomes higher/stays longer in the dorsal ectoderm (green) and Heartless and Dof expression is upregulated in the dorsal-most mesodermal cells (not shown here). The germ band is in a fully extended stage.

Mesodermal fate is adopted through the action of a series of maternal genes of the so-called dorsal group. It results in a nuclear concentration gradient of the Dorsal transcription factor. Specific expression pattern of zygotic genes is established based on the different binding affinity of their promoters to Dorsal. On the ventral side of the embryo the highest nuclear levels of Dorsal turn on *twist* and *snail*, the two major sequester transcription factors of mesoderm formation and development (Figure 3a). Dorsal participates with Twist in activating *dof* and the FGF receptor *heartless* (*htl*) ventrally (reviewed in (Michelson et al., 1998)). The recently identified Htl specific FGF ligands *FGF8-like1/pyramus* and *FGF8-like2/thisbe* are activated laterally by lower levels of Dorsal (Gryzik and Muller, 2004; Stathopoulos et al., 2004). It is likely that Snail represses their

expression in the ventral cells thereby generating a strict complementary expression pattern of FGF ligands and their receptor (Figure 3b,c). FGF signalling is immediately needed after invagination to establish initial contact between ectodermal and mesodermal cells and then later for effective and coordinated spreading. It remains to be elucidated how Htl ligands act: in a permissive or in an instructive manner.

In addition to its migratory role Htl has a second function in mesodermal cell fate specification (Michelson et al., 1998). Different regions of the ectoderm send different inductive signals to the contacting mesoderm. The dorsal-most mesodermal cells are induced by Dpp that gives them the competence to differentiate into visceral, cardiac or dorsal somatic muscle derivatives. Superimposed activation of Heartless or Heartless and *Drosophila* EGF receptor together serves to distinguish the fate of a subgroup of three cells in each hemisegment characterised by Even-skipped (Eve) expression (Figure 3d): two pericardial progenitors and a single dorsal somatic muscle cell respectively. Thus, the presence of Eve-positive mesodermal clusters relies on the dual function of FGF signalling. First, it controls cell migration allowing that mesodermal cells acquire a precise position relative to the ectoderm and second, contributes to cell fate determination.

1.3 Molecular mechanism of FGF signalling

FGF receptors (FGFR) belong to the family of receptor tyrosine kinases (RTK). FGFRs are monomers in the cell membrane. Ligand binding induces dimerisation of the receptors resulting in trans-autophosphorylation of their cytoplasmic domains on several tyrosine residues. Autophosphorylation is necessary to “wake up” kinase activity, but most auto-phosphorylated tyrosines are located in non-catalytic regions of the receptor molecule. These sites function as binding sites for SH2 or PTB domain containing signalling molecules which then upon recruitment activate diverse signalling pathways commonly used by RTKs (reviewed in (Schlessinger, 2000)). A key mediator for FGF receptor functions is the adaptor protein FRS2. It binds constitutively to FGF receptors and is tyrosine phosphorylated upon FGFR activation (Ong et al., 2000). It links the receptor to downstream signalling cassettes by recruiting multiple protein complexes through phospho-tyrosine based interactions (Figure 4). Grb2-dependent interaction of FRS2 leads to the activation of PI3K by the recruitment of the FRS2-Grb2:Gab1-PI3K complex to the FGF receptor (Ong et al., 2001). FRS2-Grb2:Sos-Ras interaction activates the MAPK cassette (Kouhara et al., 1997), but FRS2 is also able to bind to the protein phosphatase SHP2 (Hadari et al., 1998) providing an alternative route for the recruitment of Grb2:Sos-Ras through SHP2. FGF receptors can directly interact with and phosphorylate the adaptor molecule Shb, which contributes to FRS2 phosphorylation in an SHP2 dependent manner (Cross et al., 2002) – a potential additional regulation for FRS2 activation and recruitment.

Beside adaptor molecules FGFR can interact directly with and activate enzymes: phospholipase C γ (PLC γ), thereby directly activating the PLC γ signalling cascade (Falasca et al., 1998) and RasGAP (Cross et al., 2002), a Ras family specific GTPase activating protein. The fact that FGFR can activate Ras and also recruit RasGAP, an inhibitor of Ras signalling shows tightly coupled regulatory tools for the timing, amplitude and duration of FGF signalling. Studies on Torso, a *Drosophila* RTK showed that SHP2 dephosphorylates RasGAP binding site on Torso thereby increasing MAPK activity (Cleghon et al., 1998). This might be also utilised in FGF signalling supported by the fact that phosphatase activity of SHP2 is necessary for the activation of the MAPK cascade.

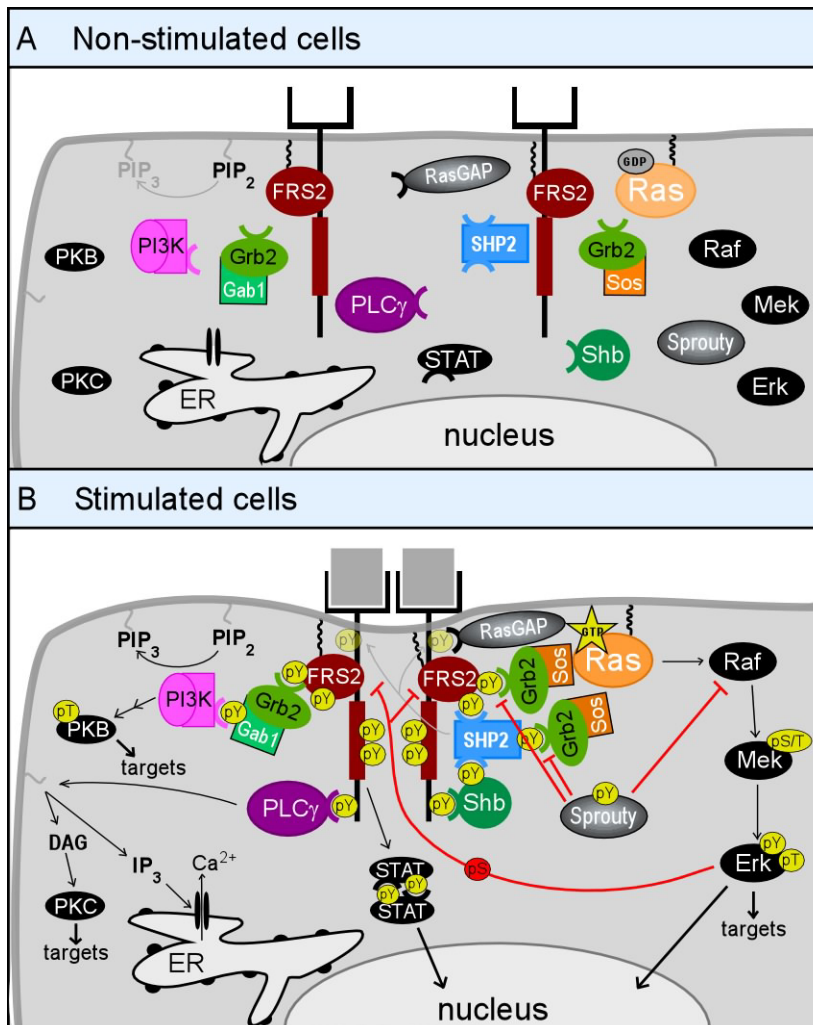


Figure 4. Model for the molecular mechanism and regulation of FGF signalling. (A) In non-stimulated cells FGFR is in monomeric form and constitutively binds the adaptor molecule FRS2. The adaptor molecule Grb2 forms constitutive cytosolic complexes with the adaptor Gab1 or the RasGEF protein Sos. Ras GTPase is in an inactive, GDP bound form. (B) In FGF stimulated cells FGFRs dimerise, trans-autophosphorylate each other and phosphorylate FRS2 and other signalling molecules which are recruited to the plasma membrane. These molecules form signalling complexes via phospho-tyrosine-SH2 domain mediated interactions. Grb2:Sos complexes are membrane recruited by phosphorylated FRS2 or SHP2 and activate Ras that in turn activates the MAPK cascade. As a result phosphorylated Erk enters the nucleus. FRS2 binds also to Grb2 in complex with Gab1 which then activates the PI3K cascade. PLCγ binds directly to phosphorylated FGFR and initiates a cascade that leads to the activation of Protein kinase C (PKC). JAK-STAT pathway is activated by the FGFR and phosphorylated STAT is translocated to the nucleus. Negative regulatory

feedback loops depend also on phosphorylation: (i) RasGAP binds to the phosphorylated FGFR and interferes with Ras signalling, (ii) phosphorylated Sprouty inhibits MAPK signalling at several levels and (iii) activated Erk inhibits interaction of FRS2 with the FGFR by phosphorylating FRS2.

1.3.1 Downstream signalling cassettes activated by FGFR

Activated FGFRs transmit the signal to numerous signalling cascades: the JAK/STAT pathway, PLCγ, PI3K and Ras-MAPK cassettes and thus activate effector molecules in the nucleus and in the cytoplasm (Figure 4) (reviewed in (Schlessinger, 2000)). Signalling cassettes work in cross talk creating a network of stimulatory and inhibitory modifying signals. This work focuses on signalling events transmitted by Ras from two reasons: first, the Ras-MAPK pathway is essential for transduction of the FGF signal in *Drosophila* and second, activated form of Ras can circumvent many effects caused by mutations of *Drosophila* FGF receptors or Dof (Gisselbrecht et al., 1996; Imam et al., 1999; Michelson et al., 1998; Reichman-Fried et al., 1994; Vincent et al., 1998) indicating that Ras activity is important for effective FGF signalling.

Ras mediated signalling

Ras is a small molecule with GTP hydrolysing activity localised to the plasma membrane through lipid modification. It has the capacity to recruit effector molecules in a GTP-dependent

manner. FGF signalling activates it by relocating the guanine nucleotide exchange factor Sos into its vicinity thereby enriching its GTP bound, active form. Up today there are seven Ras effectors known: the serine/threonine kinase Raf, the phospholipid kinase PI3K, the guanine nucleotide dissociation stimulator for Ral GTPases RalGDS (White et al., 1995), phospholipase C ϵ (Wing et al., 2003), the polarity protein AF6/Canoe (Kuriyama et al., 1996), the Ras inhibitor RIN1 (Wang et al., 2002) and IMP, a regulator of MAPK signalling (Matheny et al., 2004).

The Ras-MAPK cascade

The core structure of the MAPK signalling cascade is a module of three kinases: Raf (MAPK kinase kinase), Mek (MAPK kinase) and Erk (MAPK), which become sequentially activated. Phosphorylated Erk enters the nucleus and activates set of transcription factors. The cascade is effectively assembled and localised to the appropriate subcellular site by scaffolding proteins (reviewed in (Morrison and Davis, 2003)). In quiescent cells Raf as well as Mek in complex with the scaffolding protein Kinase suppressor of Ras (Ksr) are bound to 14-3-3 proteins through phosphorylated serine residues which keep them in the cytoplasm (Figure 5A). Upon Ras activation they become partially dephosphorylated that allows Raf to relocate to the membrane via interacting with GTP bound Ras. The Ksr complex released from the inhibitory binding of 14-3-3 recruits Erk and also translocates to the membrane where Raf-Mek-Erk activation occurs (Figure 5B). The protein phosphatase PP2A is responsible for the Ras stimulation-dependent dephosphorylation of Raf and Ksr, thus acting as a regulator at the level of Raf activation (Ory et al., 2003). Another regulator of the MAPK cascade at this level is the Ras effector RIN1. It directly competes with Raf for Ras binding (Wang et al., 2002). RKIP (Raf Kinase Inhibitor Protein) and the Ras effector IMP regulate MAPK cascade at the level of Mek activation. RKIP uncouples Raf-Mek signal transmission by directly inhibiting Raf-Mek interaction (Yeung et al., 2000; Yeung et al., 1999) whereas IMP inactivates the scaffolding protein Ksr. Binding of IMP to Ras blocks its inhibitory activity on Ksr (Matheny et al., 2004). Thus, Ras has dual effector inputs on the MAPK cascade: initiating Raf activation while derepressing Ksr-dependent Raf-Mek complex formation.

Other Ras effectors

Ras is a branching point in signalling since it can activate effectors with diverse functions. Beside the Raf-MAPK cascade discussed above it activates the lipid modifying enzymes PI3K and PLC ϵ . They produce second messengers by lipid phosphorylation and hydrolysis respectively to propagate signalling.

The Ras effector RalGDS activates the Ras family small GTPase Ral. Ral is involved in membrane trafficking by regulating the formation of the exocyst complex (Lipschutz and Mostov, 2002) and the ligand-induced endocytosis of growth factor receptors (Nakashima, 1999). It influences cytoskeletal organisation and filopodia formation through filamin binding and by controlling the activity of Rac/Cdc42 (Ohta, 1999; Sugihara, 2002). It can also influence gene expression by interacting with the Ras-MAPK cascade (Okazaki, 1997). Thus, Ral GTPase might be a good candidate to interconnect signalling cascades downstream of FGF signalling.

Rap1 – a parallel pathway to activate known Ras effectors

Ras signalling is not the exclusive way to activate known effectors of Ras. In some cases Ras effectors are reported to be recruited through the activation of Rap1 to the site of action. Rap1 is a Ras family GTPase with a virtually identical effector domain to that of Ras, suggesting that both proteins theoretically interact with similar effectors. Yeast two hybrid and *in vitro* binding

assays showed that both can bind Raf, RalGDS and AF6/Canoe (Boettner et al., 2003; Ohtsuka et al., 1996). In overexpression situations they behaved antagonistically, but evidence is accumulating that Rap1 functions independently of Ras protein signalling *in vivo*, utilising effectors similar or identical to those of Ras (Asha et al., 1999; Boettner et al., 2003; York et al., 1999). The activation is also independent of the two proteins. Ras is activated by the Grb2:Sos complex. Although Rap1 activation follows the same mechanism and thus, requires the binding of GEF proteins, which are recruited upon phosphorylation-dependent interactions to the plasma membrane, it uses different molecules. The Crk adaptor:C3G Rap1GEF complex is recruited to activate Rap1 upon stimulation (Ishimaru et al., 1999). The specific and exclusive affinity of the GEFs to the respective GTPases ensures independent activation of the two pathways. The *in vivo* role of Rap1 is of emerging importance in the activation of Ral and AF6/Canoe upon EGFR stimulation (Boettner et al., 2003; Mittar et al., 2004; Mott et al., 2003). However, a direct role of Rap1 in FGF signalling remains to be elucidated.

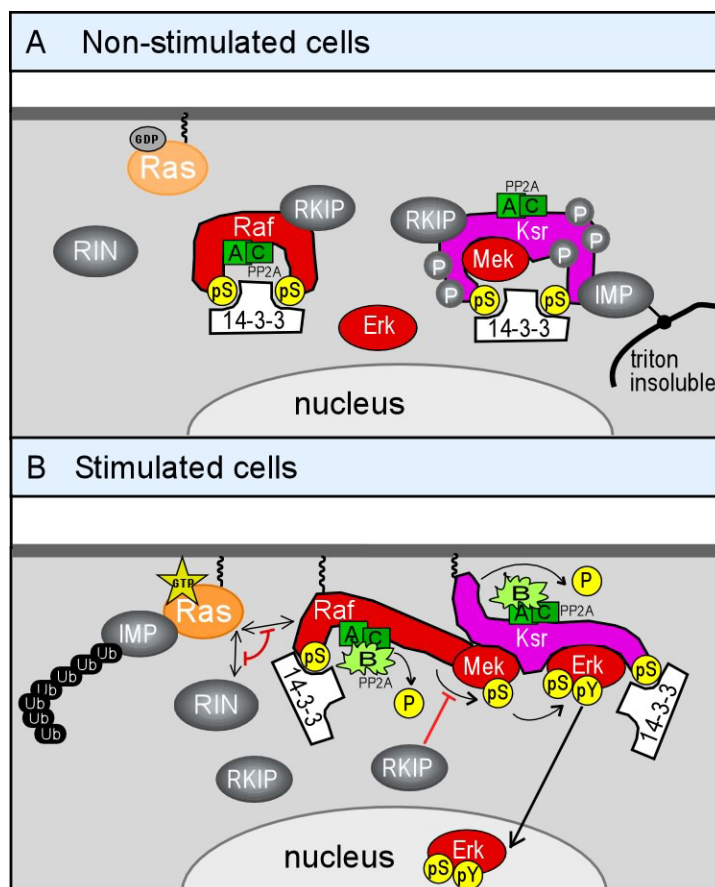


Figure 5. Model for the regulation of the MAPK pathway by Ras activation. (A) In non-stimulated cells, binding of 14-3-3 to phosphorylated Raf and Ksr retains both complexes in the cytoplasm. In addition, Ksr is docked to Triton-insoluble cellular fraction via IMP interaction which also triggers the inhibitory phosphorylation of Ksr. The PP2A core enzyme (A and catalytic C subunit) is bound to Raf and Ksr. Inactive Mek is also part of the Ksr complex. RKIP inhibits Raf-Mek interaction. (B) Stimulation of the cells results in the assembly of the active PP2A holoenzyme leading to dephosphorylation of particular residues in Raf and Ksr. 14-3-3 is displaced from these sites which facilitates the membrane recruitment of both proteins and enables Raf to interact with Ras. Simultaneously, IMP releases Ksr upon binding to activated Ras. Interaction of IMP with Ras induces IMP auto-ubiquitination. Liberated Ksr mediates the complex formation between Raf, Mek and Erk. RKIP is competitive inhibitor of the Raf-Mek interaction. Ras activation recruits an additional negative regulator RIN1. It competes with Raf for the same binding site in GTP-bound Ras.

1.3.2 Negative regulators in FGF signalling

Negative regulators ensure the correct cellular fate by enabling to manipulate signalling levels over a time course or to tune the sensibility of cells to a common signal source. Sprouty was discovered in flies and owes its name to prevent excessive tracheal branching (Hacohen et al., 1998). Meanwhile a whole family of Sprouty(-like) molecules has been identified as general inhibitors of RTK signalling. They can uncouple Ras signalling on multiple levels based on their capacity to interact with Grb2, Gap1 and Raf (Figure 4B). Tyrosine phosphorylation is essential for Sprouty function indicating that it represents a negative feedback loop of the system (reviewed in (Dikic and Giordano, 2003)).

Negative regulatory elements of the MAPK cascade itself were discussed above. MAPK activation induces a negative feedback mechanism that has general consequences on FGF signalling: Erk dependent phosphorylation of the key adaptor FRS2 leads to the downregulation of FGF signalling (Figure 4B) (Lax et al., 2002).

1.3.3 Learning from the fly - specialities of FGF signalling in *Drosophila*

Results of the mammalian cell culture studies helped to reconstitute FGF signalling in flies. The example of Sprouty shows that reverse inputs were similarly fruitful to brighten our knowledge on signalling regulation. *In vivo* studies in *Drosophila* mesoderm and tracheae indicated that cells respond with different levels of FGF-dependent MAPK activity based on their position in the migrating tissue. In the mesoderm only the first 3-4 row of cells contacting the underlying ectoderm show MAPK activity (Wilson, 2004). In the tracheae - after an initial broad activation - MAPK activity is restricted to the cells that form the leading front of migration (Gabay et al., 1997). It shows that intercellular cross talk regulates differential downregulation of FGF signalling in order to shape the developing tissue.

Most fly homologues of the conserved FGF signal transduction machinery have been identified. However there was a missing link in *Drosophila* FGF signal transduction for a long time, namely the functional equivalent of FRS2. The *Drosophila* homolog of FRS2 does not have the function of an FGF receptor specific adaptor (personal communication to M. Krasnow). First hint for a solution came with the identification of Dof. Genetic and biochemical studies provided evidence that Dof might be the FGF receptor specific adaptor in *Drosophila*. It is essential for all known functions of *Drosophila* FGF receptors but at present it is not fully understood how and to what extent it “copies” FRS2 functions and what is a solely Dof specific contribution in FGF signalling.

1.4 The protein Dof

dof is a fast evolving gene (Wilson et al., 2004). Two weak vertebrate homologues of Dof are known, B-cell adaptor for PI3K (BCAP) (Okada et al., 2000) and B-cell scaffolding protein with ankyrin repeats (BANK) (Yokoyama et al., 2002). Highest level of conservation is found in their DBB domain, a unique domain of the family (Wilson et al., 2004). Two ankyrin repeats and a coiled coil region as well as a C-terminal proline rich sequence stretch are also common structural elements of the three proteins. Dof contains several potential sites of post-translational modifications: (i) eight tyrosine motifs which could serve as binding sites for different SH2 domains in their phosphorylated form, (ii) four consensus sites for sumoylation and (iii) a caspase cleavage site (Figure 6). Dof is able to form a complex with the activated FGFR and is tyrosine phosphorylated in an FGFR dependent manner (Petit et al., 2004; Wilson et al., 2004). Phosphorylation of Tyr 515 recruits the protein phosphatase Corkscrew to Dof (Petit et al., 2004). Dof has several further potential phosphorylation-dependent interaction partners like Grb2, PI3K, RasGAP and Crk and many phosphorylation-independent putative interactors identified in a yeast two hybrid screen, e.g. components of the sumoylation machinery (Battersby et al., 2003). Dof has the capability for self-association (Battersby et al., 2003). Two regions are important in the protein for interaction: the most conserved DBB domain in the N-terminal half of the molecule and the C-terminal part including the coiled coil region. Whereas the DBB domain is able both to dimerise with itself and to bind the C-terminal part, the C-terminal part fails to bind itself and can only trans-interact with the DBB domain. This gives rise to several possible assembly models based either on intermolecular or on intramolecular interactions or on both. It was found in yeast that the C-terminal

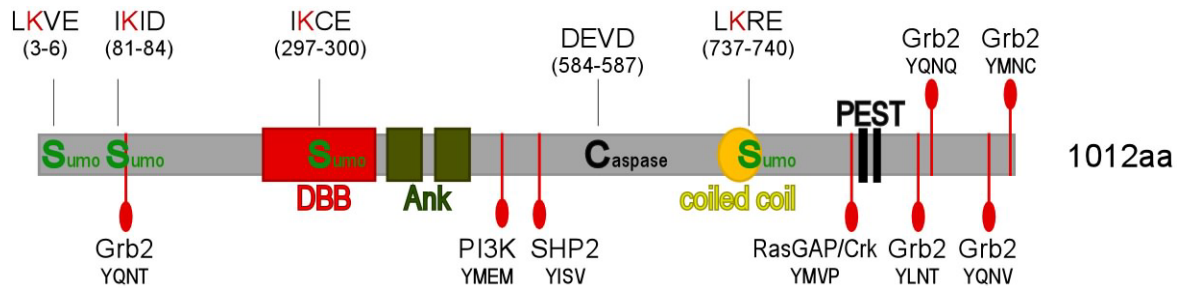


Figure 6. Domain organisation and consensus sequence motifs of Dof. Coloured boxes mark DBB (Dof, BCAP and BANK) domain (red), Ankyrin repeats (green) and coiled coil region (yellow). Thick black lines label recognition sequences for protein degradation (PEST). Consensus tyrosine motifs are marked by red lines on the scheme. In addition the potential binding partners as well as the amino acid sequence of each motif are depicted. The four potential SUMO attachment sites are labelled with a green S on the drawing, amino acid sequence and position of the consensus sites is marked. Lysine residue of SUMO attachment is labelled in red. Amino acid position and sequence of the consensus caspase cleavage site is given and the corresponding position is labelled with a black C on the scheme.

part weakened self-association compared to C-terminally truncated forms, supporting the presence of a head to tail, “closed” structure relying exclusively on intramolecular interaction. A subset of the yeast interactors showed conformation dependent binding to Dof in such a way, that a model for the existence of both a “closed”, and an “open” molecule conformation has been proposed (Battersby et al., 2003). Beside self-interaction the DBB domain is also essential for efficient receptor binding, and the presence of the C-terminal tail weakens also this interaction in yeast (Wilson et al., 2004). This is an additional indication that trans-interaction between DBB domain and C-terminal part is utilised in Dof self-association.

1.5 Specific aim of this thesis

Different cellular responses are triggered by FGF signalling in *Drosophila*, all of them seem to require Dof activity. How can Dof transmit all the different branches of FGF signalling?

One way to achieve such a diverse function is to interact with many different molecules. In this work potential Dof interactors found in an earlier yeast two hybrid screen were studied for their Dof-binding capability with special attention on the *Drosophila* FGF receptor Heartless. Common feature of adaptor proteins is that signalling-dependent post-translational modifications influence the dynamics of their interaction behaviour. Predicted post-translational modifications of Dof - phosphorylation, sumoylation and caspase cleavage - were identified in this work with the aim to find their functional relevance in the regulation of signal transduction.

Alternatively or in parallel to this FGF signalling might raise its diversity downstream of Dof. Given that Ras can partially circumvent upstream dysfunctions in FGF signalling and has many known effectors, it might provide the major branching point in FGF signalling. This work also aimed to dissect known Ras functions and to analyse their contribution in FGF signalling.

2. Results

2.1 Revisiting yeast Dof-interactors in *Drosophila* S2 cells

To validate the biological relevance of interactions found in a yeast two hybrid screen using a truncated form of Dof as bait (Battersby et al., 2003) interaction studies were performed in *Drosophila* cell culture with a subset of candidates identified in the screen. We selected 16 out of the 41 interactors of the yeast two hybrid screen (Figure 7). Our major point was at the selection the structural-functional aspects of the gene products the candidates coded for. Candidates were chosen that are known to be involved in FGF dependent developmental processes or encode signalling molecules or their regulators, cytoskeleton proteins, enzymes with potential roles on modification of Dof or candidates with interesting protein domains but unknown function. Among the 41 interactors were several nuclear proteins and transcription factors, a fact which was unexpected, since Dof is localised in the cytosol with enrichment at the cell membrane (Vincent et al., 1998). To investigate this apparent contradiction two known nuclear proteins were also chosen for further analysis. In addition, we selected preferentially candidates represented with more than one independent clone in the yeast two hybrid screen. On the other hand we have not sorted out interacting clones being out of the correct reading frame. Yeast two hybrid screen was carried out under two different growth selection conditions. Although it is believed, that difference in growth under different selection conditions might represent difference in strength of interaction we did not chosen this as a criterion of the selection. Selected candidates are sorted in Figure 1. based on their growth behaviour in yeast. Ade2 positives are the candidates which grow also at higher stringency, whereas Ade2 negative stands for growth only at less stringent conditions.

To perform interaction studies we applied a transient transfection system in *Drosophila* S2 cells and expressed full length Dof together with the candidate protein with different epitope tags under the control of a copper inducible promoter. Dof had a FLAG epitope tag on its N-terminus (FLAG-Dof) whereas interacting candidates had an N-terminal HA epitope tag. If more than one interactor clone was found in yeast coding for the same gene the longest cDNA clone was expressed in S2 cells, even if it represented only C-terminal parts of the protein. If 5' UTR was included in the yeast clone it resulted than additional N-terminal amino acids. As only alteration the reading frame of interactor clones was changed if the original yeast clone was not in frame with the Gal4 activation domain. Immunoprecipitation assay was carried out on lysates of S2 cells expressing FLAG-Dof together with the listed candidates. Interaction was tested by precipitating the interacting candidate via the HA epitope tag and monitoring the co-precipitation of FLAG-Dof and *vice versa*. To precipitate FLAG-Dof, in addition to the FLAG epitope tag also Dof specific antibody was used, thereby increasing the reliability of the assay.

As an independent analysis for potential interaction, co-localisation studies were performed with some of the candidates in transiently transfected *Drosophila* S2 cells.

2.1.1 Summary of Dof interaction data

The immunoprecipitation results of Dof with the yeast interactors are summarised in Figure 7. An interaction was verified as positive if co-precipitation of FLAG-Dof or the interactor clone (i-clone) was detected at least in one immunoprecipitation reaction. A positive interaction was defined as strong if FLAG-Dof co-immunoprecipitated the interactor and *vice versa*. If co-precipitation was observed only from the side of the one protein it was defined as weak interaction. No interaction complete failure of co-precipitation.

		Yeast two hybrid interaction			Co-immunoprecipitation assay					
i-clone	gene	number of clones	frame	domain structure (length of i-clones)	expression in S2 cells	HA-i-clone co-precipitation with FLAG-Dof	FLAG-Dof co-precipitation with HA-i-clone	co-IP interactor class		
						FLAG	Dof	HA		
Ade2 positive clones	I-6	beta spectrin	4	0		-	-	-	n.d.	
	I-8	sinuous	1	+2		✓	-	-	±	weak
	I-14	rpS10	10	+1		✓	?	+	+	strong
	I-19	CDEP	3	0		✓	+	+	+	strong
	I-28	dof	9	0		✓	+	-	+	strong
	I-77	ubc9	5	0		✓	?	+	+	weak***
	I-150	heartless	5	0		✓	+	+	+	strong
	I-25	gustavus	1	0		✓	?	+	+	strong
	I-51	GAP69C	1	0		✓	+?	+	+	strong
	Ade2 negative clones	I-133	bap60	5	0		✓	-	±	-
I-163		bip2	2	0		low	-	-	-	n.d.
I-173		rack1	1	+2 (5'UTR)		-	-	-	-	n.d.
I-184		Su(var)2-10	1	0		✓	-	-	-	no
I-188		CG11275	1	0		✓	+	+	+	strong
I-234		PP2A-B'	1	0		✓	+	?	+	strong
I-249		racGAP	1	+2		low	-	-	-	n.d.

*** interacted only if SUMO was co-transfected

Figure 7. Interaction of Dof with yeast two hybrid interactors in immunoprecipitation assay in S2 cells. Clones isolated in the yeast two hybrid screen and selected for Dof interaction assay are listed on the left. The gene represented by the interaction clone is given in the next column. Number of clones: the number of clones of the corresponding gene found in the screen. Frame: open reading frame of the i-clone transcript relative to the Gal4 activation domain in the yeast expression vector. 0 denotes the reading frame identical with that of the activation domain (in-frame fusion). Domain structure is represented of the protein encoded by the interacting clone. The reference transcript is shown as a thin bar, the open reading frame as a thick bar, described protein domains and PFAM motifs are shown as rectangles indicating their names above the diagram. Part of the transcript coded by the interacting clone is shaded in blue; if more than one clone was identified of the gene, the position of the first amino acid of the shortest clone is indicated by an arrow. Immunoprecipitation assay of i-clones with Dof: first column shows if i-clone was successfully expressed in S2 cells. For immunoprecipitation three antibodies (columns FLAG, Dof and HA) were used. + in columns FLAG and Dof means that the i-clone co-precipitated with FLAG-Dof. + in column HA means that FLAG-Dof co-precipitated with the i-clone. Minus indicates no co-precipitation, ± weak co-precipitation and question mark stays if the co-precipitation could not be reliably detected. Strong interaction, co-precipitation was

Out of the 16 candidates selected for cell culture experiments three did not give conclusive results due to insufficient protein levels for biochemical analysis (i249, i6, and i163). The clone i173 was not finally included in the analysis (for details see Subchapter i173). The twelve candidate proteins, which showed high expression levels in S2 cell lysates divided into three groups according to their interaction behaviour with Dof. Eight of them showed strong interaction (Dof, Heartless, i19, i51, i234, i25, i188 and i14). Three candidates – i8, i77 and i133 – fell into the weak interaction category. Although i77 belonged to the class of strong interactors upon the criteria defined above, we considered it as weak interactor because the co-expression of SUMO protein was required for efficient interaction with Dof. i184 was the only candidate that clearly failed to interact with Dof in the cell culture assay.

In summary, none of the candidates of the Ade2 positive group, thus, interactors which grow also at more stringent selection conditions in yeast belonged to the no interaction category. Furthermore, many of the Ade2 negative yeast interactors showed strong interaction with Dof in S2 cells. Thus, members of both groups behaved as equally potent candidates. In general, the yeast two hybrid screen appeared as a reliable pre-selection for proteins that were able to form a complex with Dof in *Drosophila* S2 cells.

The analysis of the individual candidates is presented in the following subchapters.

2.1.2 Signalling molecules

Dof self-association

In the yeast two hybrid screen Dof as bait interacted with 9 clones representing C-terminal fragments of Dof itself (Battersby et al., 2003).

In our experiments full length Dof was taken with N-terminal FLAG and HA epitope tags and co-expressed in S2 cells. In Western blot of lysates we always detect a band of the size predicted for Dof (113 kDa), and additional bands that migrate at about 72 kDa and 100 kDa (Figure 8), which represent N-terminal fragments of Dof, since they are recognised by antibodies directed against the N-terminal epitope tags. Chapter 2.5 discusses a detailed analysis of these fragments. We performed immunoprecipitations of cell lysates with antibodies against Dof or the epitope tags and analysed them on Western blots (Figure 8). The FLAG and HA antibodies precipitated the appropriately tagged proteins, and the Dof antiserum precipitated both constructs. In addition, immunoprecipitation made with anti-FLAG antibody also contained proteins marked with the HA-tag and *vice versa*. This indicates that FLAG-Dof and HA-Dof were present in one complex. Yeast two hybrid data showed that the presence of the C-terminal tail of Dof weakened self-association and proposed a model of “closed” conformation where self-association is based on intramolecular interaction. Our result shows that even in the presence of the C-terminal part of the protein, Dof is able to form dimers or multimeric complexes in S2 cells. However, this was an overexpression situation, and *in vivo* protein levels are not known for comparison. The probability of complex formation and the existence of different conformations might be concentration dependent. Nor can be excluded that certain factors, which are absent in *Drosophila* S2 cells might influence Dof self-association *in vivo*.

observed in both directions; weak, only the one protein pulled down successfully the other; no interaction, the proteins did not co-precipitate. n.d., no data. Modified and extended from (Battersby et al., 2003).

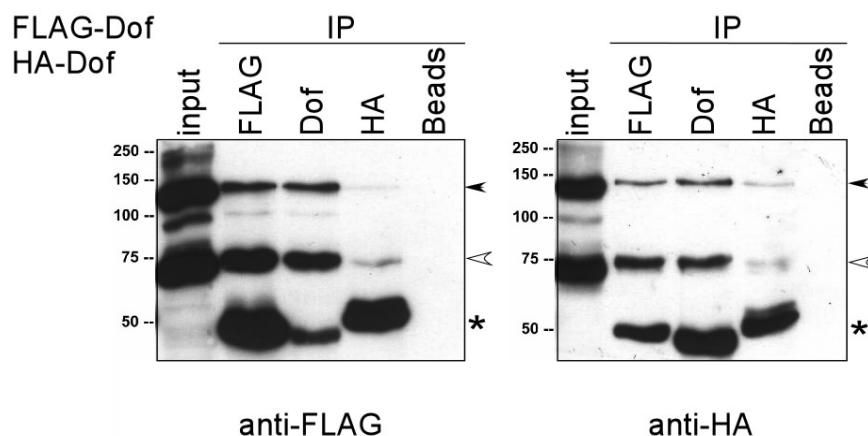


Figure 8. Interaction of Dof with itself. N-terminally FLAG and HA epitope-tagged Dof proteins were expressed transiently in *Drosophila* S2 cells under the control of the metallothionein promoter. At 24 hours after induction, cell lysates were immunoprecipitated with anti-FLAG, anti-Dof or anti-HA antibodies. Western blots were probed for the tagged proteins by anti-FLAG (left) or by anti-HA (right) antibodies. The “input” lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each “IP” lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy chain of the antibodies used for immunoprecipitation. Black arrowhead marks full length Dof, white arrowhead N-terminal Dof fragment.

Heartless

Five clones representing four different C-terminal fragments of Heartless, the one *Drosophila* FGF-receptor homologue were found to interact with Dof in yeast (Battersby et al., 2003). Breathless, the other FGF-receptor homologue in flies was not found in the screen, but in a direct test it also showed positive interaction with Dof (Wilson et al., 2004).

In the cell culture assay Heartless was tested for interaction. In this case we did not use the same fragment found in the screen, but co-expressed FLAG-Dof with a constitutively active form of Heartless bearing a double HA epitope tag on its C-terminus. This construct contained the entire intracellular part and transmembrane domain of Heartless fused to the dimerisation domain of the C1 protein of the λ phage. It forms dimers in the cell membrane and therefore is constitutively in an autophosphorylated, activated form (Wilson et al., 2004). Immunoprecipitation was performed from cell lysates with antibodies against Dof or the epitope tags and analysed on Western blots (Figure 9.). The FLAG and Dof antibodies precipitated Dof, and the HA antibody precipitated Heartless. In addition, the FLAG and Dof antibodies co-precipitated the HA-tagged receptor and the HA antibody pulled down the Dof specific bands detectable with FLAG antibody. This confirms the yeast two hybrid data and indicates that FLAG-Dof and activated Heartless form a complex in S2 cells. A detailed study of Heartless-Dof interaction is presented in Chapter 2.2.

i19

Three independent clones were isolated in the screen that code for the gene CG31536, also named as CDEP. CDEP is a putative guanine nucleotide-exchange factor (GEF) for Rho family GTPases. Its close homologue, human CDEP (chondrocyte derived ezrin protein) represents a novel GEF for Rho family GTPases with transforming activity and may play a role in mediating or modulating the action of cAMP-elevating hormones on maturing chondrocytes (Koyano et al., 2001). Both proteins show the same domain architecture. They contain a RhoGEF/Dbl/DH domain,

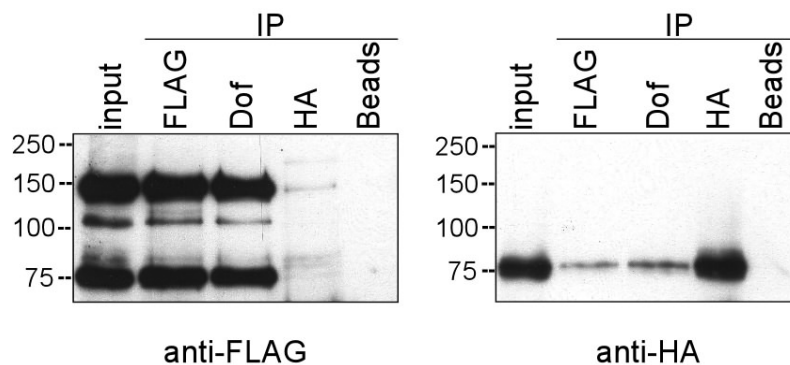


Figure 9. Constitutively active form of Heartless interacts with Dof in S2 cells. Lysates from *Drosophila* S2 cells coexpressing a constitutively active, HA-tagged form of Heartless and a FLAG-tagged full-length Dof protein were used for immunoprecipitation (IP) with antibody against FLAG, Dof or HA or control beads without antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (left) or by anti-HA (right) antibodies. The “input” lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each “IP” lane one third of an immunoprecipitation is loaded.

two C-terminal Pleckstrin-homology (PH) domains and a FERM (4.1-ezrin-radixin-moesin) domain at their N-terminus, which potentially mediates association to the cytoskeleton.

The i19 clone lacks the major part of the FERM domain (see Figure 7). The clone is detected at 85 kDa on Western blots of S2 cell lysates expressing the HA-tagged form of i19. It behaved as strong interactor in the immunoprecipitation assay (Figure 10A). Interestingly, the HA antibody pulled down a FLAG specific band corresponding the size of full length Dof but not the other, even more intense band about 72 kDa detectable in lysate and FLAG/Dof immunoprecipitates. It is a unique behaviour among the interactors tested and might indicate that i19 binds to a C-terminal part of Dof, which is not present in the 72 kDa N-terminal fragment. Nevertheless it has to be considered that the N-terminal fragment contains the DBB domain (see chapter 2.5), which is required and sufficient for dimerisation (Battersby et al., 2003). Therefore an N-terminal Dof fragment could be simply present as a consequence of Dof self-association. Since there is no N-terminal Dof fragment detectable, it cannot be excluded that binding of i19 to a C-terminal part of Dof simultaneously inhibits Dof self-association. In yeast interaction studies i19 was mapped to bind either to a region in the N-terminus or a region within or in the vicinity of the coiled coil motif in Dof (Battersby et al., 2003).

i51

One single clone belonging to the Ade2 negative group was identified in the screen which coded for the gene GAP69C. It codes a homologue of the adenosine diphosphate-ribosylation factor 1 (Arf1) GTPase activating protein (GAP) ASAP1 (Brown et al., 1998). Arf GTPases are best characterised to regulate membrane trafficking but they have also role in cytoskeletal organisation. ASAP1 localises to focal adhesions and regulates cytoskeletal remodelling (Randazzo et al., 2000). Loss of GAP69C function resulted gaps in the embryonic tracheal network (Battersby, 2001), an indication that it might act on same developmental processes as Dof. In that assay the gene was removed as a part of a big deletion on the left arm of the 3. chromosome. Null alleles of the gene generated by imprecise P-element excision are homozygous viable and fertile and show no apparent abnormal phenotype (Frolov and Alatortsev, 2001), suggesting that the phenotype of the deficiency was caused by the removal of other genes of the region and indicating that Gap69C is not essential for tracheal development.

i51 cDNA lacks the first 29 amino acids of the predicted GAP69C protein. The protein is detected at 50 kDa on Western blots of lysate from S2 cells expressing HA-i51 (calculated

molecular size 47 kDa). Western blots of the immunoprecipitation assay are shown on Figure 10B. The FLAG and Dof antibodies precipitate Dof, and the HA antibody precipitates i51 (please note, the heavy chain of the HA antibody used for IP has similar molecular size, and so overlaps with the HA-i51 specific band). It is plausible to detect any HA specific bands in the protein fraction precipitated by the FLAG antibody because it might be masked by the heavy chain of the FLAG antibody used for IP. Nevertheless the Dof antibody co-precipitates HA-i51 and the HA antibody pulls down preferentially the N-terminal fragment of FLAG-Dof. At very long exposure time the FLAG specific band corresponding to the full length Dof is also detectable (data not shown), but it is no more in a range, that statements could be given about quantitative ratios of the two bands in the different IPs. This result confirms the yeast data and indicates that Dof is able to form a complex with GAP69C in cells.

i249

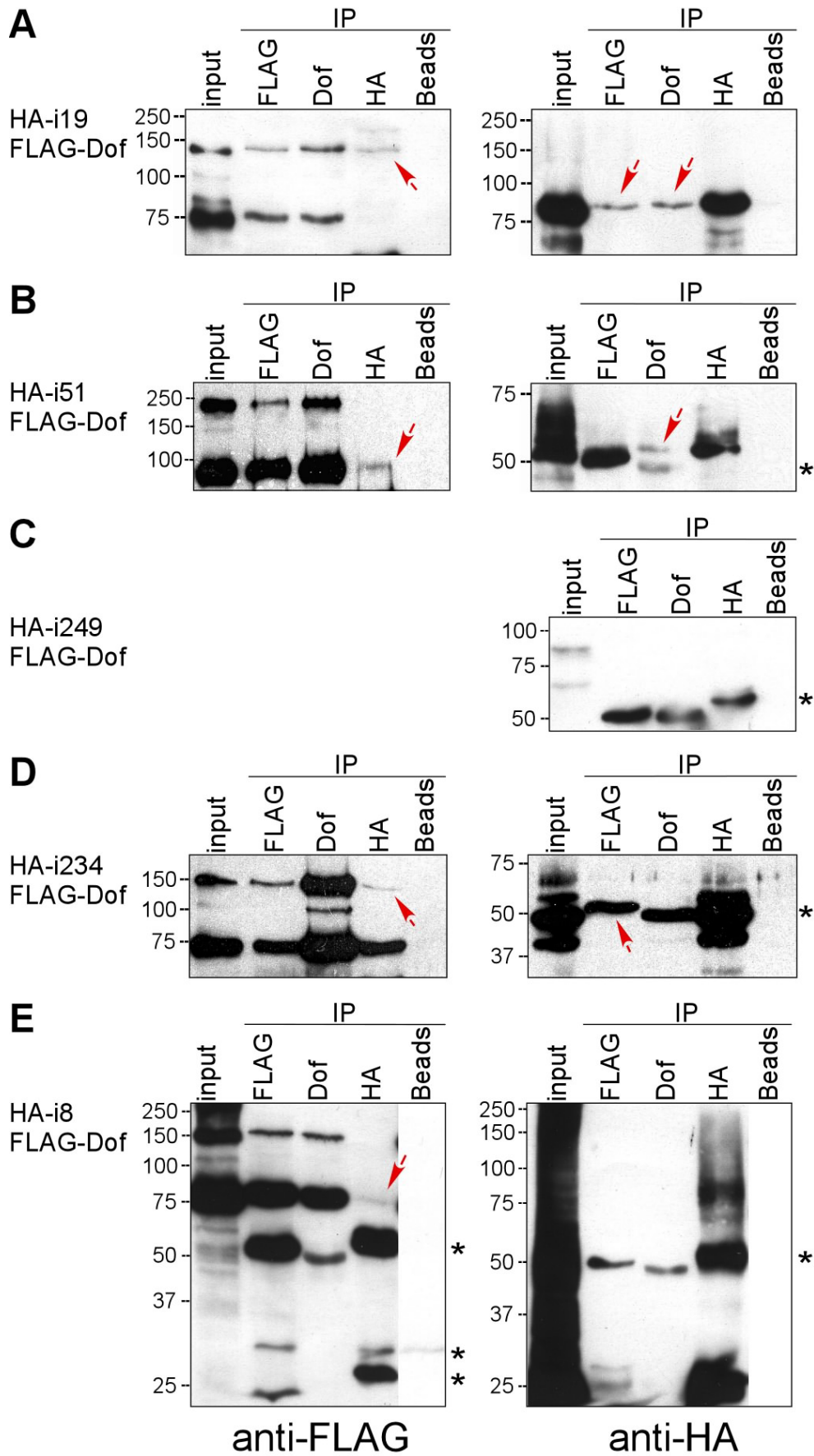
A single clone was identified in the yeast two hybrid screen that encoded the gene *racGAP50C*, also known as *DracGAP*. It is member of the Ade2 negative interactor group and furthermore it was not in the same reading frame as the Gal4 activation domain - two features, which weaken the validity of yeast interaction data. What curious our interest was that it was described as negative regulator of Rho family GTPases DRac1 and DCdc42 and showed genetic interaction with the EGFR/Ras pathway during wing development and morphogenesis (Sotillos and Campuzano, 2000).

The N-terminally HA epitope tagged, frame corrected version of i249 was expressed together with FLAG-Dof in S2 cells. Two weak bands were detected on Western blots of cell lysate stained with HA specific antibody: the smaller one with a size corresponding to the calculated molecular size of i249 (62 kDa) (Figure 10C). In immunoprecipitation experiments the protein failed to precipitate to a detectable level on Western blots. Thus, i249, a truncated form of *DracGAP* lacking the first 66 amino acids of the protein might be unstable due to incorrect folding in the cells or cells might not tolerate and so downregulate high levels of the protein by degradation. In consistent with this, very weak expression of i249 was detectable in immunostained S2 cells (data not shown).

i173

A single clone was identified in the yeast two hybrid screen that encoded the gene *RACK1* (Receptor for Activated C Kinase). It belonged to the Ade2 negative interactor group. The cDNA contained 5'UTR regions of the gene and was not in frame with the Gal4 activation domain. In a retest with different mutant Dof constructs in yeast it showed no interactions (Battersby et al., 2003). Several *Rack1* clones were isolated in an independent yeast two hybrid screen using Dof as bait (M. Affolter, personal communication). Therefore it seemed initially to be a good control for

Figure 10. Immunoprecipitation assay of Dof with i-clones encoding different signalling molecules and the *Drosophila* claudin sinuous. FLAG epitope tagged Dof and HA epitope tagged i-clones were co-expressed in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG, anti-Dof or anti-HA antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (left) or by anti-HA (right) antibodies. The "input" lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each "IP" lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy and light chains of the antibodies used for immunoprecipitation. Arrows mark co-precipitating FLAG-Dof and HA-i-clones respectively.



the validity of the yeast data of our laboratory. The 5' sequence of the i173 cDNA contains two STOP codons in the 5' UTR in the first reading frame, one STOP codon in the 5' UTR in the second frame (RACK1 ORF), and the third frame gave rise a peptide of 45 amino acids in length and completely unrelated to RACK1. Hence, even if there is a real interaction existing between the molecules it had been hazardous to base any further biochemical analysis on these yeast data. Thus, interaction assay was not performed with this clone in S2 cells.

i234

One single Ade2 negative clone was identified in the screen that coded a small C-terminal fragment of the B' regulatory subunit of Protein Phosphatase type 2A (PP2A-B') (see Figure 7). PP2A has been shown to be involved in the regulation of signal transduction, cell proliferation, apoptosis and establishment of planar polarity (Lechward et al., 2001; Ory et al., 2003; Van Hoof and Goris, 2003). The regulatory subunits determine substrate specificity and subcellular localisation of the holoenzyme.

The N-terminally HA epitope tagged version of i234 was expressed together with FLAG-Dof in S2 cells. On Western blots of lysates several bands were detected with the HA antibody, one with the predicted molecular size of i234 (45 kDa) (Figure 10D). Western blots of immunoprecipitation experiments are shown in Figure 10D. FLAG and Dof antibodies precipitated Dof, but it is not detectable if an HA specific band was present with a corresponding size for i234 in these protein fractions, because the heavy chains of the antibodies used for immunoprecipitation masked this part of the blot. The HA antibody precipitated all HA specific bands detected in the lysate and in addition, pulled down proteins recognised by the FLAG antibody. It indicates that FLAG-Dof was present in a complex containing the HA-tagged i234.

2.1.3 Cytoskeleton proteins

i6

Four clones showed interaction with Dof in the yeast two hybrid screen that encoded the β -spectrin gene. β -spectrin is essential component of the cortical cytoskeleton which confers mechanical stability to the membrane by establishing a bridge between the plasma membrane and the actin cytoskeleton (Bray, 2000). Its N-terminal calponin homology domains bind actin, while the C-terminal PH domain anchors it to the plasma membrane. A long stretch of 17 spectrin repeats space the two different binding motifs.

The i6 clone contains the last 3 spectrin repeats and the PH domain. We expressed the N-terminally HA epitope tagged version of i6 with FLAG-Dof in *Drosophila* S2 cells. On Western blots of lysates we failed to detect any bands with the HA antibody (data not shown). Hence, the clone might be a structurally unstable truncation of the molecule. Alternatively HA-i6 might be insoluble under the lysis conditions used preparing the samples for immunoprecipitations.

i8

A single clone of the Ade2 positive group of the yeast two hybrid screen encoded the gene sinuous. The clone was out of the reading frame of the Gal4 activation domain and contained

5'UTR region of the gene. After cloning into the correct reading frame (5'UTR was retained) it failed to interact with Dof in yeast (Battersby, 2001).

Sinuous was initially found in a screen for genes, which control epithelial tube size in the *Drosophila* tracheal system (Beitel and Krasnow, 2000), and recently described as a *Drosophila* claudin required for septate junction organisation in other epithelial tubular compartments (Wu et al., 2004). Arthropod septate junctions function as paracellular barriers and are considered to be analogous to the vertebrate tight junctions (Anderson, 2001; Tepass et al., 2001).

The N-terminally HA epitope tagged, in-frame version of i8 was expressed together with FLAG-Dof in S2 cells. To elucidate the specific expression of the tagged proteins, immunocytochemistry was carried out on these cells (Figure 11C-F). i8 was visualised via its HA tag and showed tight membrane localisation. It was consistent with the fact, that claudins are four-transmembrane domain proteins. In addition, FLAG-Dof showed a higher membrane enrichment in the presence of HA-i8, than if it was expressed alone (compare to Figure 11B). On Western blots of lysates stained with HA specific antibody despite our expectations to detect i8 at its predicted molecular size of 32 kDa, numerous bands of diverse sizes appearing as a "smear" were observed (Figure 10E). This phenomenon was dependent on the expression of i8 and not unspecific binding of the HA antibody to proteins of the S2 cell lysate (compare lysates containing other HA-tagged interactors). In immunoprecipitation experiments the proteins precipitated by the HA antibody contained an intense band, which might correspond to HA-i8, but still several higher molecular weight bands were detected by the HA antibody (Figure 10E). In addition, this precipitate contained a band with a corresponding size of a FLAG-Dof N-terminal fragment detected by the FLAG antibody. FLAG and Dof immunoprecipitates contained bands of the FLAG-Dof construct, but no bands recognised by the HA antibody.

Thus, these results show, that HA-i8 was specifically recognised in cells by the HA antibody. The "smear" like appearance on Western blots might be a consequence of unsatisfactory solubilisation of the transmembrane protein, trapped in micelli which might not have been resolved on SDS-PAGE. It might have trapped other proteins, which made interpretations of this interaction assay unreliable.

2.1.4 Components of the SUMOylation machinery

SUMOylation is a related enzymatic mechanism to ubiquitination. The covalent attachment of SUMO (small ubiquitin-like modifier) to lysine side-chains of substrates requires E1 activating enzymes, E2 conjugating enzymes and E3 ligases, which provide selectivity to the system through their diversity (reviewed (Johnson, 2004)). Dof contains 4 potential consensus SUMO binding sites (see introduction), therefore it was exciting to find known or potential components of the SUMOylation pathway among the yeast interactors. Here are discussed only aspects of interaction of these proteins with Dof, a detailed study about Dof SUMOylation, as potential posttranslational modification is described in Chapter 2.4.

i77

Five transcripts of the *Drosophila* Ubiquitin-conjugating enzyme 9 (Ubc9) gene, also known as *lesswright/semushi* (Apionishev et al., 2001; Epps and Tanda, 1998) were isolated in the yeast two hybrid screen representing one to five amino acid shorter clones than the full length protein. They were in-frame, Ade2 positive interactors (see Figure 7). Ubc9 encodes a SUMO-conjugating enzyme.

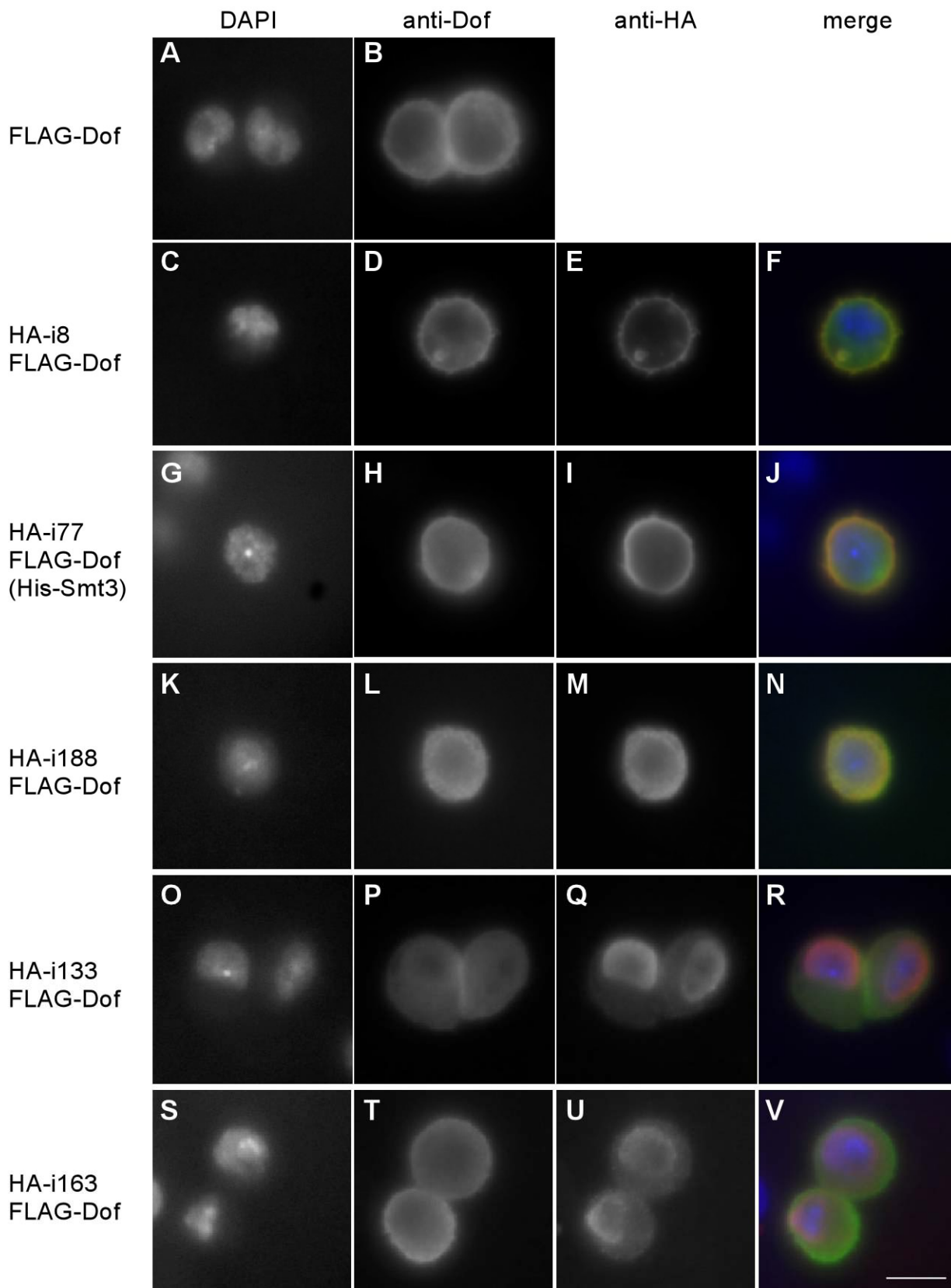


Figure 11. Subcellular localisation of Dof and several i-clones in *Drosophila* S2 cells. S2 cells overexpressing FLAG epitope tagged Dof (A,B) alone or together with HA epitope tagged (C-F) i-8, (G-J) i-77, (K-N) i-188, (O-R) i133 or (S-V) i-163 are stained with (A,C,G,K,O and S) DAPI to visualise nuclear DNA and with antibodies directed against (B,D,H,L,P and T) Dof and the (E,I,M,Q and U) HA epitope to detect localisation of Dof and of the i-clones respectively. F,J,N,R and V are merged images of the three stainings in case of each cell co-expressing Dof and an i-clone. Scale bar 2 μ m.

Expressing the N-terminally HA epitope tagged version of i77 with FLAG-Dof in *Drosophila* S2 cells we failed to detect any interaction of the proteins (data not shown). SUMOylation is a conserved posttranslational modification from yeast to human. We wondered whether *Drosophila* Ubc9 might utilise Smt3, the yeast SUMO peptide to modify and thereby interact with Dof. In other words, a potential additional essential component might be present in yeast to allow effective enzyme-substrate interaction. Based on this hypothesis we extended the cell culture co-transfection assay with an activated form of *Drosophila* Smt3. To elucidate the localisation and specific expression of the tagged proteins immunocytochemistry was carried out on these cells (Figure 11G-J). i77 showed cytosolic localisation highly enriched at the membrane. FLAG-Dof showed a diffuse cytosolic distribution in the presence of HA-i77. Western blots of immunoprecipitation experiments are shown in Figure 12A. The Dof antiserum co-precipitated HA-i77 but in case of the FLAG antibody it could not be reliably detected because of the similar running behaviour of the light chain of the FLAG antibody. The HA antibody pulled down a band, which was recognised by the FLAG antibody and might correspond to an N-terminal fragment of the FLAG-Dof protein. This confirms the yeast two hybrid data and indicates that FLAG-Dof and HA-Ubc9 interacted in S2 cells in the presence of the activated form of Smt3. This initiated further analysis to reveal if Dof is a potential Ubc9 substrate (see Chapter 2.4).

i184

A single clone of the Ade2 negative interactor group was isolated in the screen that encoded the gene *su(var)2-10*, synonymous with *dzimp* or *dpias*. The transcript represents a new, uncharacterised C-terminal isoform of *dpias* (G. Karpen, personal communication). *dpias* is the *Drosophila* homologue of the mammalian protein inhibitor of activated stat (PIAS) gene family, which has been shown to encode a SUMO E3 ligase (Kotaja et al., 2002).

Western blots of immunoprecipitation experiments from lysates of S2 cells expressing the N-terminally HA epitope tagged version of i184 together with FLAG-Dof in *Drosophila* S2 cells are shown on Figure 12B. Both proteins could be detected in lysates via their epitope tags and immunoprecipitates of the antibodies contained bands with corresponding size of the appropriately tagged proteins (HA-i184 15 kDa), but we failed to detect any co-immunoprecipitation of the proteins. It is in contradiction with the yeast data and indicates that FLAG-Dof is not able to form a complex with HA-i184 in S2 cells under the conditions we tested.

2.1.5 Other candidates

i25

One clone was identified among the Ade2 negative interactors that encoded the gene *gustavus*. It has been shown that *gustavus* directly interacts with VASA, a key protein in establishing the specialised translational activity of the *Drosophila* pole plasm and so is essential for the posterior localisation of VASA (Styhler et al., 2002). Like VASA, it localises to cytoplasmic ribonucleoprotein particules. *Gustavus* is primarily expressed in the ovaries and its transcript is detectable in up to 8 hours old embryos by Northern analysis (Styhler et al., 2002). *Gustavus* is highly conserved and apparent orthologs are present in a wide range of animal species with so far unknown function. It contains a SOCS domain at its C-terminus, initially discovered in SH2 domain-containing proteins of the suppressor of cytokine signalling (SOCS) family (Hilton et al., 1998). In mammalian cells, SOCS box can bind the elongin BC complex, an association proposed

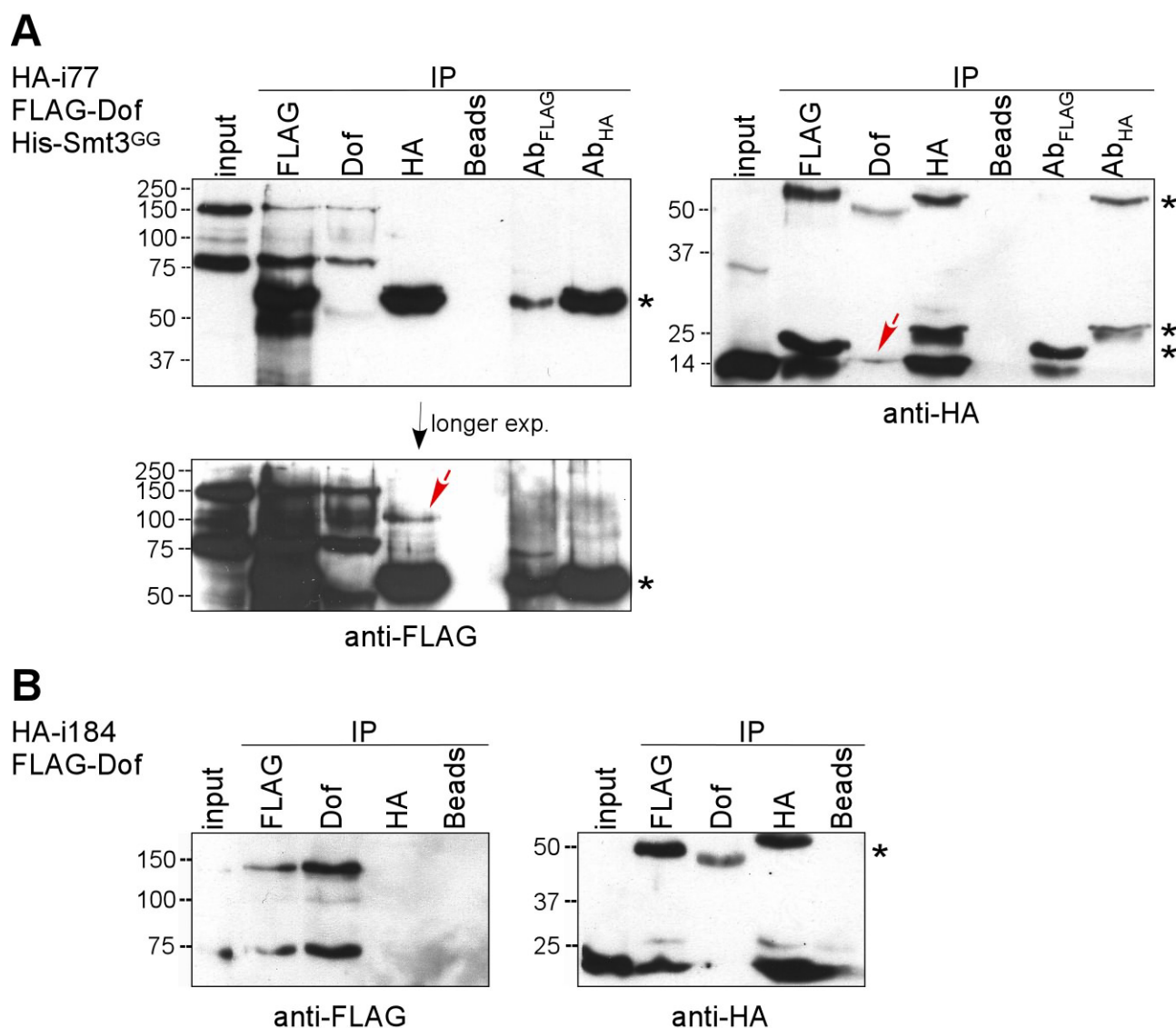


Figure 12. Immunoprecipitation assay of Dof with i-clones encoding components of the sumoylation machinery. FLAG epitope tagged Dof was co-expressed (A) with HA epitope tagged i-77 (Ubc9) and activated form of SUMO or (B) with HA epitope tagged i-184 (dpias) in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG, anti-Dof or anti-HA antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (left) or by anti-HA (right) antibodies. The “input” lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each “IP” lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy and light chains of the antibodies used for immunoprecipitation. Arrows mark co-precipitating FLAG-Dof and HA-i-77(Ubc9) respectively.

to target proteins to ubiquitination (Kamura et al., 1998). It has a SPRY domain, first identified in ryanodine receptors, which mediate Ca^{2+} release from the sarcoplasmic reticulum (Ponting et al., 1997).

Western blots of immunoprecipitation experiments from lysates of S2 cells expressing N-terminally HA epitope tagged version of i25, which contained the full length coding sequence of gustavus together with FLAG-Dof are shown in Figure 13A. The proteins precipitated by the HA antibody contained a band of 30 kDa recognised by the HA antibody, corresponding to the size of HA-i25. In addition, the FLAG and Dof antibodies co-precipitated HA-i25 and the HA antibody pulled down specifically a band, which was recognised by the FLAG antibody and might

correspond to an N-terminal fragment of the FLAG-Dof protein. This confirms the yeast two hybrid data and indicates that FLAG-Dof and HA-i25 were present in a complex in S2 cells.

i188

A single in-frame clone of the Ade2 negative interactor group was found in the screen that encoded the predicted gene CG11275. The gene is primarily expressed in midembryonic stages in the head region and in the hindgut during *Drosophila* embryogenesis (Battersby et al., 2003). Sequence based structural analysis predicts an N-terminal BTB/POZ (Bric à brac-Tramtrack-Broad/Pox virus-Zinc finger) protein-protein interaction domain in the molecule. BTB domain containing proteins can be subgrouped according other domains present in the molecule. Nuclear BTB proteins contain in addition zinc finger or Pipsqueak type DNA binding motifs and are transcription factors. An actin binding kelch domain is present in actin-associated BTB proteins of the *Drosophila* Kelch family (Stogios and Prive, 2004). Many BTB proteins, like CG11275 do not have other recognisable conserved domains. It has been shown that BTB proteins are substrate-specific adaptors in an SCF-like (Skp1-Culin1-F-box protein complex) modular ubiquitin ligase (Furukawa et al., 2003; Xu et al., 2003). They bind directly Cullin3, but not other cullins via the BTB domain, whereas their C-terminal part serves for substrate specificity of the ubiquitin ligase complex. Thus, BTB proteins merge the functional properties of Skp1 and F-box proteins into a single polypeptide.

The N-terminally HA epitope tagged version of i188, which lacked most part of the BTB domain of CG11275 was expressed together with FLAG-Dof in S2 cells. To study the localisation and specific expression of the tagged proteins, immunocytochemistry was performed on these cells (Figure 11K-N). Both HA-i188 and FLAG-Dof showed diffuse cytosolic distribution and furthermore complete co-localisation. In immunoprecipitation experiments using lysates from these cells both proteins co-precipitated each other (Figure 13B). These experiments were repeated with full length CG11275 and showed, that the BTB domain does not alter the localisation of the protein; CG11275 co-localised with FLAG-Dof in the cytosol (data not shown). It also showed interaction with FLAG-Dof in the immunoprecipitation assay of S2 cell lysates (data not shown). These results are consistent with the yeast two hybrid data and indicate that FLAG-Dof and HA-CG11275 formed a complex in S2 cells. In addition, we showed that CG11275 is present in the cytosol of S2 cells.

i14

10 clones were found that contained cDNAs of the gene RpS10b. None of the clones were in-frame fusion of the Gal4 activation domain, and all contained 5'UTR region of the gene. RpS10b encodes for the fly ribosomal subunit S10. The *Drosophila* genome contains another homologue for RpS10, RpS10a. Both are predicted genes without functional studies (<http://www.flybase.org>).

The N-terminally HA epitope tagged, in-frame version of i14 was expressed together with FLAG-Dof in S2 cells. Western blots of immunoprecipitation experiments are shown in Figure 13C. The FLAG and Dof antibodies precipitated Dof, and the proteins precipitated by the HA antibody contained a band of smaller than 20 kDa recognised by the HA antibody, corresponding to the size of HA-i14. In addition, the Dof antiserum co-precipitated HA-i14. In case of the precipitate with the anti-FLAG antibody the HA-i14 specific band was masked by the light chain of the antibody. The HA antibody pulled down bands, which were recognised by the FLAG antibody. This indicates that FLAG-Dof was present in a complex with HA-tagged i14 in S2 cells. A detailed analysis of Dof - RpS10 interaction is described in Chapter 2.5, where potential translational regulation of Dof is discussed.

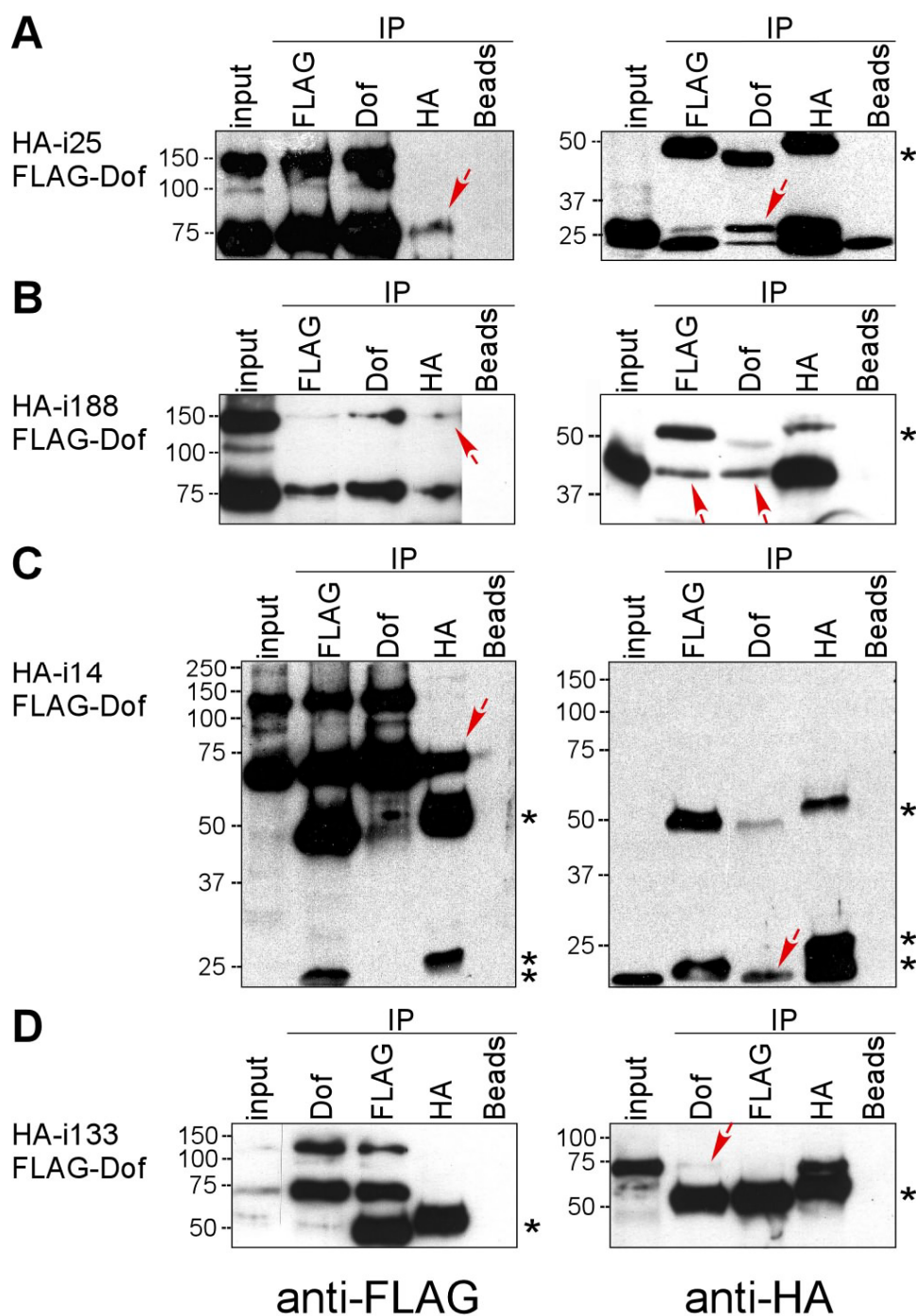


Figure 13. Immunoprecipitation assay of Dof with i-clones encoding for diverse proteins. FLAG epitope tagged Dof was co-expressed with HA epitope tagged (A) i-25, (B) i-188, (C) i-14 or (D) i-133 in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG, anti-Dof or anti-HA antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (left) or by anti-HA (right) antibodies. The “input” lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each “IP” lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy and light chains of the antibodies used for immunoprecipitation. Arrows mark co-precipitating FLAG-Dof and HA-i-clones respectively.

2.1.6 Nuclear proteins

Next we validated Dof-interaction with nuclear proteins by choosing two Ade2 negative interactors representing components of two basic chromatin binding complexes.

i133

Five independent clones were isolated in the screen that coded for the gene *bap60*. It is an essential component of the Brahma complex, the *Drosophila* homolog of the yeast SWI/SNF nucleosome remodelling complex (Moller, 2003).

The N-terminally HA epitope tagged version of i133, a full length cDNA clone of *bap60* was expressed together with FLAG-Dof in S2 cells. In immunostained cells HA-i133 co-localised with nuclear DNA and FLAG-Dof showed diffuse distribution (Figure 12O-R). We performed immunoprecipitations of lysates with antibodies against Dof or the epitope tags and analysed them on Western blots (Figure 13D). The FLAG and Dof antibodies precipitated Dof, and the proteins precipitated by the HA antibody contained a band of 70 kDa recognised by the HA antibody, corresponding to the size of HA-i133. In addition, the Dof antibody, but not the FLAG antibody pulled down a weak protein band, which was recognised by the HA antibody. The HA antibody immunoprecipitate did not contained bands detectable by FLAG antibody. Considering that FLAG-Dof is processed in S2 cells C-terminal fragments of Dof might exist which can be precipitated only with the Dof antiserum. This suggests that HA-Bap60 might bind to C-terminal fragments of FLAG-Dof, which could not be precipitated and detected via the N-terminal FLAG epitope tag.

i163

Two independent interactor clones encoded the gene *bip2/dTAF_{II}155*. Based on sequence homology, conserved domain structure (N-terminal Histon fold and C-terminal PHD finger) and biochemical interaction assays *bip2* belongs to the family of TATA-box binding protein (TBP) associated factors (Taf_{II}) that together with TBP form TFIID, a general factor required for accurate and regulated transcription initiation by RNA polymerase II (Gangloff et al., 2001). Bip2 was found to interact with the BTB/POZ domain of the transcription factor Bric à brac, highlighting a direct link between transcriptional regulators and the basic transcription machinery (Pointud et al., 2001). The N-terminally HA epitope tagged version of i163, which lacked the N-terminal third of Bip2 was expressed together with FLAG-Dof in S2 cells. Nuclear accumulation of HA-i163 was confirmed by immunocytochemistry (Figure 11S-V). Although it was expressed in cells, we failed to detect any bands on Western blots of lysates with HA antibody (data not shown). It indicates that the protein was not soluble under the lysis conditions used to prepare the sample for immunoprecipitation. We did not follow up the later possibility.

2.2 Interaction study of Dof with the FGF receptor Heartless

It has been shown that Dof interacts with both *Drosophila* FGF receptors in yeast (Battersby et al., 2003). Heartless as well as Breathless constructs lacking the N-terminal smaller lobe of the kinase domain were found as minimal region for Dof binding (Battersby et al., 2003). This region of the molecules on its own has no known catalytic activity and is unlikely to be tyrosine-phosphorylated in yeast indicating that Dof interacted with an unphosphorylated fragment of the two FGF-receptors. Cell culture interaction assays indicated that Dof forms a complex with the constitutively active form of Heartless (λ Htl) (see Chapter 2.1). These suggest that FGF receptors might bind Dof independently from their activated state in a constitutive manner. Nevertheless the two data sets derived from different experimental approaches, which might not be directly comparable. To bypass this problem cell culture interaction studies were extended with different Heartless constructs.

2.2.1 Interaction of different forms of Heartless with Dof

Full length wild type Heartless in C-terminal fusion with a double HA epitope tag was expressed with FLAG-Dof in *Drosophila* S2 cells. Immunoprecipitation experiments showed that wild type

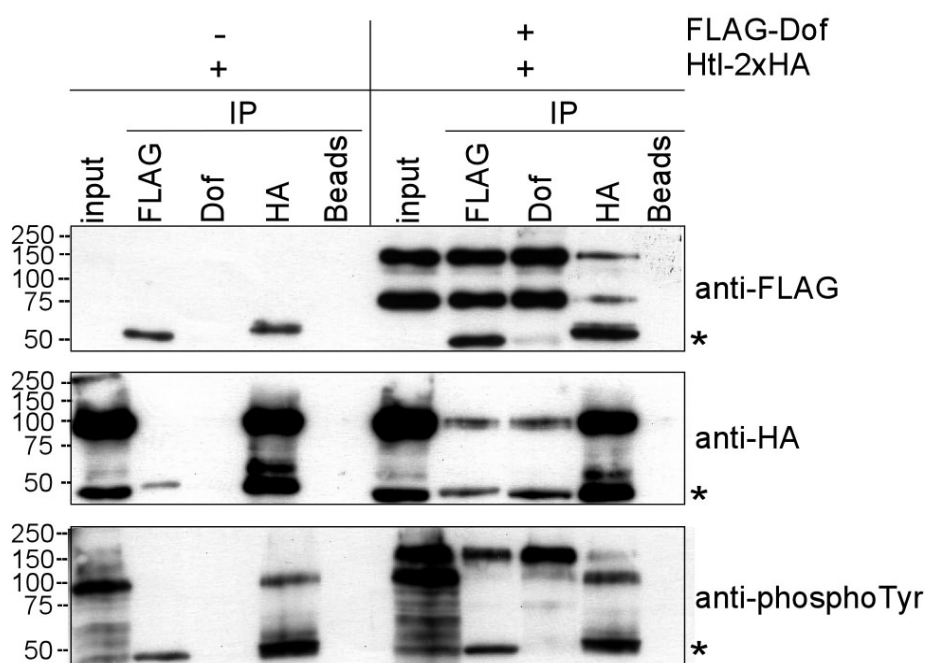


Figure 14. Interaction of Dof with wild type Heartless in S2 cells. Wild type Heartless bearing a double HA epitope tag on its C-terminus (Htl-2xHA) was expressed alone (left panel) or together with FLAG epitope tagged Dof (right panel) in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG, anti-Dof or anti-HA antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (top) or by anti-HA (middle) antibodies and phosphorylation state of the precipitated proteins was checked by an antibody directed against phospho-tyrosine residues (bottom). Note the intense tyrosine phosphorylated protein band at approximately the size of wild type Htl. Note that in addition to the predicted size of full length Dof FLAG antibody detects a smaller fragment of similar intensity representing a breakdown product. Note in contrast, that the intensity of the two tyrosine phosphorylated protein bands with corresponding size to the two bands recognised by the FLAG antibody is different. The "input" lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each "IP" lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy chains of the antibodies used for immunoprecipitation.

Heartless interacted with FLAG-Dof (Figure 14), however, expression of this receptor construct led to ligand-independent kinase activation, possibly due to dimer formation caused by overexpression (Figure 14, bottom panel). The proteins precipitated by HA antibody contained a band recognised by phospho-tyrosine antibody with a size corresponding to full length Heartless (~100kDa). In addition, FLAG- and Dof-IPs contained a strong and HA-IP a weak phospho-tyrosine specific band representing phosphorylated forms of FLAG-Dof. It was consistent with the finding, that Dof is phosphorylated in the presence of an activated FGF receptor (Petit et al., 2004; Wilson et al., 2004).

To find an alternative way to assay the interaction of Dof with a nonactivated form of FGF receptor a kinase-dead version of the constitutively active Heartless construct was expressed with FLAG-Dof in *Drosophila* S2 cells. The specificity of this construct was that it appeared as dimer due to the dimerisation domain of the lambda phage C1 protein fused to the transmembrane and intercellular part of Heartless but due to a pointmutation in the ATP binding pocket kinase activity was blocked. Immunoprecipitation of cell lysates were performed (Figure 15). The kinase-dead version of Heartless still interacted with Dof, despite its inability to phosphorylate itself or the associated Dof protein. Thus, activation of Heartless by phosphorylation is not required for interaction with Dof, but the involvement of dimerisation still cannot be excluded.

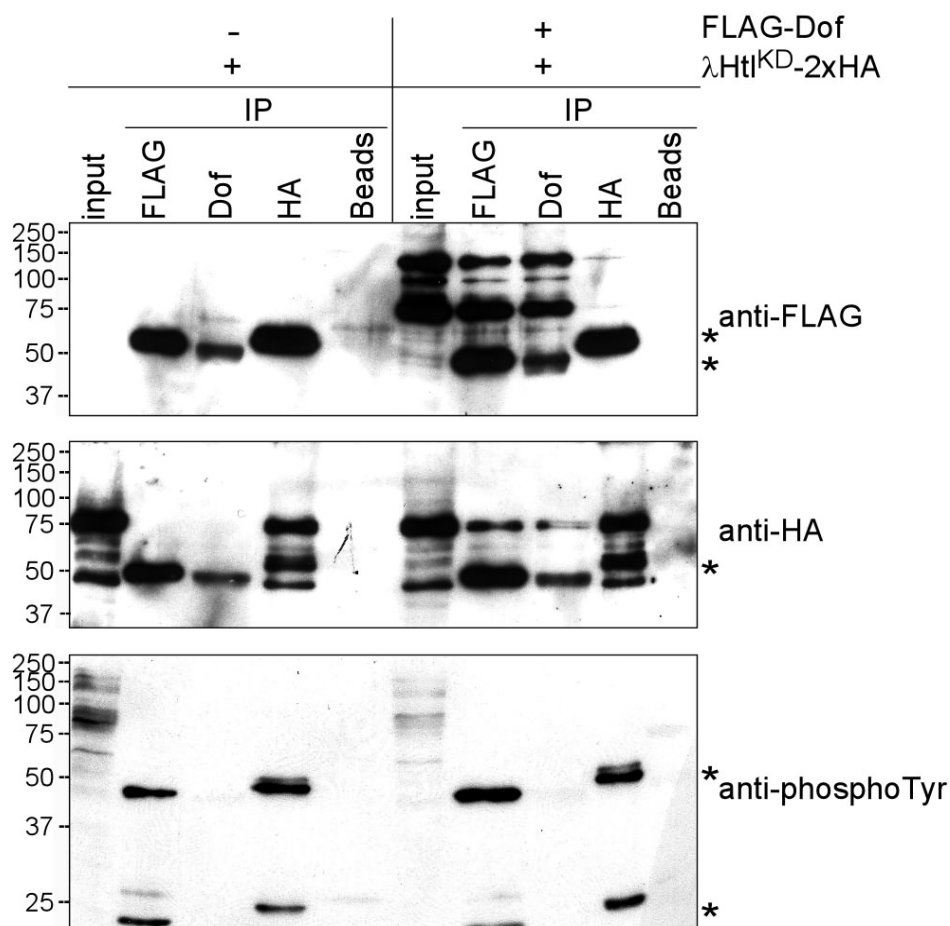


Figure 15. Interaction of Dof with kinase dead Heartless in S2 cells. A kinase dead form of constitutively dimerising Heartless bearing a double HA epitope tag on its C-terminus (λ Htt^{KD}-2xHA) was expressed alone (left panel) or together with FLAG epitope tagged Dof (right panel) in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG, anti-Dof or anti-HA antibodies. IPs were analysed by SDS-PAGE together with cell lysate (input). Western blots were probed for the tagged proteins by anti-FLAG (top) or by anti-HA (middle) antibodies and phosphorylation state of the precipitated proteins was checked by an antibody directed against phospho-tyrosine residues (bottom). Note the lack of tyrosine phosphorylated protein bands in the IPs. The “input” lanes contain 15 μ l of lysate (one-sixth of the amount used for each immunoprecipitation); in each “IP” lane one third of an immunoprecipitation is loaded. The asterisks mark the heavy and light chains of the antibodies used for immunoprecipitation.

2.2.2 Domain mapping in Dof for receptor interaction

In order to map the FGF receptor interaction domain in Dof a series of FLAG-Dof deletion constructs were co-expressed with constitutively active Heartless in *Drosophila* S2 cells and tested for their ability to co-immunoprecipitate with the FGF receptor (Figure 16). Deletions that did not affect the DBB region had minimal effects on the interaction. The first 446 amino acids were

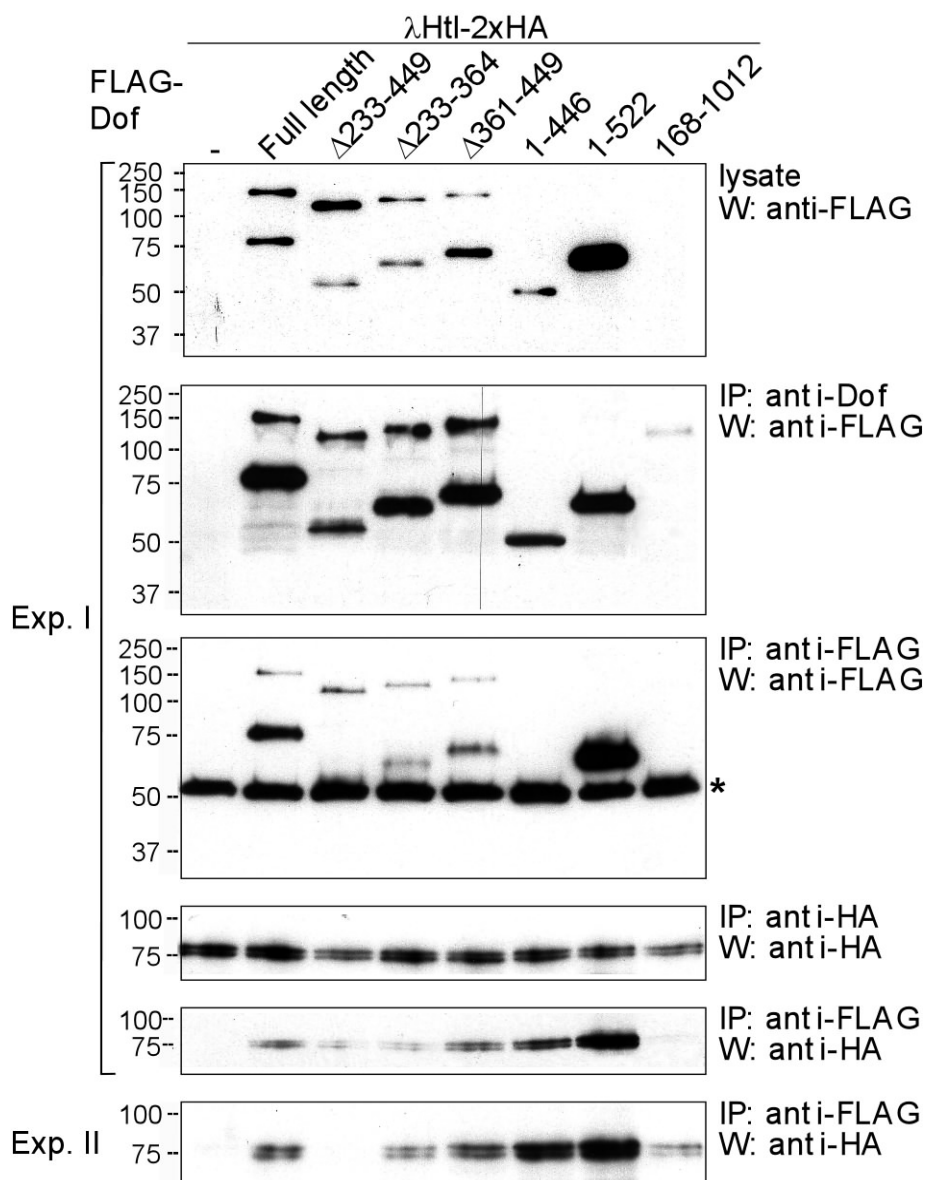


Figure 16. Interaction of activated Heartless with mutant forms of Dof in S2 cells. Western blots of whole cell lysates and immunoprecipitations (IP) from S2 cells expressing a constitutively active form of HA epitope-tagged Heartless (λ Htl-2xHA) and mutant versions of Dof bearing N-terminal FLAG epitope tags. Protein complexes were immunoprecipitated from the lysates using antibody against Dof, FLAG or HA, and the precipitated proteins were detected on the Western blots using anti-FLAG or anti-HA antibodies. The asterisk marks the heavy chain of the FLAG antibody used for IP. In addition to a protein of approximately the size predicted for each of the constructs, in each lane there is also a smaller fragment representing a breakdown product. The sizes of these smaller fragments are in all cases consistent with a cleavage of Dof at the same position between ankyrin repeat and the coiled-coil region (see also Chapter 2.5). The five top panels are from the same experiment. The bottom panel is from a separate experiment to show the variation in the amount of Dof $\Delta 233-449$ associated with the receptor. The expression levels in this experiment were the same as in Experiment (Exp.) I. Note that although Dof[168-1012] is not detectable in the lysate at this exposure, a certain amount is precipitable and (in experiment II) is able to co-precipitate the receptor.

sufficient for binding (FLAG-Dof¹⁻⁴⁴⁶) and even FLAG-Dof¹⁶⁸⁻¹⁰¹², although expressed at very low levels, co-precipitated the receptor, albeit at similarly low levels. By contrast, constructs lacking the DBB region alone or in combination with the ankyrin repeats showed strongly reduced binding with certain experimental variations. It indicates that the region between amino acids 233 and 364 is required for efficient interaction.

The DBB domain contains a consensus SUMOylation site at amino acid positions 296-299. The

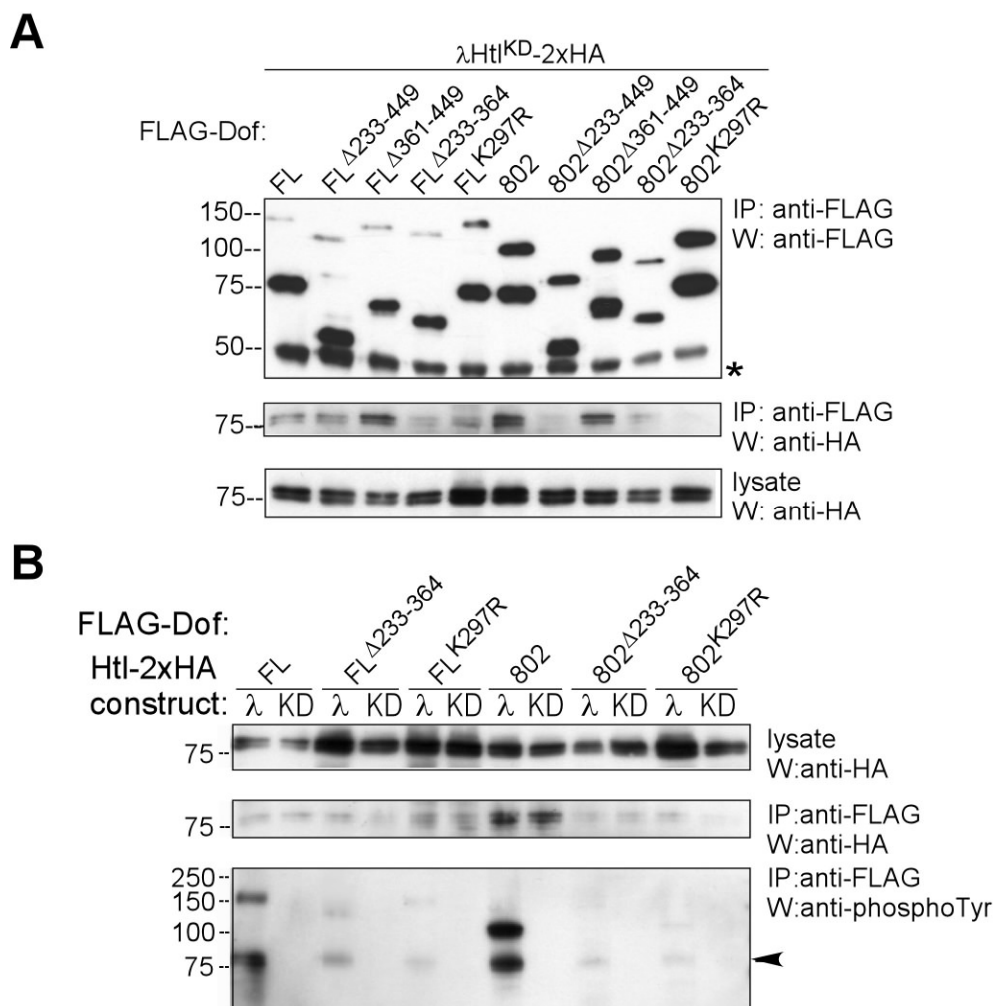


Figure 17. Lysine at position 297 in Dof is involved in efficient interaction of Dof with the FGF receptor Heartless. (A) Western blots of whole cell lysates and immunoprecipitations (IP) from S2 cells expressing a kinase dead version of the constitutively active HA epitope-tagged Heartless (λ Httl^{KD}-2xHA) and mutant versions of Dof bearing N-terminal FLAG epitope tags. Protein complexes were immunoprecipitated from the lysates using antibody against FLAG, and the precipitated proteins were detected on Western blots using anti-FLAG or anti-HA antibodies. The asterisk marks the heavy chain of the FLAG antibody used for IP. In addition to a protein of approximately the size predicted for each of the constructs, in each lane there is also a smaller fragment representing a breakdown product. Note that full length Dof had a weaker interaction with the mutant Heartless protein than the Dof construct lacking the last 200 amino acids (FLAG-Dof802).

(B) Mutant Dof constructs lacking the DBB domain or bearing a point mutation in the DBB domain were co-expressed with constitutively active (λ) or with kinase dead (KD) HA-tagged Heartless in S2 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody. IPs were analysed by SDS-PAGE together with whole cell lysate. Western blots were probed for the tagged Heartless proteins by anti-HA antibody and the presence of tyrosine phosphorylated proteins in the IPs was detected by an antibody directed against phospho-tyrosine residues. The amount of precipitated mutant FLAG-Dof proteins on the Western blot is not shown here but was similar as in the experiment shown in panel A. Arrowhead marks phospho-protein bands with corresponding size of constitutively active Httl. Note that full length Dof interacted weaker with both forms of Heartless than the Dof construct lacking the last 200 amino acids (FLAG-Dof802).

mutation of this site by exchanging lysine 297 to an arginine in the context of Dof¹⁻⁸⁰² resulted in the complete elimination of interaction with the receptor in yeast (Battersby, 2001). This indicates that Lys 297 is critical in receptor binding. We tested the effect of this mutation in immunoprecipitation assay in the context of full length Dof as well as of Dof¹⁻⁸⁰² (Figure 17). Kinase dead version of the constitutively active Heartless construct was expressed with these mutant versions of FLAG-Dof in S2 cells. Both, full length and C-terminally truncated Dof constructs with the point mutation lysine to arginine at position 297 showed strongly reduced binding to the kinase dead receptor (Figure 17A). Their interaction affinity to Heartless was as weak as that of the Dof constructs lacking the DBB domain (Dof Δ 233-364). In addition, wild type Dof interacted much weaker with Heartless than the construct lacking the last 200 amino acids of Dof suggesting that this region might have an inhibitory effect on the binding of Dof to the FGF receptor.

To reveal if lysine 297 plays also an essential role in the interaction with the activated receptor immunoprecipitation assay was extended to the constitutively active form of Heartless (Figure 17B). K297R mutant Dof constructs showed as weak interaction with both receptor forms as the constructs lacking the DBB domain. Wild type Dof showed weak binding compared to the C-terminally truncated Dof construct. Therefore the weak interaction of mutant Dof constructs containing the last 200 amino acids can not be interpreted as an effect of the mutations in the DBB region. However, the K297R point mutation caused significant reduction in receptor binding in the context of Dof[1-802]. Reduced receptor binding correlated with reduced tyrosine phosphorylation of the mutant Dof constructs (Figure 17B, bottom panel). Thus, these results indicate that Lys 297 in Dof is required for efficient interaction with the FGF receptor Heartless in S2 cells. These results are consistent with earlier yeast data, including the fact that the presence of the C-terminal 200 amino acids in Dof weakens interaction with the FGF receptors (Battersby, 2001).

The fine-mapping of the interaction domain in Dof for receptor binding is summarised in Table 2. In cell culture assays there was no difference in the behaviour of the constitutively active and the kinase-dead versions of Heartless indicating that ATP binding and phosphorylation of the receptor does not influence Dof interaction. No Dof deletion constructs were found that completely failed to interact with Heartless in S2 cells. Wild type Dof showed weaker interaction with Heartless than the truncated Dof form lacking the last 200 amino acids. Strongly reduced binding was observed in Dof constructs lacking regions including amino acids 233-364 as well as in Dof constructs bearing a point mutation at position 297. In yeast two hybrid experiments two FGF receptor fragments with different length were tested for interaction. Whereas the Dof construct with a 167 amino-acid N-terminal truncation efficiently interacted with both receptor fragments, Dof ^{Δ 233-364} as well as constructs bearing the point mutation K297R or lacking the first 226 or 276 amino acids showed no or only weak interaction with the two receptor fragments, although a slight difference was observed in case of the two receptor fragments. These suggest that lysine 297 is an important residue in the interaction of Dof with Heartless but more N-terminal regions appeared also essential for receptor binding. Thus, the integrity of the region between amino acids 168 and 364 which spans the DBB domain is required for efficient receptor interaction.

2.2.3 The effect of Dof binding on the activation level of Heartless in S2 cells

Interaction of Dof with the FGF receptor could have the function to facilitate receptor activation. Dof could trigger receptor activation *in vivo* via two mechanisms: either by propagating the FGF receptors to the cell surface or by stabilising conformational changes for autophosphorylation. Observations that subcellular localisation of Heartless is not influenced by Dof would support the second way of action (Wilson et al., 2004). Alternatively or in parallel Dof could be involved in the transmission of the signal from the receptor. The phosphorylation of Dof in the presence of an

Table 2. Interaction of the FGF receptor Heartless with mutant forms of Dof

Dof constructs	Yeast two hybrid assay		Immunoprecipitation	
	Htl 349-730	Htl 491-730	λ Htl-2xHA	λ Htl ^{kinase dead} -2xHA
Full length	+/-	+/-	+/(+)	+/(+)
1-802	+	+/-	+	+
1-522	n.d.	n.d.	+	+
1-446	+	+	+	+
168-1012	+	+	+	n.d.
227-1012	-	(+)/-	n.d.	n.d.
277-1012	-	+/-	n.d.	n.d.
FL^{Δ233-364}	-	(+)/-	(+)	(+)
1-802^{Δ233-364}	n.d.	n.d.	(+)	(+)
FL^{Δ233-449}	n.d.	n.d.	(+)	(+)
1-802^{Δ233-449}	n.d.	n.d.	(+)	(+)
FL^{Δ361-449}	n.d.	n.d.	+	+
1-802^{Δ361-449}	n.d.	n.d.	+	+
FL^{K297R}	n.d.	n.d.	(+)	(+)
1-802^{K297R}	-	-	(+)	(+)

Mutant Dof constructs studied in the interaction assays are listed on the left. FL marks full length Dof protein. Numbers in the name mark either the position of the first and last amino acids present in the construct or beyond a “ Δ ” sign the position of the first and last amino acids of an internal deletion. K297R labels a lysine to phenylalanine exchange at amino acid position 297 in the protein.

Yeast two hybrid interaction data are summarised in the next two panels. Two fragments of the FGF receptor Heartless were analysed. Htl 349-730 lacks only the N-terminal first 12 amino acids of the intracellular domain of the receptor, Htl 491-730 lacks the N-terminal lobe of the kinase domain. Interactions were tested under two selection conditions. If yeast showed different growth behaviour under the two conditions, both results are represented in the table: first that of the less stringent than that of the more stringent selection separated by a slash. Note, the smaller receptor fragment showed weak interaction to a subset of Dof deletion constructs which failed completely to bind to the larger Htl fragment.

Immunoprecipitation data are listed in the two right columns. Interactions were analysed with a constitutively active Heartless construct containing the entire intracellular part of the receptor (λ Htl-2xHA) and with a kinase dead version of the first construct (λ Htl^{kinase dead}-2xHA).

“+” marks strong, “(+)” weak and “-” no interaction. “n.d.” marks no data.

activated FGF receptor supports this model (Wilson et al., 2004).

To see if the physical interaction of Dof with the FGF receptors influences the activation level of the receptors, phosphorylation state of constitutively active Heartless was monitored in S2 cells in the presence of Dof constructs bearing different affinity for receptor interaction (Figure 18). Receptor phosphorylation was slightly enhanced in the presence of Dof constructs showing high binding affinity for Heartless, indicating that Dof interaction might trigger receptor activation. However, these preliminary data require further precise quantitative analysis. This experiment did not reflect *in vivo* situation since the receptor molecules were artificially forced to dimerise by a

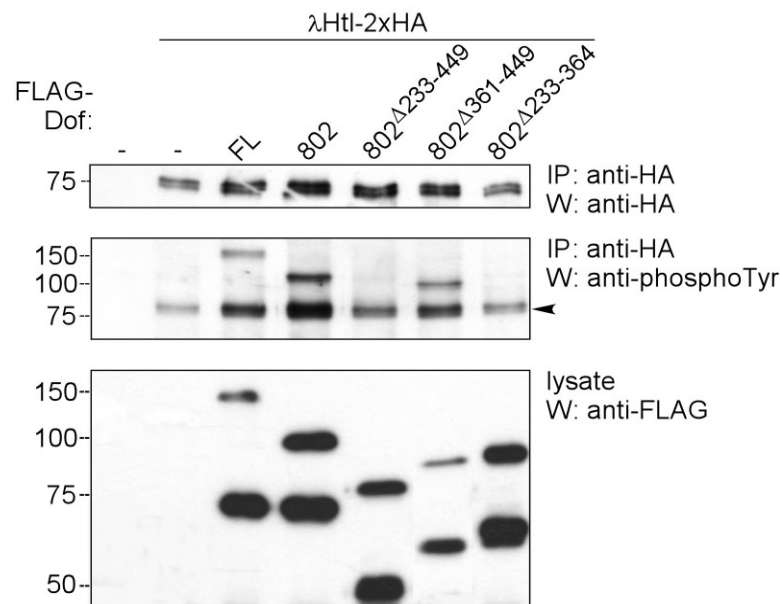


Figure 18. Effect of Dof on the phosphorylation/activation levels of the FGF receptor Heartless. Western blots of whole cell lysates and immunoprecipitations (IP) from S2 cells expressing constitutively active HA-tagged Heartless (λ Htl-2xHA) alone or together with mutant Dof constructs. Protein complexes were immunoprecipitated from the lysates using antibody against HA, and the precipitated proteins were detected on the Western blots using antibodies directed against the HA epitope or phospho-tyrosine residues. Arrowhead marks phospho-protein bands with corresponding size of constitutively active Htl.

heterologous domain, which might circumvent at least partially the need of other potential factors to facilitate activation and thus, might underscore the effect of such factors.

2.2.4 Large Dof-FGF receptor complexes are present in S2 cells

To determine the size of the protein complex Dof forms with an activated FGF receptor in S2 cells gel filtration experiments were performed. Whole cell lysates of stable transfectant *Drosophila* S2 cells overexpressing FLAG-Dof alone or together with a constitutively active FGF receptor were fractionated on a size exclusion chromatography column (Figure 19). Overexpressing FLAG-Dof alone the protein accumulated at a complex size of 600-750 kDa (fractions 13-14) and its continuous presence up to fraction 19 indicated that it was present in diverse smaller complexes (Figure 19B). Monomeric full length molecule would be expected in fractions 20-21 (calculated molecular weight of Dof is 114 kDa). This indicates that Dof molecules without protein-protein association were not present in the lysate. Dof was able to interact with itself in S2 cells (see Chapter 2.1). This might propose that the detected complexes are Dof oligomers with different numbers of Dof units (tetramers or pentamers as maximum). Alternatively or in addition Dof might form a complex with diverse cellular components. The wide spectra of complex sizes might be either a reflection of independent complexes with different protein composition or the consequence of overexpression situation and therefore components of the complex which are expressed at lower levels in the cells were titrated out sequentially.

Cell lines co-expressing FLAG-Dof and a constitutively active FGF receptor showed changes in the size of Dof complexes with slightly differences in the case of the two different *Drosophila* FGF receptors (Figure 19C, D). In the presence of activated Heartless Dof accumulated in fraction 16 corresponding to a complex size of about 500 kDa, whereas in the presence of activated Breathless it accumulated in a somewhat larger complex with a size of about 550 kDa (fraction 15) with a remarkably broader distribution on the column. Both FGF receptors were detected in complexes,

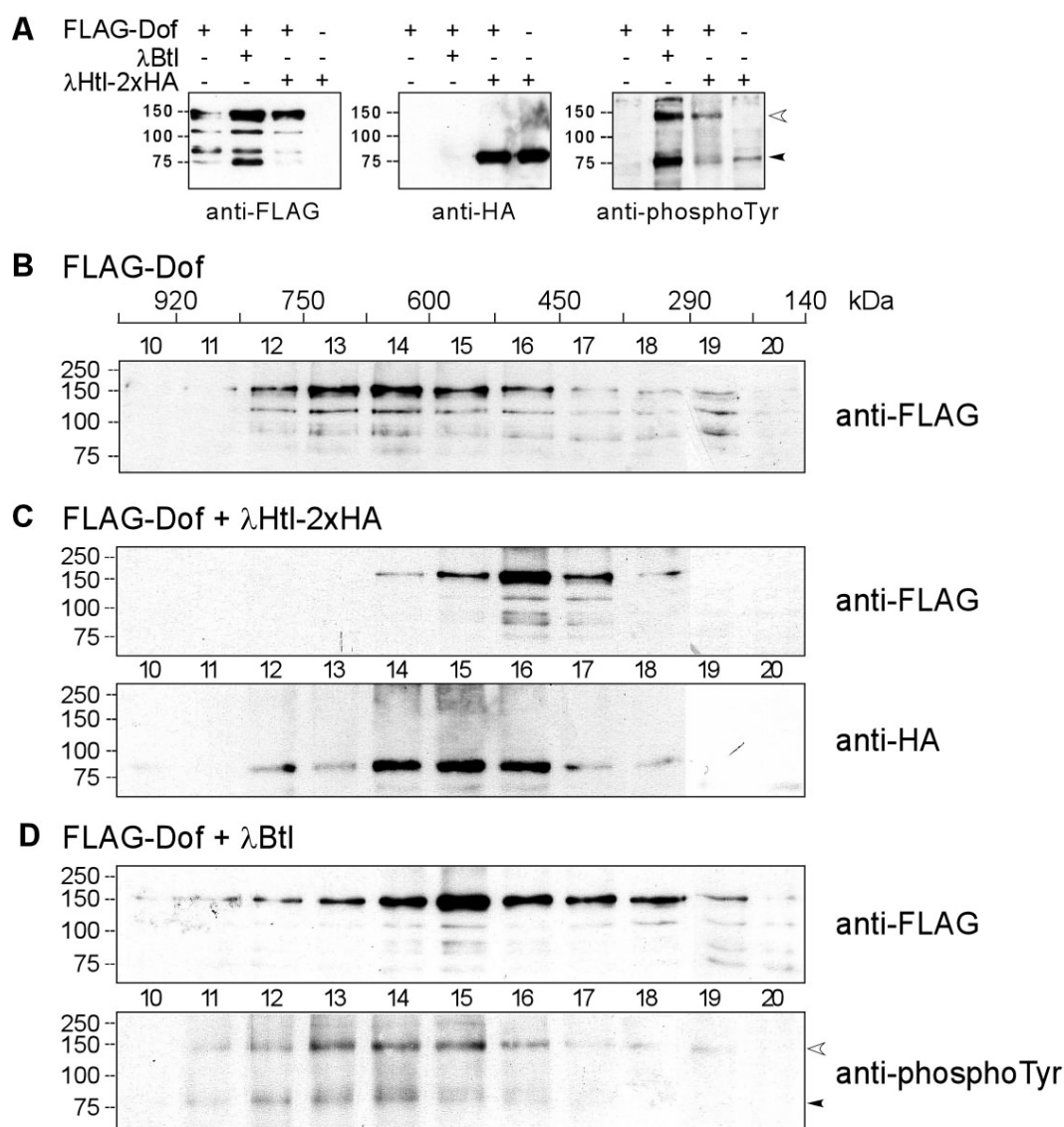


Figure 19. The size of Dof complexes formed in S2 cells. (A) Western blots of whole cell lysate of S2 cell lines stably transformed as indicated above each lane with different combinations of the activated form of the FGF receptor Breathless (λ -Btl), the constitutively active HA-tagged Heartless (λ -Htl-2xHA), both under the control of a heat shock promoter and Dof with an N-terminal FLAG epitope tag under the control of the actin 5C promoter (FLAG-Dof). The presence of the proteins following heat shock induction was monitored using antibodies directed against the FLAG and HA epitope tags and phospho-tyrosine residues. Black and white arrowheads mark phospho-tyrosine bands corresponding to the activated receptors and phosphorylated Dof respectively. (B-D) Size exclusion chromatography of whole cell lysates from stably transformed S2 cells expressing (B) FLAG-Dof, (C) FLAG-Dof and λ -Htl-2xHA or (D) FLAG-Dof and λ -Btl. Proteins from each fraction (fraction numbers are labelled above each lane) were collected and precipitated with trichloric acid, separated by SDS-PAGE and detected by Western blotting analysis using anti-FLAG antibody to detect Dof, anti-HA antibody for detecting λ -Htl and anti-phospho-tyrosine antibody to visualise λ -Btl as well as phosphorylated form of Dof, marked by black and white arrowheads on the blot respectively. Standard proteins were used to estimate the size of protein complexes collected in the fractions and calculated standard curve is given as a scale bar above the fraction numbers in B.

which were 50-100 kDa larger in size compared to the major peak of FLAG-Dof complexes of the same lysate and so co-fractionated only partially with FLAG-Dof. Interestingly, the profile of tyrosine phosphorylated Dof also did not completely overlap with the general FLAG-Dof protein profile (Figure 19D). Its accumulation peak on the column was at about 50-100 kDa higher molecular weights than that of “bulk” FLAG-Dof detected via the FLAG epitope tag and thereby exactly co-fractionated with tyrosine phosphorylated activated Breathless.

These data show that activated FGF receptors co-fractionate preferentially with tyrosine phosphorylated Dof indicating that Dof phosphorylation might require complex formation with activated FGF receptors and not only their presence in the cells. The fact that non-phosphorylated Dof protein accumulated in fractions where FGF receptors were underrepresented suggests that Dof was present in receptor independent complexes. The expression level of FLAG-Dof relative to that of the activated receptors is not known therefore the stoichiometry of the complexes is not understood. Nor is understood if the FGF receptor-independent fraction of Dof is only due to different abundancy of the two proteins in the cells or might have physiological relevance.

In summary, Dof is able to form high molecular weight complexes in resting cells. These complexes might be re-organised upon signalling, when Dof forms a complex with activated FGF receptors and becomes phosphorylated probably in this complex. This complex is large enough to contain two or more Dof molecules and probably other components in addition to the receptor dimer.

2.3 Tyrosine phosphorylation of Dof

FGF receptors do not have the capability to directly bind Grb2, like other receptor tyrosine kinases (RTK). In order to activate the MAPK cascade they utilise adaptor molecules to bridge this gap. The adaptor molecule Dof forms a complex with both *Drosophila* FGF receptors and is tyrosine phosphorylated in the presence of activated FGF receptors. To see if Dof phosphorylation could contribute in transmitting the signal from the FGF receptor towards downstream effector cascades, like the MAPK pathway our first aim was to map the tyrosine phosphorylation sites in the molecule.

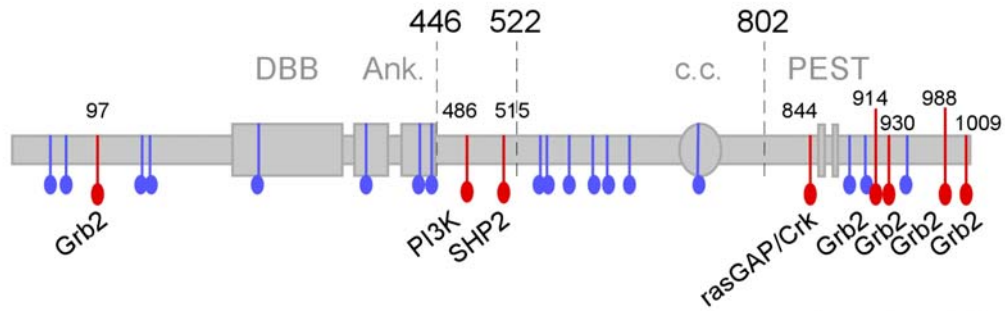
This might then help to identify phosphorylation-dependent binding partners of Dof.

2.3.1 Identification of tyrosine residues phosphorylated in Dof

There are 19 tyrosines in the molecule (Figure 20A) and eight of them are located in conserved sequence motifs, which could serve as docking sites for the *Drosophila* homologues of signal transducer molecules Grb2, PI3K, SHP2, RasGAP or Crk in their tyrosine phosphorylated state (Vincent et al., 1998). We focused our attention on tyrosines of these consensus binding sites. We applied an indirect way to map phosphorylated tyrosines. Dof constructs bearing point mutations at positions of tyrosine residues were co-expressed with the constitutively active FGF receptor Heartless in S2 cells. The phosphorylation state of the mutant Dof proteins was monitored in cell lysates by an antibody specifically recognising phosphorylated tyrosine residues. Mutations that resulted the production of proteins containing phenylalanine residues in place of particular tyrosines or deleted parts of the molecule were analysed in different combinations. *In vivo* analysis of truncated forms of Dof indicated that the last 210 amino acids containing one potential RasGAP or Crk and four potential Grb2 docking sites (see breakpoint 802 in Figure 20) are not essential for the function of the molecule (Wilson et al., 2004). However, additional mutation of the three remaining consensus tyrosine residues at positions 97, 486 and 515 to phenylalanine resulted in a dramatic reduction in Dof activity *in vivo* suggesting that at least one of the three tyrosines is important for Dof function (Wilson et al., 2004). We found that the phosphorylation of this protein was not completely abolished in S2 cells (Figure 21B). This pointed out that tyrosines other than those lying within recognisable consensus binding sites for known adaptor molecules can also be phosphorylated.

Figure 20. The distribution of tyrosine residues in Dof and a summary of tyrosine phosphorylation of mutant Dof constructs used in the phosphorylation assays. (A) Schematic representation of the Dof protein. Grey boxes and an oval label protein domains, the name of each domain is marked above the boxes/oval. DBB, Dof-BCAP-BANK domain; Ank., ankyrin repeats; c.c., coiled coil region; PEST, protein degradation motif. Each tyrosine in the protein is labelled with a coloured line+oval on the scheme; blue lines mark tyrosines not located in known consensus tyrosine motifs, red lines highlight tyrosine residues located in consensus tyrosine motifs. Signalling molecules for which these consensus tyrosine motifs could serve as binding sites if the tyrosine is phosphorylated are listed below each of the tyrosines. Amino acid position of these tyrosines is marked above each red line. Amino acid positions 446, 522 and 802 – breakpoints of Dof deletion constructs – are marked with grey dashed lines on the scheme. (B) Schematic representation of mutant Dof constructs used in the phosphorylation assays. Tyrosine residues of consensus motifs and non-consensus tyrosines which were mutated in some of the constructs are marked as red and blue lines respectively and their amino acid position is given above each line. The letters F stay for tyrosine to phenylalanine exchanges in the marked positions. The name of the constructs is listed on the left. Each name marks the position of the first and last amino acids present in the construct and the positions of the point mutations. FL labels full length Dof protein. Each construct was fused to the FLAG epitope tag on its N-terminus (green oval). Right column summarises the results of the phosphorylation assay of the mutant constructs. “+”, the construct was tyrosine phosphorylated, “(+)”, weak tyrosine phosphorylation, “-”, no tyrosine phosphorylation.

A



B

Construct	Phosphorylation State
1-446	-?
1-522	+
1-522(F_)	+
1-522(_F_)	+
1-522(_F)	+
1-522(FF_)	+
1-522(F_F)	+
1-522(_FF)	-
1-522(FFF)	-
1-802	+
1-802(F_)	+
1-802(_FF)	+
1-802(FFF)	+
1-802 ^{592F}	+
1-802 ^{613F}	+
1-802 ^{629F}	+
1-802 ^{592F,613F,629F}	(+)
1-802 ^{654F,726F}	+
1-802 ^{486F,515F,592F}	+
1-802 ^{486F,515F,613F}	+
1-802 ^{486F,515F,629F}	+
1-802 ^{486F,515F,592F,613F}	(+)
1-802 ^{486F,515F,592F,629F}	(+)
1-802 ^{486F,515F,613F,629F}	(+)
1-802 ^{Y5F}	-
FL(_FF)	+
FL ^{Y5F}	+

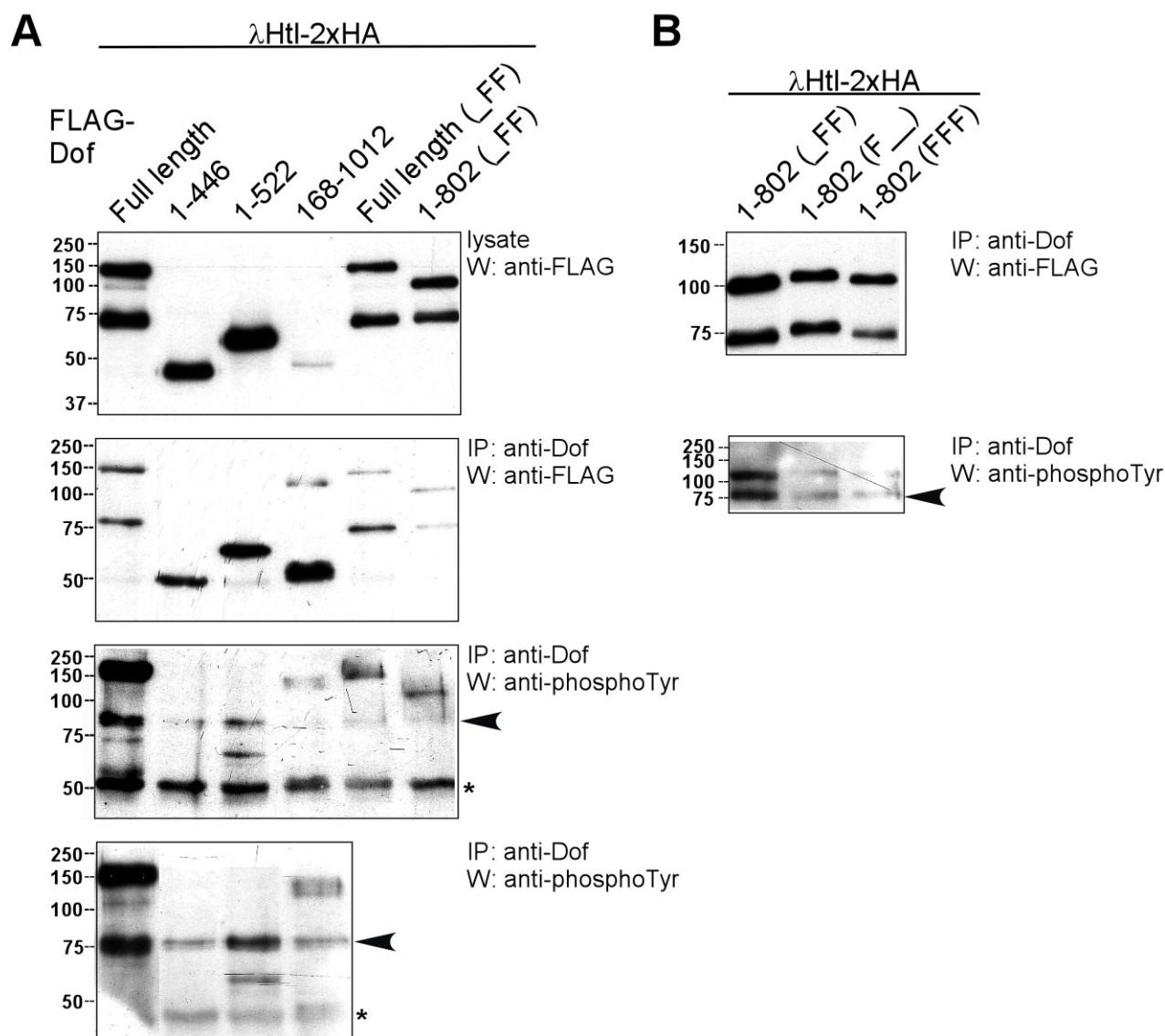


Figure 21. Comparison of the phosphorylation states of mutant Dof proteins in the presence of an activated FGF receptor. FLAG epitope-tagged mutant Dof constructs were expressed together with an activated form of HA-tagged Heartless (λ Htl-2xHA) in S2 cells. Whole cell lysates were separated by SDS-PAGE, or used for immunoprecipitation with anti-Dof antibody. Western blots were probed using antibodies directed against FLAG or phospho-tyrosine residues. Black arrowheads mark the tyrosine phosphorylated protein bands corresponding to the activated Heartless construct. The asterisks mark the heavy chain of the Dof antibody used for IP. (A) With the exception of Dof[168-1012] all constructs are expressed at high levels and are precipitable with antibody against Dof. Note, the full length Dof protein is strongly phosphorylated, whereas the phospho-tyrosine signal is reduced in all of the mutants. The band for Dof[1-446] cannot be judged in this panel, as it might be obscured by the heavy chain of the Dof antibody. However, in the bottom panel, which is from a separate experiment, the signal of the heavy chain is very weak, but yet no signal for Dof[1-446] is visible. (B) Note, the C-terminally truncated version of Dof in which the tyrosines in the potential Grb2, PI3K and SHP2 consensus sites are mutated can still be phosphorylated.

Analysis of further Dof deletion constructs indicated that tyrosine residues between amino acids 446 and 802 are substrates for kinase activity (Figure 21A). Dof¹⁻⁵²² was the smallest N-terminal fragment that was efficiently phosphorylated whereas deletion of a further 76 amino acids (construct Dof¹⁻⁴⁴⁶) abolished detectable phosphorylation. Since other protein bands were detected at the same molecular weight position on the Western blot as construct Dof¹⁻⁴⁴⁶ we could not exclude its phosphorylation completely. Therefore we tested Dof¹⁻⁵²², which still contained the three consensus tyrosines for potential interaction with Grb2, PI3K and SHP2 to what extent the mutation

of these residues influences phosphorylation state of the molecule in S2 cells (Figure 22). All constructs containing different combinations of point mutations at the position of the three consensus tyrosines showed comparable expression levels. The introduced mutations did not influence the ability of the Dof constructs to interact with the constitutively active FGF receptor Heartless. However, mutation of two tyrosines at the positions of the potential PI3K and SHP2 binding sites was sufficient to abolish phosphorylation completely. Thus, this indicates that tyrosine residues at position 486 and 515 are substrates for phosphorylation in Dof.

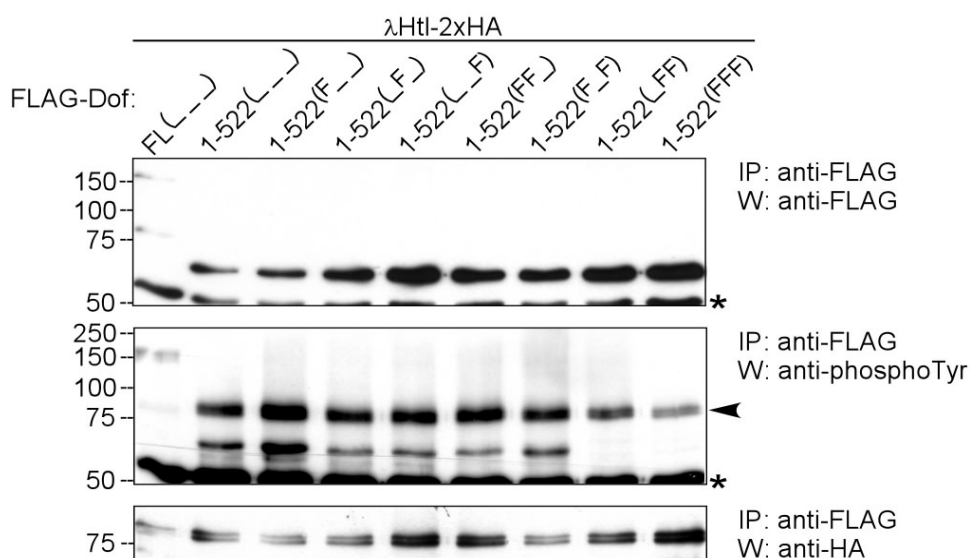


Figure 22. Mapping of phosphorylated tyrosine residues in the first 522 amino acids of the Dof protein. FLAG epitope-tagged Dof constructs containing the first 522 amino acids of Dof and different combinations of the tyrosine to phenylalanine point mutations at amino acid positions 97, 486 and 515, marked as (F___), (___F) and (___F) respectively were expressed together with an activated form of HA-tagged Heartless (λ Htl-2xHA) in S2 cells. Protein complexes were immunoprecipitated from whole cell lysates using antibody against the FLAG epitope, and the precipitated proteins were detected on the Western blots using antibodies directed against FLAG, HA or phospho-tyrosine residues. Black arrowhead marks the tyrosine phosphorylated protein bands corresponding to the activated Heartless construct. The asterisk marks the heavy chain of the FLAG antibody used for IP.

Since residues 486 and 515 were the only two tyrosines phosphorylated in the first 522 amino acids of Dof, the phosphorylated non-consensus tyrosine residue(s) observed in the Dof 1-802(FFF) construct might lie in the region between amino acids 522 and 802, which contains seven tyrosines. From earlier experiments we learned that the N-terminal 75 kDa Dof fragment was less strongly phosphorylated than the full length form (see Figure 14 in Chapter 2.2). Therefore we focused on five tyrosine residues at positions 592, 613, 629, 654 and 726, which are not present in the N-terminal cleavage product of Dof (for details on Dof cleavage site see Chapter 2.5). Mutation of these five residues to phenylalanine in different combinations in a Dof¹⁻⁸⁰² context drew attention to three tyrosines at positions 592, 613 and 629, the simultaneous mutation of which caused dramatic reduction on the phosphorylation level of the protein (Figure 23A), indicating that they might be the major phosphorylation targets in the region between amino acids 522 and 802 in Dof. We aimed to see if all three of these tyrosines were involved in phosphorylation and if the residual phosphorylation observed in Dof [1-802]^{592F,613F,629F} was due to the phosphorylation of the tyrosines at positions 486 and 515. Therefore, different combinations of additional point mutations were introduced at positions 592, 613 and 629 into the construct Dof [1-802]^{486F,515F} and the phosphorylation state of the derived Dof proteins was monitored (Figure 23B). All constructs appeared stable in cells, albeit with slightly different expression levels, and retained their capacity

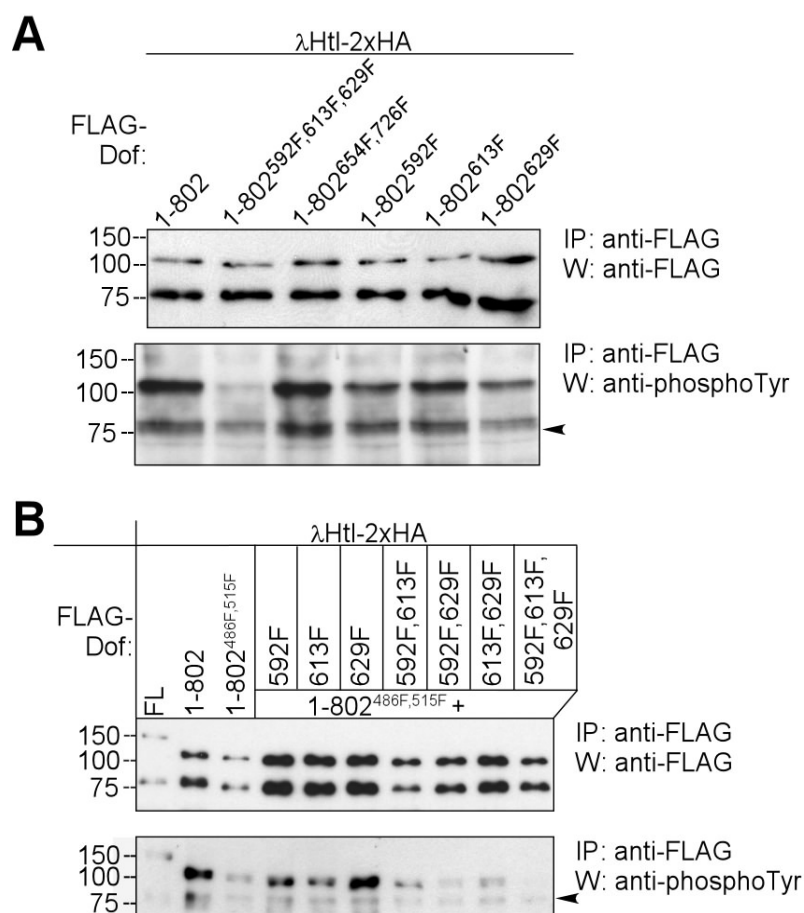


Figure 23. Mapping of phosphorylated tyrosine residues between amino acids 522 and 802 in the Dof protein. (A) FLAG epitope-tagged Dof constructs containing the first 802 amino acids of Dof and different combinations of the tyrosine to phenylalanine point mutations at amino acid positions 592, 613, 629, 654 and 726 or (B) FLAG epitope tagged mutant Dof constructs containing the first 802 amino acids with tyrosine to phenylalanine point mutations at amino acid positions 486 and 515 and in addition different combinations of the tyrosine to phenylalanine point mutations at amino acid positions 592, 613 and 629 were expressed together with an activated form of HA-tagged Heartless (λ Htl-2xHA) in S2 cells. Protein complexes were immunoprecipitated from whole cell lysates using antibody against the FLAG epitope, and the precipitated proteins were detected on Western blots using antibodies directed against the FLAG epitope or phosphotyrosine residues. As control for phosphorylation levels construct containing the first 802 amino acids of Dof without further mutations was used. Black arrowheads mark the tyrosine phosphorylated protein bands corresponding to the activated Heartless construct.

to interact efficiently with Heartless. Increasing the number of mutated tyrosines resulted in stepwise reduction in the phosphorylation state of Dof leading to a complete abolishment of phosphorylation if all three tyrosines were exchanged. This indicates that the tyrosine residues at positions 592, 613 and 629 are substrates for phosphorylation.

To test whether tyrosine residues of the last 210 amino acids of Dof contribute to the phosphorylation state of the protein, the five tyrosines identified as kinase substrates were exchanged to phenylalanine in full length Dof and the mutant construct was tested for the presence of phosphorylated tyrosines in the same assay as above (Figure 24). Despite the removal of the five potential phosphorylation sites the protein was phosphorylated at comparable levels with wild type Dof, suggesting that the C-terminal tail might contain multiple phosphorylation sites. The abundance of tyrosines within consensus binding sites for Grb2 (4 out of the 8 tyrosines of the region) enhances the probability that Grb2 binding sites are utilised for phosphorylation. Three of the other four tyrosines are in non-consensus positions and one is part of a potential RasGAP or Crk docking site.

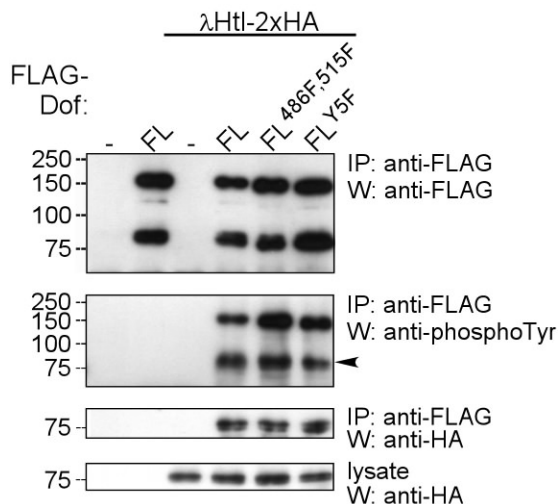


Figure 24. Tyrosine residues of the last 200 amino acids contribute to the phosphorylation of the Dof protein. Full length wild type Dof (FL) or full length Dof constructs with tyrosine to phenylalanine point mutations at amino acid positions 486 and 515 (FL^{486F,515F}) or in addition at amino acid positions 592, 613 and 629 (FL^{Y5F}) were expressed together with an activated form of HA-tagged Heartless (λ Htl-2xHA) in S2 cells. Protein complexes were immunoprecipitated from whole cell lysates using antibody against the FLAG epitope, and the precipitated proteins were detected on Western blots using antibodies directed against the FLAG or HA epitopes or phospho-tyrosine residues. Black arrowhead marks the tyrosine phosphorylated protein bands corresponding to the activated Heartless construct.

In summary, we identified five distinct tyrosine residues in Dof at positions 486, 515, 592, 613 and 629 that might be phosphorylated in the presence of activated FGF receptors. In addition, we found that the C-terminal part of Dof between amino acids 802 and 1012 is also phosphorylated. Tyrosines 486 and 515 are located in consensus binding sites for PI3K and Corkscrew (*Drosophila* homologue of SHP2) respectively, whereas 592, 613 and 629 are not part of known motifs. Whether the consensus binding sites for RasGAP or Crk and Grb2 in the last 210 amino acids of Dof are targets of phosphorylation needs more detailed analysis. It similarly requires further studies to determine whether all of the tyrosines identified by site directed mutagenesis are *in vivo* kinase substrates in a wild type Dof molecule or phosphorylation was partially an artefact of kinase activity in the absence of endogenous substrate sites in the molecule.

2.3.2 Conservation of Dof phosphorylation sites in Dof homologues

Comparative sequence analysis of the identified phosphorylation sites in molecules homologous with Dof might help to reveal if these sites are evolutionarily conserved and so potentially might confer conserved functions. Dof is a fast-evolving gene as even its closest known homologue in *Anopheles gambiae* shows significantly lower similarity than the average degree of conservation between clear orthologs in the two insect genomes (Wilson et al., 2004). In vertebrates two proteins that function in B-cell signalling, BCAP and BANK share structural domains with Dof (Okada et al., 2000; Yokoyama et al., 2002). In all three homologues conservation is mainly restricted to the DBB domain, ankyrin repeats and coiled coil region (Wilson et al., 2004). Sequence analysis revealed that tyrosines located in consensus binding sites for Grb2, PI3K, SHP2 and RasGAP or Crk in Dof are also present in the homologues. Whereas the RasGAP or Crk binding site is restricted to Dof and its *Anopheles* homologue, potential SHP2 binding sites are present in all three related molecules. The PI3K docking site is lost in hBANK, but present abundantly in BCAP. Similarly, the Grb2 consensus binding site is lost only in hBANK (Figure 25A). *Anopheles* Dof is the closest known homologue of *Drosophila* Dof, and consistent with this we find the most reserved positioning of the consensus binding sites compared to Dof, although no data are available about the phosphorylation and the functional relevance of these motifs. While in both mBCAP and hBANK novel potential SHP2 consensus binding sites appeared at the N-terminus of the proteins, mBCAP still contains an SHP2 site in the middle of the molecule like *Drosophila* Dof. In addition a multiplication of the PI3K binding sites could be observed in mBCAP. This might be in correlation with the function of BCAP in PI3K activation in B-cell receptor signalling. However, the tyrosines of the four PI3K consensus binding sites contribute to the phosphorylation of BCAP and are

required for the recruitment and activation of PI3K in their phosphorylated state are not the exclusive sites of tyrosine phosphorylation in the molecule (Inabe and Kurosaki, 2002). In hBANK only SHP2 sites are conserved among the consensus binding sites Dof contains. Nevertheless it has been shown that not the N-terminal part, but the region between amino acids 367 and 653 with its eight tyrosines spanning between the conserved ankyrin repeats and coiled coil domain is required for phosphorylation of hBANK (Yokoyama et al., 2002).

The three non-consensus tyrosines identified as phosphorylation targets in Dof are part of sequence patches which are highly conserved in *Anopheles* Dof (Figure 25B). While the conserved regions around the three tyrosines are in close proximity in *Drosophila* Dof they are interrupted with 11 and 16 amino acid long insertions in the *Anopheles* sequence indicating that they might represent three independent motifs in the molecule. Whereas Tyr592 is in a conserved but unique sequence environment the conserved amino acids around Tyr613 and Tyr629 show remarkable similarity to each other. This motif is found two more times in the C-terminal 200 amino acids in both molecules, one overlapping with the potential consensus RasGAP or Crk binding site (Figure 25B). A minimal consensus profile derived from these eight conserved sites is depicted in (Figure 25C). This motif is absent in BCAP, but interestingly, present in hBANK in the region between ankyrin repeats and coiled coil domain at amino acid position 458, which marks the position of the tyrosine (Figure 25D). Protein database search with this novel motif scored several known adapter and scaffolding molecules and tyrosine kinases both in *Drosophila* and higher vertebrates; a shortlist of examples from mouse and *Drosophila* is given in Figure 25E. These molecules are partially known to be tyrosine phosphorylated upon signalling, but tyrosine residues that are located in this motif were not object of molecular studies so far.

Figure 25. Conservation of consensus tyrosine motifs among Dof homologues and the identification of two phosphorylation sites in Dof as novel consensus tyrosine motifs conserved in several signalling molecules. (A) Schematic representation of *Drosophila* Dof and related proteins *Anopheles* Dof, mouse BCAP and human BANK. Conserved protein domains of the four proteins and consensus tyrosine motifs are labelled on the scheme. White box, DBB domain; black box, ankyrin repeat and white oval, coiled coil region. Signalling molecules that could bind to the marked conserved tyrosine motifs if tyrosine is phosphorylated are listed above the position of each motif with the amino acid position of the tyrosine within the motif. Red stars mark the positions of a novel consensus tyrosine motif identified in Dof (see below). (B) Protein sequence alignment over the C-terminal half of *Drosophila* Dof (DmDof) and *Anopheles* Dof (AgDof) starting from the end of the ankyrin repeats (accession numbers O96757 and Q8T5J9). Asterisks denote identical amino acids whereas dots mark similar amino acid residues. Black shaded sequence corresponds to the coiled coil region. Each tyrosine of both sequences is marked in red and tyrosine phosphorylation sites identified in Dof are labelled with a blue “P” above the particular residues. Consensus tyrosine motifs are underlined and shaded grey in both sequences. Positions of the novel tyrosine motif are shaded yellow and numbered 1-4 above the sequence. Note that one consensus tyrosine motif is part of the third novel tyrosine motif in both sequences. CLUSTAL W alignment was modified by hand. (C) Alignment of the novel tyrosine motif in Dof, present four times in both the *Drosophila* and the *Anopheles* proteins. Numbering of the sequence patches is as in panel B. Residues with blue shading denote positions that are absolutely conserved. Four or more identical or similar residues shared by the sequences are shaded black and grey respectively. Consensus sequence is depicted below the alignment. Alignment was made using CLUSTAL W, and output file was formatted using BOXSHADE. (D) Alignment of *Drosophila* Dof and human BANK (accession number Q8WYN5) over the sequence patch of the novel tyrosine motif. Start and end positions of the sequences are marked on the alignment. Residues of the tyrosine motif are shaded yellow. (E) A conserved pattern of the eight tyrosine motifs in panel C was generated using Pratt (www.ebi.ac.uk/pratt). ScanProsite (www.expasy.org/tools/scanprosite) was used to find other proteins containing the conserved motif. Hits were sorted taxonomically and signalling molecules in mouse and *Drosophila* were short listed as example. The alignment over the novel consensus tyrosine motif of these proteins is shown here. Conserved residues are shaded blue. 3BP2, SH3 binding protein 2; Ap2a1, alpha-adaptin A; Ap2a2, alpha-adaptin C; ACK1, Activated CDC42 kinase 1; Ppp1r13b, Protein phosphatase 1 regulatory subunit 13B; CNK2, Connector enhancer of kinase suppressor of Ras 2; Dok1, p62(dok) adaptor; Dvl1-3, dishevelled 1-3 (homologue); Dsh, dishevelled (*Drosophila*); Dab, Disabled; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*.

Thus, we postulate the sequence Y-[X]3-P-[X]3-P as a novel evolutionarily conserved motif for potential tyrosine phosphorylation and thereby probably serving as docking site for so far unidentified regulator(s) of tyrosine kinase activity-linked signalling. Functional analysis is required to confirm the biological role of these conserved tyrosine residues in Dof. These studies might help to identify new regulatory components of tyrosine kinase coupled signalling in general.

2.4 The role of sumoylation in Dof function

Small ubiquitin-related modifier (SUMO) family proteins function by becoming covalently attached to and thereby modify the effect of other proteins. Not only SUMO itself but components of the whole conjugation pathway are closely related to the corresponding members of the ubiquitination machinery. SUMO modifies many proteins that participate in diverse cellular processes, including transcriptional regulation, maintenance of chromosomal integrity, nuclear transport and signal transduction. However, the functional consequences of sumoylation are different from ubiquitination. How sumoylation functions is not fully understood and might differ from substrate to substrate. In many cases it alters subcellular localisation or interaction of the substrate with other proteins or with DNA, but it acts also as inhibitor of proteosomal degradation by blocking ubiquitin binding sites (reviewed in (Johnson, 2004)). The majority of identified proteins modified by SUMO attachment is nuclear or becomes sumoylated upon entering the nucleus or assists at nuclear import. Thus, most known functions of this modification are linked to the nucleus. Nevertheless, there are some examples where SUMO modification is needed for cytoplasmic function. The MAPK kinase Mek1 becomes sumoylated after cAMP stimulus in *Dictyostelium* and the initially nuclear Mek1 relocates to the plasma membrane upon SUMO attachment where it probably activates membrane recruited MAPK. Strikingly, by 3 min after pathway activation Mek1 was desumoylated and disappeared from the plasma membrane (Sobko et al., 2002). This indicates a very dynamic regulation of SUMO modification at the plasma membrane which makes sumoylation a potent sensitive regulator of signal transduction but also challenges for a hard hunt to catch such events. Recent work showed that *Drosophila* Ulp1, the SUMO deconjugating enzyme localises to the nucleoplasmic face of the nuclear pore complex and prevents accumulation of cytoplasmic SUMO conjugates probably by inhibiting proteins from leaving the nucleus in a SUMO attached form (Smith et al., 2004). These indicate that the nucleus might be the major but not the exclusive permissive place in the cell for a sumoylated state and suggest a tight spatio-temporal regulation of cytoplasmic SUMO modification to prevent sumoylation of the many nuclear SUMO target proteins in the cytosol but allow temporary modification of cytosolic SUMO substrates.

Most SUMO substrates contain at least one copy of the short SUMO attachment consensus sequence I/V/L-K-x-E, in which the lysine residue is the site for modification. However, the abundance of this sequence motif in proteins, together with the fact that proteins were identified with alternative SUMO attachment sites indicate that probably not all proteins with consensus sumoylation site are SUMO targets and that interactions other than those between the SUMO conjugating enzyme and the consensus motif are critical in determining SUMO modification *in vivo*.

Dof contains four SUMO attachment consensus sites with lysine residues at amino acid positions 3, 82, 297 and 737 respectively (see Figure 6 in Introduction). Yeast two hybrid data provided additional indication for potential SUMO modification of Dof. Ubc9, the E2 SUMO conjugating enzyme and dPIAS, the *Drosophila* homologue of the mammalian PIAS family E3 SUMO ligases interacted with Dof in yeast (Battersby et al., 2003) and Ubc9-Dof interaction was confirmed in co-immunoprecipitation experiments in *Drosophila* S2 cells (see Chapter 2.1 for details). To test directly if Dof is able to become SUMO modified biochemical experiments were performed.

2.4.1 Dof sumoylation assay in *Drosophila* S2 cells

Ubc9 was shown to be sufficient to sumoylate substrates without additional E3 SUMO ligase activity under *in vitro* conditions and in cell culture overexpression studies (Bhaskar et al., 2000;

Lee et al., 1998). Therefore, we took the minimal critical components of SUMO modification including Ubc9 and SUMO itself to establish sumoylation assay in *Drosophila* S2 cells. Full length Dof with an N-terminal FLAG epitope tag was transiently co-expressed with an HA-tagged form of Ubc9 and with the activated form of *Drosophila* SUMO. Activated SUMO lacks the last two C-terminal amino acids of the protein which have to be cleaved off by SUMO-cleaving enzymes to make the molecule active for binding with E1 enzymes. The three proteins were overexpressed in different combinations in the cells. To analyse the sumoylation state of FLAG-Dof the protein was purified from whole cell lysates by immunoprecipitation using antibody against Dof followed by Western blot analysis using antibodies against the FLAG epitope tag and *Drosophila* SUMO protein. The FLAG antibody detected protein bands only in immunoprecipitates of cell lysates where FLAG-Dof was expressed (Figure 26, upper panel). In each of these protein fractions at least three bands were recognised by the FLAG antibody. They migrated at about 140 kDa, 100 kDa and 72kDa which corresponds to the size of full length Dof and N-terminal fragments of FLAG-Dof respectively. In immunoprecipitates of cell lysates containing FLAG-Dof, Ubc9 and SUMO a weak fourth band was detected by the FLAG antibody, migrating with an apparent size of about 85 kDa. This band must represent an additional N-terminal fragment of Dof since the FLAG epitope tag was attached to the N-terminus of the protein. To assess the presence of sumoylated proteins in Dof immunoprecipitates SUMO antibody was used in Western blot analysis. A protein band migrating at about 85 kDa was recognised by the SUMO antibody specifically in Dof immunoprecipitates of cell lysates where FLAG-Dof, Ubc9 and SUMO were expressed simultaneously but not of other lysates that lacked at least one of the three proteins (Figure 26, lower panel). Considering that covalent attachment of SUMO to a substrate causes a shift of about 10 kDa in the migratory behaviour of that protein the most likely explanation might be that the detected protein band represents the sumoylated form of the 72 kDa N-terminal fragment of Dof. This is strongly supported by the presence of an N-terminal Dof fragment of similar size (ca. 85 kDa) specifically and exclusively in the same precipitate where the protein band was detected with the SUMO antibody.

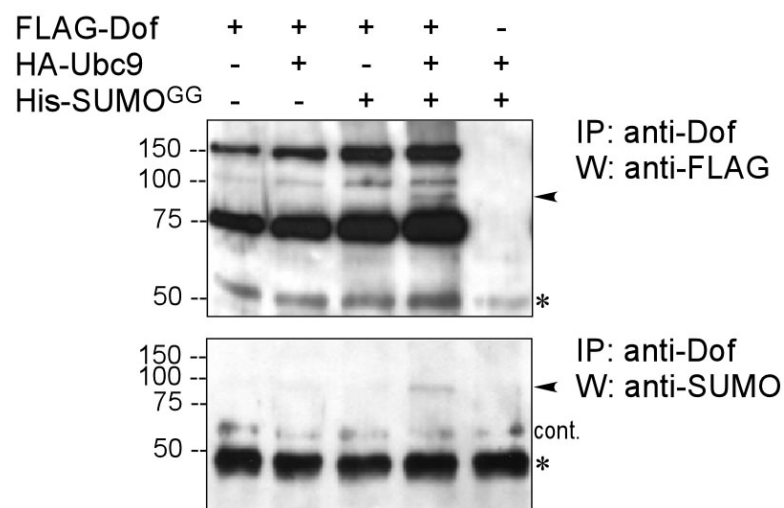


Figure 26. Sumoylation of Dof in *Drosophila* S2 cells. FLAG epitope-tagged full-length Dof, HA epitope-tagged dUbc9 and the activated form of dSUMO with an N-terminal 6xHis epitope tag (His-SUMO^{GG}) were transiently transfected in the labelled combinations in *Drosophila* S2 cells. Whole cell lysates of transfected cells were immunoprecipitated with Dof antibody and analysed on Western blots. Blots were stained with FLAG antibody to visualise Dof protein (upper panel) or with SUMO antibody raised against *Drosophila* SUMO to detect sumoylated proteins in Dof antibody immunoprecipitates (lower panel). Asterisks mark the heavy chain of Dof antibody used for immunoprecipitation. “cont.” marks contaminating protein bands detectable with SUMO antibody. Arrowheads indicate FLAG and SUMO antibody reactive bands at similar molecular weight positions.

Three SUMO attachment consensus sites with lysine residues at amino acid positions 3, 82 and 297 are present in the 72 kDa N-terminal Dof fragment (for details of Dof processing see Chapter 2.5). Strikingly, lysine to arginine exchange of amino acid 297 in the molecule leads to the complete loss of Dof function *in vivo* (R. Wilson personal communication) probably by abolishing effective interaction of the molecule with the FGF receptor (see Chapter 2.2). Important questions of future works are whether lysine 297 is the target for sumoylation in the N-terminal Dof fragment and whether sumoylation is involved in the interaction of Dof with the FGF receptors. No less importance has the analysis why only the N-terminal fragment of Dof became sumoylated in our assay but not the full length molecule. An effort to increase or stabilise the proportion of sumoylated Dof in S2 cells might be also useful for further experimental work.

In summary, this result shows that Dof is able to become sumoylated in S2 cells but further work is required to identify the site of modification in the molecule and to analyse the functional relevance of SUMO attachment of Dof.

2.4.2 Analysis of the role of the SUMO conjugation pathway in FGF dependent developmental processes

If sumoylation plays a role in the regulation of Dof function *in vivo* the sumoylation machinery itself should be also involved in Dof-dependent developmental processes. To test this hypothesis, mutant alleles of the genes encoding Ubc9 and SUMO were analysed for genetic interaction with FGF signalling during embryogenesis. Our assays involved the monitoring of the formation of Eve-positive clusters in the mesoderm and the development of the tracheal network.

Ubc9

First we studied the gene coding for *ubc9*, called *lesswright* (*lwr*) or *semushi* (*semi*) in *Drosophila*. Figure 27 shows the *lwr* locus in the *Drosophila* genome. Several P element insertion and excision alleles are located in this genomic region. The *lwr* alleles *lwr5486* and its derivative *lwr4-3* dominantly suppress the non-disjunction and cytological defects of female meiotic mutations that influence spindle formation (Apionishev et al., 2001). The lethal P element insertion *semi2858* and its derivative *semi107* are located downstream to the coding region of both, the *lwr* and the distal neighbouring *u-shaped* genes. The *semi* alleles block nuclear import of Bicoid by affecting *ubc9* rather than *u-shaped* function and as a consequence embryos have multiple defects in anterior segmentation (Epps and Tanda, 1998). Although each of these alleles is linked to *ubc9* gene activity, they form two distinct complementation groups, that of the *lwr* alleles and that of the *semi* alleles. The different effects of the alleles and their relevance to Ubc9 function is not understood. Thus, the genetics of this genomic region is not worked out.

Since the relationship of the two allele groups is not clear we decided to analyse mutant alleles of both complementation groups. Embryos homozygous for the mutant alleles *lwr5486*, *lwr4-3* and *semi2858* showed no detectable deviation from wild type in the formation of mesodermal Eve-positive clusters and in the development of the tracheal network (data not shown). On the other hand *semi107* embryos showed early segmentation defects with incomplete penetrance. These embryos showed reduced numbers of Eve-positive clusters and trachea metamers often combined with tracheal branching defects (Figure 28). A few embryos showed defects in germ band retraction occasionally combined with segmentation abnormalities and/or tracheal branching defects (Figure 28G). Thus, it indicates that the *semi107* allele has pleiotropic effects. Defects in FGF signalling dependent processes were considered as secondary effects since they were often combined with

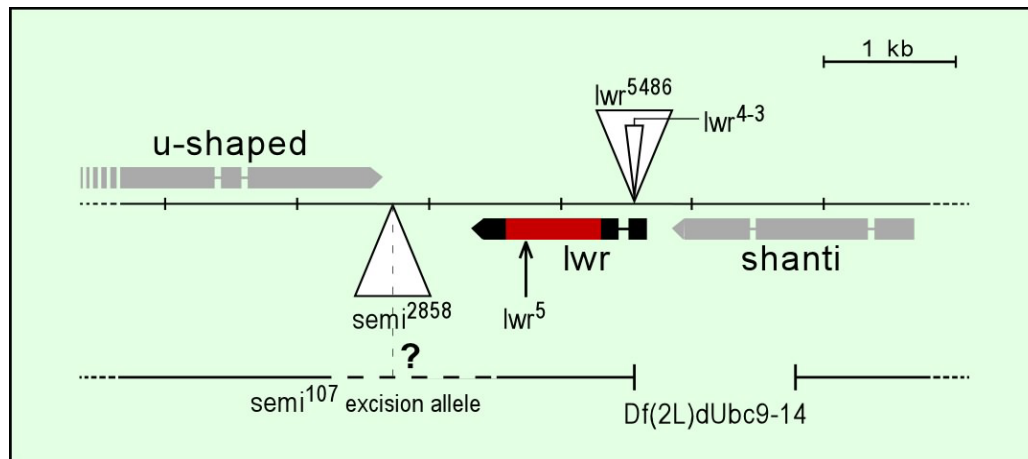


Figure 27. Characterisation of lesswrite (*lwr*)/semushi (*semi*) mutations in the *lwr* genomic locus. The *lwr*/*semi* gene is located at cytological position 21C on the second chromosome. It has two exons (black boxes) separated by a short intron (black bar). The second exon contains the entire coding region (red rectangle). The gene is in the same orientation as the adjacent proximal gene *shanti* but reads in the opposite direction than the distal neighbouring gene *u-shaped*. The lethal P{PZ} insertion *lwr*⁵⁴⁸⁶ is located at nucleotide position 62 in the 5'UTR of *lwr*. *lwr*⁴⁻³, a lethal excision of *lwr*⁵⁴⁸⁶ contains 76 bp residual foreign sequence at the *lwr*⁵⁴⁸⁶ insertion site. The deficiency *Df(2L)dUbc9-14* was generated by imprecise excision of *lwr*⁵⁴⁸⁶. *lwr*⁵ is an EMS induced lethal allele and contains a single R to H point mutation at amino acid position 104 in the coding region of *lwr*. The lethal P{PZ} insertion *semi*²⁸⁵⁸ is positioned 3' to the coding sequence of both genes, *lwr* and *u-shaped* with a distance to the putative poly-adenylation signals of 560 and 47 nucleotides respectively. *semi*¹⁰⁷ is a lethal excision of *semi*²⁸⁵⁸ without published molecular data (Epps and Tanda, 1998). Schematic map is modified from (Apionishev et al., 2001) and <http://www.flybase.org>.

severe segmentation defects, consistent with the data that *semi* alleles cause reduced segment numbers (Epps and Tanda, 1998). Surprisingly and in contrast to earlier findings, *semi*²⁸⁵⁸ embryos did not show detectable defects including segmentation defects, although homozygosity for this allele caused embryonic lethality (summarised in Table 3B).

The lack of abnormal development in the analysed tissues of *lwr*⁵⁴⁸⁶, *lwr*⁴⁻³ and *semi*²⁸⁵⁸ embryos might be due to the high *lwr* mRNA levels deposited in the eggs maternally. Although this maternal contribution is not sufficient for the embryo to develop to larval stages it can mask early requirement of Ubc9 and potential differences in Ubc9 expression levels needed in different tissues. To overcome this problem we generated germ line clones (GLC) of the *lwr*⁴⁻³ allele and as a control of the *semi*¹⁰⁷ allele. Females with mutant germ line clones were crossed to *lwr*⁵⁴⁸⁶, *lwr*⁴⁻³, *semi*²⁸⁵⁸ or *semi*¹⁰⁷ heterozygous males. To control paternally rescued phenotypes females were also crossed to wild type males. *lwr*⁴⁻³ GLC females produced very few or practically no eggs. The 25 eggs laid in total by 1500 females were unfertilised and showed fused dorsal appendages (Figure 29A-C), summarised in Table 3A), indicating that the *lwr* gene is required during oogenesis. This is in agreement with the finding that *lwr* plays a role in female meiotic events (Apionishev et al., 2001). *semi*¹⁰⁷ GLC females laid fertilised eggs with wild type morphology indicating that the *semi*¹⁰⁷ mutation represents a hypomorphic allele, which does not interfere with oogenesis. One copy of a wild type or *lwr*⁵⁴⁸⁶ paternal chromosome completely rescued embryonic lethality consistent with the reports that *lwr*⁵⁴⁸⁶ and *semi*¹⁰⁷ belong to separate complementation groups. The *lwr*⁴⁻³ allele failed to complement *semi*¹⁰⁷ GLCs in our assay.

Analysis of *semi*¹⁰⁷ GLC embryos showed that only the homozygous *semi*¹⁰⁷ allelic combination caused detectable defects on the formation of *Eve*-positive clusters and on the development of the tracheal network (summarised in Table 3A). These abnormalities were indistinguishable from the ones observed in zygotic mutant *semi*¹⁰⁷ embryos (data not shown) and are probably secondary consequences of segmentation defects as discussed above.

In summary, this genetic approach did not indicate an obvious requirement of Ubc9 function in FGF signalling *in vivo*. Sorting out genetics of this gene is a prerequisite of future work.

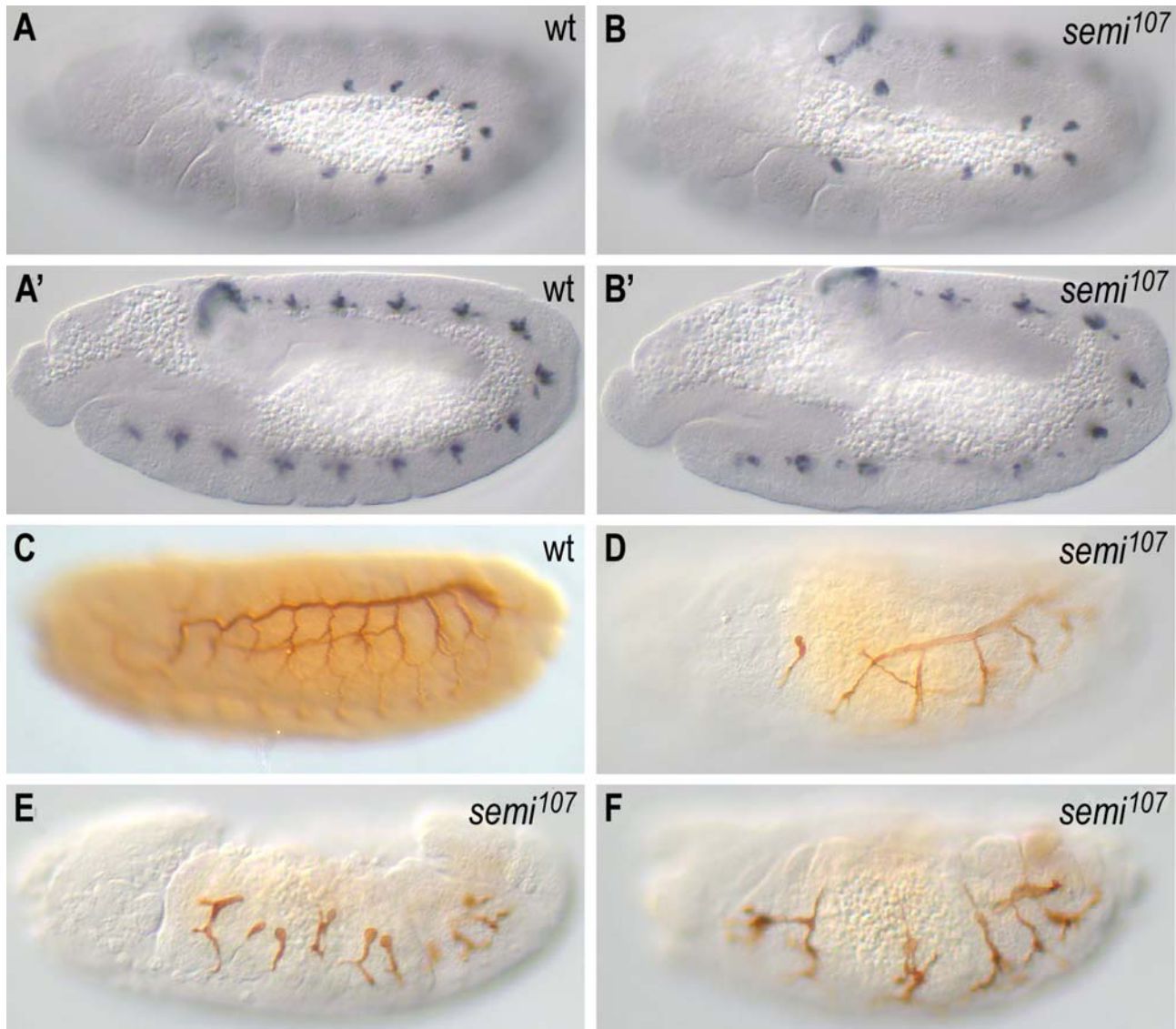


Figure 28. Homozygous mutant *semi*¹⁰⁷ embryos show pleiotropic morphogenetic defects. (A-B) stage 11 (A) wild type or (B) homozygous *semi*¹⁰⁷ embryos were stained with Even-skipped antibody to visualise Eve-positive cluster in the mesoderm. A' and B' show the same embryos as seen in A and B with Eve-positive cells of the central nervous system (CNS) in the focus. Note the overlapping positions of the lack of mesodermal Eve-positive clusters with the patterning defects in the CNS. (C-F) stage 15 (C) wild type or (D-F) homozygous *semi*¹⁰⁷ embryos stained with 2A12 antibody to visualise the lumen of the trachea network. Note (D) the reduced number of tracheal units, (E) the complete failure of tracheal branching including dorsal trunk fusion or (F) the combination of these two abnormalities. Note segmentation defects of embryos on B, D and F and germ band retraction defect of the embryo on E. Anterior to the left.

Table 3. Studies on different lethal alleles in the *lwr* locus

A	Germ line clones				B Zygotic mutations		
	paternal genotype	<i>lwr</i> ⁴⁻³ GLC	semi ¹⁰⁷ GLC		embryonic lethality	Eve-clusters	tracheae
embryonic lethality			Eve-clusters	tracheae			
<i>lwr</i> ⁵⁴⁸⁶	9 eggs (fused dorsal appendages)	> 90% hatched (339/352)	not affected	not affected	41% hatched (189/467)	not affected	not affected
semi ²⁸⁵⁸	9 eggs (fused dorsal appendages)	66% hatched (162/246)	not affected	not affected	66% hatched (321/478)	not affected	not affected
<i>lwr</i> ⁴⁻³	no eggs	63.5% hatched (306/482)	not affected	not affected	51% hatched (211/418)	not affected	not affected
semi ¹⁰⁷	7 eggs (fused dorsal appendages)	55% hatched (166/300)	42% (34/81) lower number of clusters in embryos lacking middle segments	40% (45/113) reduced number of tracheal units in embryos with fewer segments occasionally branching defects	45% hatched (180/402)	24% (19/80) lower number of clusters in embryos lacking middle segments	32% (38/120) reduced number of tracheal units in embryos with fewer segments
wild type	no eggs	> 90% hatched (268/285)	not affected	not affected	-	-	-

(A) females with germline homozygous for the *lwr*⁴⁻³ or semi¹⁰⁷ alleles were crossed to males heterozygous for mutations listed on the left. For *lwr*⁴⁻³ GLC the total number of eggs produced by 300 females in 5 days is labelled in case of each cross. To assess the effect of paternal rescue in semi¹⁰⁷ GLCs 250-500 eggs were scored for viability. Percentage of the hatching was calculated as a ratio of the number of larvae hatched over the total number of eggs analysed. Absolute numbers are given in brackets for each case. Overnight collection of embryos was stained with Even-skipped or 2A12 antibody to visualise mesodermal Eve-positive clusters or the lumen of the tracheal network respectively. Observed abnormalities are described and the calculated ratio of embryos with abnormalities noted. Not affected marks phenotypes indistinguishable from wild type.

(B) Recessive lethal alleles listed on the left were tested for zygotic effects in their homozygous form. Their severity on embryonic lethality was assessed by calculating the ratio of hatched larvae over the total number of eggs analysed. Absolute numbers are shown in brackets. Effects on mesoderm and tracheal morphogenesis were analysed the same way as described for the GLCs. GLC, germ line clone.

Smt3

A single gene coding for SUMO is present in the *Drosophila* genome, called *smt3*. Two lethal P elements, l(2)k01211 and l(2)04493 are available which have independent origin but are mapped to the same position molecularly: about 10 base pairs upstream of the first exon of the *smt3* gene (www.flybase.org). Consistent with this they do not complement each other. Work with these two alleles showed that *smt3* interacts with *ras1* in *Drosophila* eggshell patterning and morphogenesis. It is thought to act in EGF signalling downstream of the EGF receptor (Schnorr et al., 2001). EGF signalling occurs in follicle cells of somatic origin which surround the egg and produce the eggshell.

Homozygous mutant embryos of both alleles were analysed and no abnormalities were found in mesoderm or tracheal development (data not shown). *smt3* has a strong maternal contribution which might deliver sufficient protein for embryonic development. Thus, germ line clones were generated with the l(2)04493 allele. Three independent FRT l(2)04493 recombinant chromosomes were

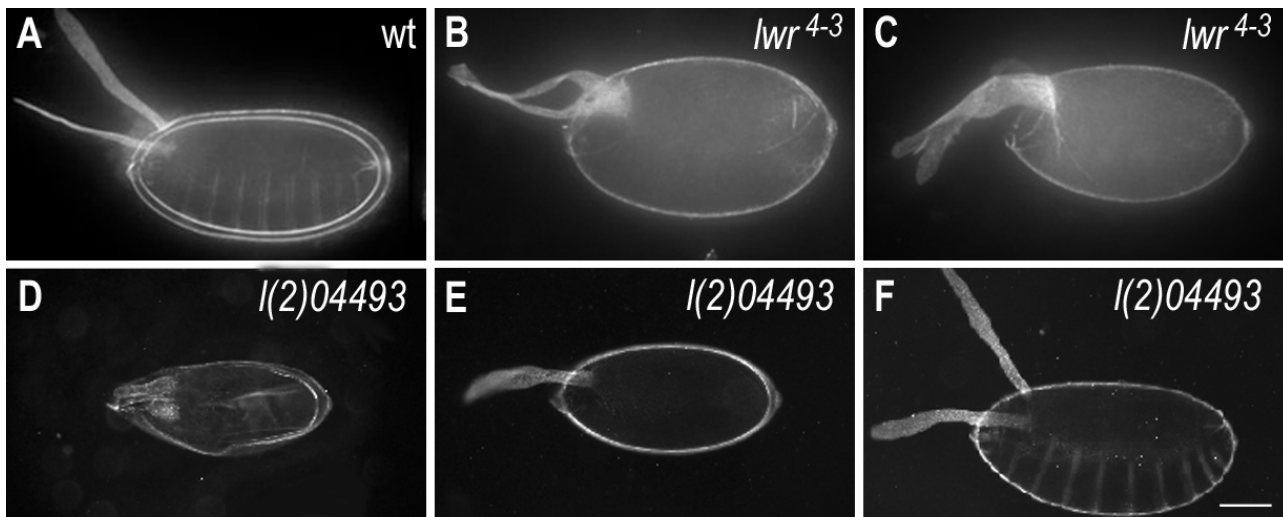


Figure 29. Dorsal appendage morphology defects of eggs derived from lwr^{4-3} and *smt3* mutant germ line clones (GLC). Eggshell preparation of eggs laid by (A) wild type females and by females with germ line clones homozygous (B,C) for the lwr^{4-3} allele or (D-F) for the lethal P-element insertion I(2)04493 in the *smt3* gene. B shows the weakest, C a representative example for the dorsal appendages fusion phenotype found among the lwr^{4-3} mutant GLC eggs. Note, that the eggs are unfertilised. (D-F) Phenotypic range of eggs laid by females with *smt3* mutant GLC. (D) dorsal appendage material appeared as short trunk, (E) the two appendages were fused on their basis or (F) were identical to wild type structures. Note the egg in F was fertilised. Anterior to the left. Scale bar 100 μ m.

analysed. GLC females were crossed to males heterozygous for the appropriate FRT I(2)04493 recombinant chromosome, the original I(2)04493 chromosome or the independent I(2)k01211 P insertion and to wild type males as a control for the paternally rescued phenotype. Eggs were produced in each cross. In each case they could be divided into three phenotypic classes: (i) eggs that showed fused dorsal appendages; in the most severe cases short trunks or bulbs labelled only the position of the appendages (Figure 29D-E). These eggs were rarely fertilised. (ii) eggs that had normal dorsal appendages (Figure 29F), but larvae did not hatch from them either because they were unfertilised or due to an arrest in embryonic development. (iii) eggs from which larvae hatched. The distributions of these phenotypes and the percentages of unfertilised eggs are shown in Figure 30 for each cross. The defects in eggshell morphology indicated a role of *smt3* during oogenesis. *Smt3* is reported to be required in the dorsal follicle cells for the formation of the dorsal appendages of the eggshell (Schnorr et al., 2001). The observed eggs with fused dorsal appendages are in concert with this. Based on the method how germline clones were induced and selected, eggs derived from GLCs are mutant for germ cells, but are derived from ovaries that can contain both mutant and wild type follicle cells. This might explain the two classes of eggs with fused or wild type dorsal appendages. Alternatively, the P insertions might represent hypomorphic alleles of *smt3* thereby causing various egg phenotypes. We also can not exclude the variable effect of perduration where SUMO protein stays due to long half life of the molecule and/or by recycling from sumoylated substrates for a sufficient period after mitotic recombination to drive mutant germ cells through oogenesis.

The three recombinant lines showed a large difference in the severity of the observed phenotypes indicating that “background” interfering mutations of the original chromosomes were differentially inherited due to different recombination sites. Eggs obtained from crosses with males bearing the recombinant mutant chromosomes or the original I(2)04493 P insertion often showed more severe phenotype than those derived from males bearing the independent I(2)k01211 P insertion, possibly due to the additional effect of homozygous chromosomal background. Therefore we concentrated on the comparative analysis of the cross with males heterozygous for I(2)k01211 and of that with wild type males. In zygotically rescued embryos (wild type male cross) about 85-90% of the

fertilised eggs of line#1 and #3 hatched to larvae whereas in line#2 it was only about 20%. The high variation between the different lines indicates that it might be an effect independent of *smt3*. In crosses with *I(2)k01211* heterozygous males where half of the progenies inherited wild type chromosome only 50-75 percent of the embryos developed to larvae. Thus, the presence of the zygotic mutation interfered with the embryonic development of eggs, which had successfully overcome oogenesis, albeit with a moderate penetrance. This supports a zygotic requirement of *smt3*.

Embryos were collected and analysed from these GLC crosses. Many embryos showed a complete lack of tissue patterning and occasionally embryos were observed with tracheal defects but no abnormalities were detected in the mesoderm or in tracheal development at statistically relevant frequency (data not shown). Thus, we concluded that these *smt3* alleles might not primarily influence FGF-dependent development in the flies.

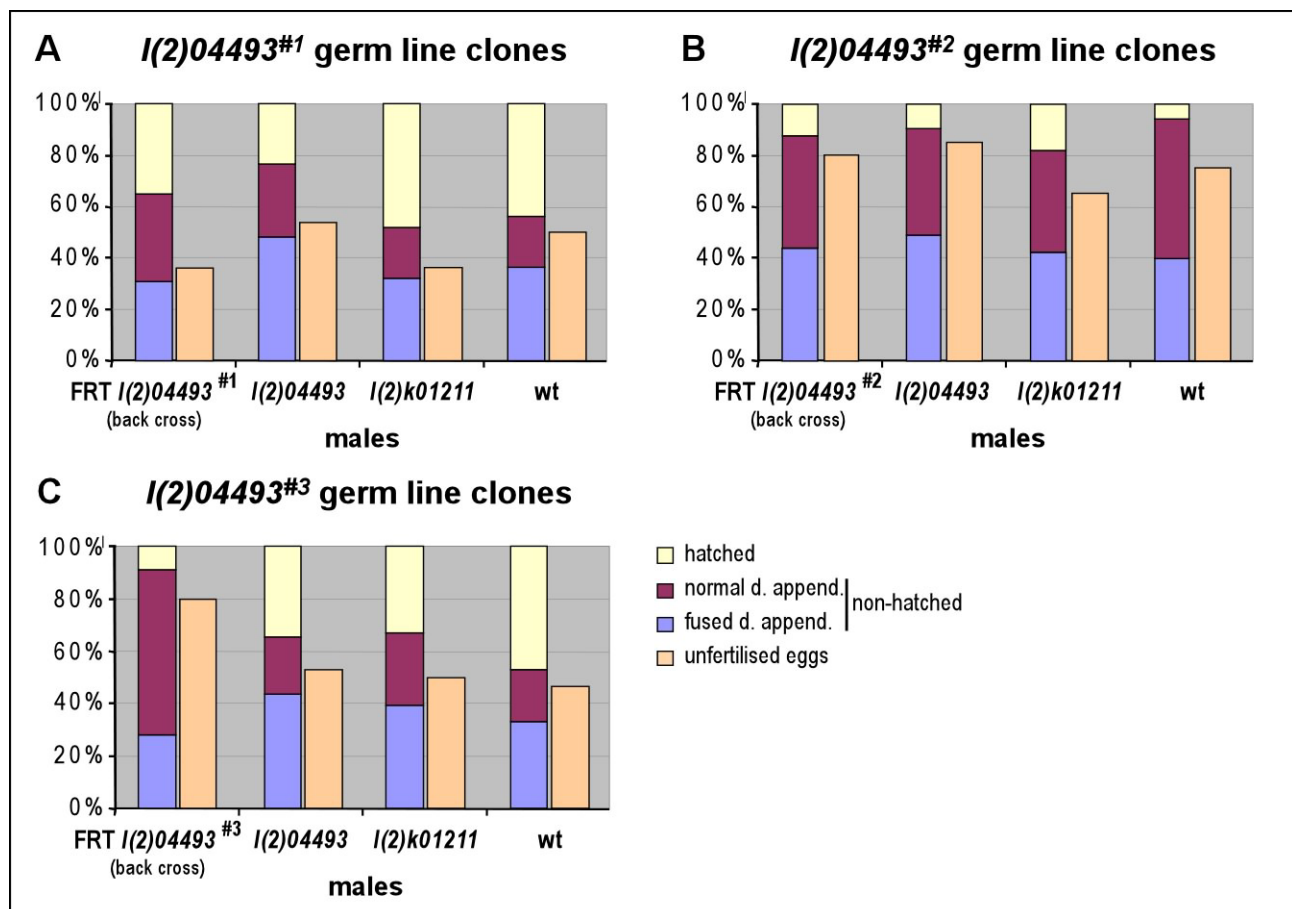


Figure 30. Germ line clone analysis of the *smt3* mutant allele *I(2)04493*. (A-C) GLC analysis of three independent FRT40A *I(2)04493* recombinant chromosomes. In each case females with homozygous *I(2)04493* clones in the germ line were crossed to males bearing the same recombinant chromosome (FRT *I(2)04493*^{#1-3}), to males with the original *I(2)04493* P{PZ} insertion chromosome, to males bearing an independent P{lacW} insertion in identical genomic position as *I(2)04493* (*I(2)k01211*) or to wild type males (wt). Eggs obtained from the crosses belonged to three different phenotypic classes: eggs, from which larvae hatched, non-hatched eggs with normal dorsal appendages or with fused dorsal appendages. The distributions of the three phenotypes among the 200-400 eggs analysed for each cross and taken as 100% is shown on the left column of each data series on the diagrams. The right columns of the data series show the percentage of unfertilised eggs in relation to the total number of eggs analysed. Colour code see in the legend next to the diagrams.

In summary, the phenotypes of mutants of the two genes Ubc9 and SUMO showed no similarities. Unlike in *semi107* mutants we did not see early embryonic segmentation defects in *smt3* mutants. Neither homozygous mutant *lwr* alleles nor *smt3* alleles showed detectable embryonic defects in our assay. GLC of these alleles indicate that they are both involved in oogenesis. The few eggs laid by *lwr4-3* GLC females were poorly developed in general but clearly showed fused dorsal appendages indicating that *lwr* might be involved in the formation of dorsal appendages but that it has an essential role in even earlier processes. Considering that the two genes encode obligatory components of the same pathway and there are no similar genes in the *Drosophila* genome *lwr* and *smt3* mutations might demonstrate differences in allelic strength or perduration. In summary, we obtained no conclusive answer if Ubc9 is required in mesoderm and tracheal development and some weak indications that SUMO might not be essential in these processes. However, the major conclusion is that alternative approaches are needed to answer this question.

2.5 Identification of Dof as caspase substrate and analysis of the role of Dof cleavage

2.5.1 Dof is cleaved in S2 cells

Dof protein bearing a FLAG epitope tag on its N-terminus was expressed in *Drosophila* S2 cells in several experiments discussed so far. A typical pattern was always observed on Western blots of whole cell lysates visualising Dof via its N-terminal FLAG tag. Intact Dof was detected at about 140 kDa on protein gels. In addition, one major degradation product at an apparent molecular weight of about 75 kDa and two minor degradation products with molecular weights of about 90 and 110 kDa were usually observed. The presence and amount of the minor products varied in different experiments whereas the major degradation product always appeared. The ratio between the intact form and the major degradation product of Dof also varied in different experiments (compare earlier experiments). Protein sequence analysis of Dof highlighted a potential caspase-3 cleavage site with the sequence DEVD at amino acid positions 584-587 in the molecule. The intact molecule has a predicted molecular weight of 113 kDa and cleavage at amino acid position 587 would yield N-terminal and C-terminal cleavage products with predicted molecular weights of 66 and 47 kDa respectively. In order to detect all cleavage products of Dof in S2 cells two different epitope tags were fused to the two ends of the protein; a FLAG tag to the N- and a 2xHA tag to the C-terminus (Figure 31A). Expressing this construct in S2 cells the described 140 kDa and 75 kDa protein bands were detected with FLAG antibody on Western blots of lysates corresponding to full length Dof and an N-terminal fragment of the molecule respectively (Figure 31B left panel). The antibody against the HA epitope detected bands with molecular weights of 140, 60, 50 and 35 kDa corresponding to full length Dof and additional C-terminal fragments of the protein respectively

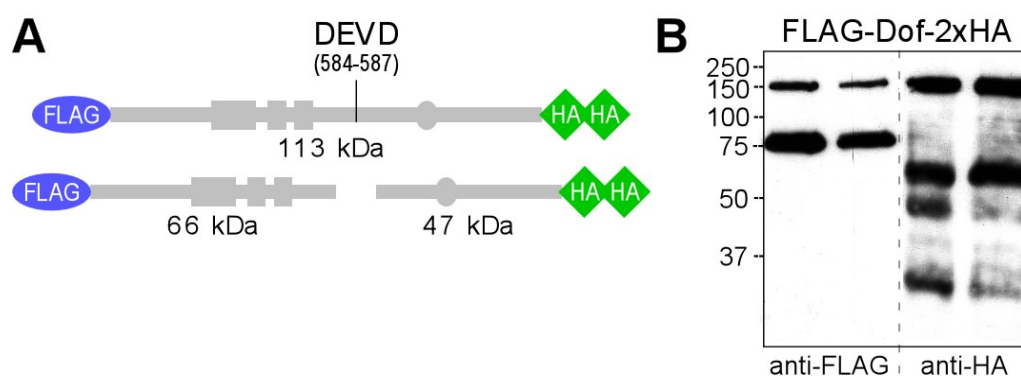


Figure 31. Detection of N- and C-terminal fragments of Dof in S2 cell lysate. (A) Schematic representation of the Dof construct bearing a FLAG epitope tag (blue oval) and a double HA epitope tag (green rhombus) fused to its N- and C-terminus respectively. Grey boxes and oval on the scheme denote protein domains DBB, ankyrin repeats and coiled coil respectively and serve for orientation. The protein sequence “DEVD” marks a potential consensus caspase cleavage site in Dof between ankyrin repeats and coiled coil region (black line). The exact amino acid position is given in brackets. 113 kDa marks the calculated molecular weight of the full length Dof molecule. Cleavage at this position would generate two separate Dof fragments with calculated molecular weights marked below the drawings. Note that full length Dof construct is detectable via both epitope tags whereas the N-terminal and C-terminal fragments would be detectable by only the FLAG tag or the HA tag respectively. (B) Western blot analysis of lysate from S2 cells expressing the Dof construct depicted in A using antibodies against FLAG (left panel) and HA (right panel). The two lanes on both panels are duplicates of the same lysate. Note that both blots show a protein band of similar size (full length Dof) and in addition smaller protein bands representing different N- and C-terminal fragments of Dof. Note the difference between the calculated molecular weight and running behaviour of Dof on protein gels.

(Figure 31B right panel). Considering that intact Dof is detected at a higher molecular weight on protein gels than predicted, the major degradation product observed at about 75 kDa with the FLAG antibody might represent the N-terminal 66 kDa cleavage product whereas the 60 kDa protein band recognised by the HA antibody might correspond to the C-terminal 47 kDa cleavage product. We think that the additional smaller C-terminal fragments might be further degradation products of the 60 kDa protein band from two reasons. First, no protein band was observed with FLAG antibody at a size that would be complementary to these bands (between 75 and 150 kDa) in the lysate; secondly, although the N-terminal 75 kDa Dof fragment was always detectable the presence of a precise band of the C-terminal 60 kDa fragment was less reliably detected in many experiments indicating that the C-terminal cleavage product might be less stable.

2.5.2 Cleavage of Dof depends on caspase activity

To see if caspases are responsible for the cleavage of Dof in S2 cells we used different ways to block caspase activity. First, to distinguish whether degradation of Dof occurs after lysis, or the protein is already processed in the cells, a pancaspase inhibitor drug was added to the lysis buffer or alternatively, cells were incubated with the same drug prior lysis. No difference was observed in the degradation pattern of FLAG-Dof on Western blots of whole cell lysates using lysis buffer with or without caspase inhibitor (Figure 32A, lanes 1 and 2). On the other hand, if cells were treated with different concentrations of the same caspase inhibitor for 2 hours before harvest, the amount of full length FLAG-Dof increased significantly in the lysate compared to untreated cells (Figure 32, lanes 6 and 7). This result indicates that Dof processing is sensitive to caspase inhibitor drugs. Furthermore it shows that cleavage occurs in the cells, before lysis. The partial effect of the caspase inhibitor Z-VAD on Dof cleavage might be an issue of concentration and timing the drug was used. Indeed we observed a dose dependent action of Z-VAD on cleavage (compare lines 6 and 7 on Figure 32). Since cells were treated with the drug only 22 hours after Dof expression was induced cleavage products might have already accumulated by that time.

To see if Dof processing could be completely abolished by blocking caspase activity the baculoviral caspase inhibitor p35 was constitutively expressed in the cells prior the induction of Dof expression. p35 blocked Dof cleavage completely (Figure 32A, lane3). In addition, the amount of intact FLAG-Dof in the lysate correlated with the amount of the transfected p35 construct (Figure 32A, compare lanes 3-5). Transfection and induction by copper causes enormous stress to the cells, which might serve as pro-apoptotic signal. Since p35 was continuously expressed in the cells after transfection (unlike FLAG-Dof, the expression of which had to be induced), more p35 might have provided higher probability for survival resulting more living cells which expressed Dof. Nevertheless, we cannot exclude the possibility that p35 affects Dof expression levels in a dose dependent manner.

The experiments above serve with strong evidence to conclude that cleavage of the Dof protein is mediated directly or indirectly by caspases in *Drosophila* S2 cells.

To reveal which caspase might be responsible for the cleavage of Dof the expression level of individual caspases was knocked down by RNA interference in S2 cells. *Drosophila* has seven different caspases three of them - Dcp-1, Drice and Dronc - are definitely expressed in S2 cells (P. Meier personal communication). We targeted these three caspases by RNAi and monitored the cleavage pattern of FLAG-Dof in whole cell lysates (Figure 32B). Decreased Dcp-1 levels in the cells resulted in a partial decrease of the N-terminal cleavage product of Dof whilst abolishing Drice had no effect on Dof cleavage. Depletion of Dronc eliminated Dof cleavage completely. Efficiency of the RNA interference was tested on overexpressed epitope-tagged forms of the caspases in S2 cells (Figure 32C). Dcp-1 and Drice proteins were eliminated by RNAi to

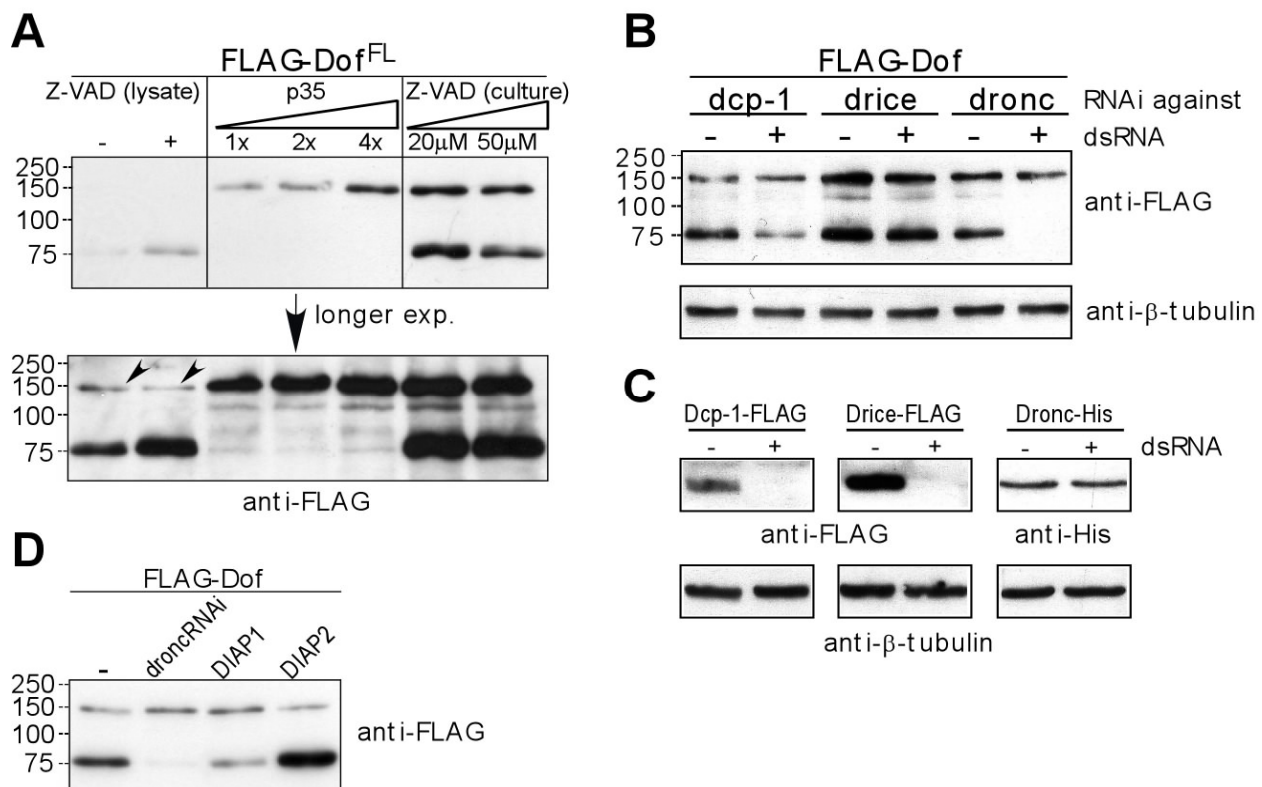


Figure 32. Effect of caspase inhibition on the cleavage of Dof in S2 cells. (A) Western blot of whole cell lysates from S2 cells expressing N-terminally FLAG-tagged Dof (FLAG-Dof) (lanes 1, 2, 6 and 7) or co-expressing FLAG-Dof and different amounts of p35 (lanes 3, 4 and 5). The relative amount of p35 plasmid DNA to that of the FLAG-Dof plasmid DNA used for transient transfection is marked above each lane. In lysates of lane 1 and 2 cells derived from the same transfection, were split during harvest and one half was lysed in a lysis buffer supplemented in addition with 20 μM Z-VAD. Cells that gave the lysates of lane 6 and 7 were incubated with the marked concentration of Z-VAD for 2 hours prior lysis. Full length Dof and the presence of N-terminal fragments were detected using antibody against the FLAG tag. Lower panel shows the same blot after longer exposure. Arrowheads denote the protein bands of full length Dof in lane 1 and 2 not detectable at shorter exposure. (B) Dcp-1, drice and dronc RNAi on S2 cells expressing FLAG-Dof. “+” marks addition of the corresponding dsRNA, lanes with “-” are mock treated. Western blots of whole cell lysates from these cells were probed with antibody against FLAG to detect full length Dof and the presence of the N-terminal Dof fragment. To control the amount of the lysates loaded in each lane Western blots were probed with antibody against β-tubulin. (C) Western blot of whole cell lysates from S2 cells expressing FLAG-tagged Dcp-1, FLAG-tagged Drice or His-tagged Dronc. Prior the transfection of the dcp-1, drice or dronc constructs half of the cells were incubated with dcp-1, drice or dronc dsRNA respectively. The presence of tagged Dcp-1, Drice or Dronc proteins was monitored using antibodies against FLAG or His epitope tags. To control the amount of the lysates loaded in each lane Western blots were probed with antibody against β-tubulin. (D) Western blot of whole cell lysates from S2 cells expressing FLAG-Dof alone following mock treatment (marked with “-”) or dronc RNAi, or co-expressing FLAG-Dof with HA epitope-tagged DIAP1 or DIAP2. Full length Dof and the presence of N-terminal Dof fragments were detected using antibody against the FLAG tag. dronc RNAi on S2 cells expressing FLAG-Dof was a separate experiment from the one shown in panel B.

undetectable levels. The inefficient knock down of the overexpressed Dronc protein might be due to the fact that this construct was under the control of an inducible promoter that might have overwhelmed the RNAi machinery. The strong effect of Dronc depletion on Dof cleavage indicates that endogenous levels of Dronc might be efficiently eliminated by RNAi. Thus, Dof cleavage in S2 cells is absolutely dependent on Dronc activity and Dcp-1 is also required for efficient cleavage.

Dronc is considered to be the major initiator caspase in *Drosophila* and is the only known caspase in flies which is insensitive to the inhibitory effect of p35 (Jabbour et al., 2002; Meier et al., 2000). Hence, these data indicate that Dronc regulates the cleavage of Dof in S2 cells not by

directly acting on it but by regulating the activity of other caspases, probably of Dcp-1, which than process Dof.

Finally, we aimed to block caspase activity by the overexpression of IAP (inhibitor of apoptosis) proteins, factors that bind to and inhibit caspases (Salvesen and Abrams, 2004). DIAP1 and DIAP2, the two known IAPs in *Drosophila* were co-expressed with FLAG-Dof in S2 cells. The overexpression of DIAP1 caused a significant decrease of the 75 kDa N-terminal cleavage product of Dof, albeit not as strong as that upon dronc RNAi (Figure 32D). DIAP2 had no effect on Dof cleavage. This is consistent with the finding that DIAP1 regulates Dronc activity (Meier et al., 2000).

In summary, we showed by four independent approaches that Dof cleavage is caspase mediated in S2 cells. We found that the initiator caspase Dronc plays an essential role in this process in S2 cells.

2.5.3 Cleavage of Dof requires an intact caspase cleavage site in the molecule

To see if the predicted caspase cleavage site DEVD at amino acid positions 584-587 in Dof is utilized for cleavage the consensus site was destroyed by mutating the aspartate to a glutamate at position 587 in the molecule. This Asp at the P1 position in the caspase cleavage site is essential in substrate recognition and cleavage for the most known caspases (Hawkins et al., 2000). To test the effect of this point mutation on Dof cleavage wild type Dof bearing a FLAG or an HA epitope tag on its N-terminus and caspase site mutant Dof^{D587E} with an N-terminal FLAG epitope tag were expressed alone or in different combinations in *Drosophila* S2 cells (Figure 33). Both, wild type FLAG-Dof and wild type HA-Dof were cleaved in the cells giving rise to the characteristic 75 kDa N-terminal Dof cleavage product. Co-expression of the two differently tagged wild type Dof proteins did not alter the cleavage pattern. On the other hand FLAG-Dof^{D587E} was not cleaved in the cells; furthermore, it had a strong inhibitory effect on the cleavage of wild type HA-Dof if the two proteins were co-expressed. This result shows that the caspase cleavage site DEVD in Dof is essential for the cleavage of the molecule. The molecular weights of the cleavage products would be also consistent with a cleavage at this position. Thus, we propose that the sequence motif DEVD at amino acid position 584-587 is a functional caspase cleavage site in Dof.

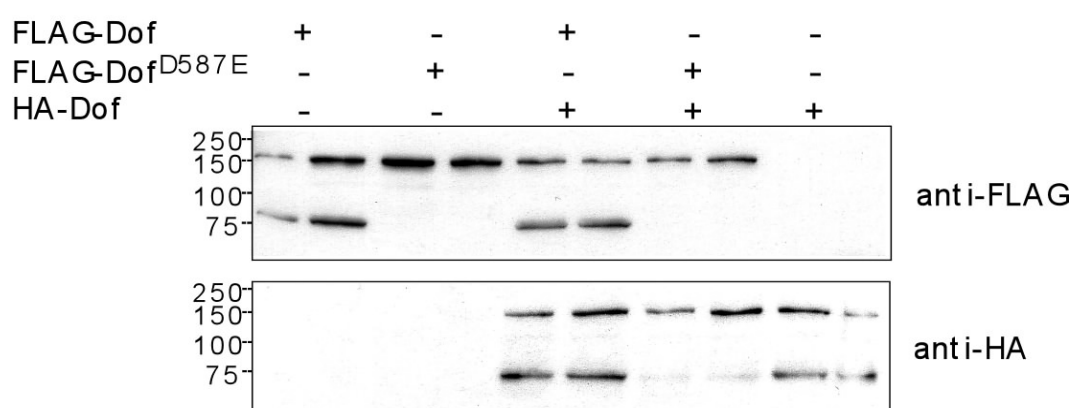


Figure 33. Effect of a point mutation in the potential caspase cleavage site in Dof on the cleavage of the protein. Western blot analysis of whole cell lysates from S2 cells expressing, as indicated above each lane, different combinations of N-terminally FLAG- or HA-tagged wild type Dof and FLAG-tagged Dof with the point mutation D to E at amino acid position 587. Each lysate is present in duplicate on the blot. Full length Dof and the presence of N-terminal Dof fragments were detected using antibody against the FLAG and HA tags.

2.5.4 Searching for the molecular role of caspase cleavage in Dof function

What might be the physiological relevance of the caspase-dependent cleavage of Dof during development? Dof might be cleaved off to downregulate FGF signalling in the cells and so acting as a survival signal to maintain signalling capacity of the cells which often antagonises with apoptosis induction. However, deletion analysis of Dof showed that the first 522 amino acids of the molecule have high biological activity, indicating that cleavage at position 587 might not be sufficient to eliminate signalling activity of Dof (Wilson et al., 2004). Thus, we analysed possibilities where Dof cleavage might have an active role in the function of the molecule.

So far we had no direct evidence if full length unprocessed Dof has the capacity to interact with the FGF receptor. Dof is able to form a complex with Heartless in S2 cells. Both, full length Dof protein and the N-terminal cleavage product of Dof co-immunoprecipitate with the FGF receptor. The first 445 amino acids of Dof are sufficient to interact with the FGF receptor Heartless (see Chapter 2.2) as well as to dimerise with the full length Dof protein (Battersby et al., 2003). Thus, these data do not distinguish if full length Dof was present in a Dof-Htl complex due to a direct interaction with the receptor or only due to Dof self-association.

To see if the presence of the N-terminal cleavage product is required for efficient receptor binding of Dof, caspase cleavage site mutant FLAG-Dof^{D587E} was co-expressed with constitutively active Heartless in S2 cells and interaction of the two proteins was analysed by immunoprecipitation (Figure 34). FLAG-Dof^{D587E} showed as efficient interaction with Heartless as wild type FLAG-Dof and both proteins were phosphorylated on tyrosine residues at comparable levels, indicating that caspase cleavage of Dof is neither required for interaction with the FGF receptor nor for efficient phosphorylation of the molecule.

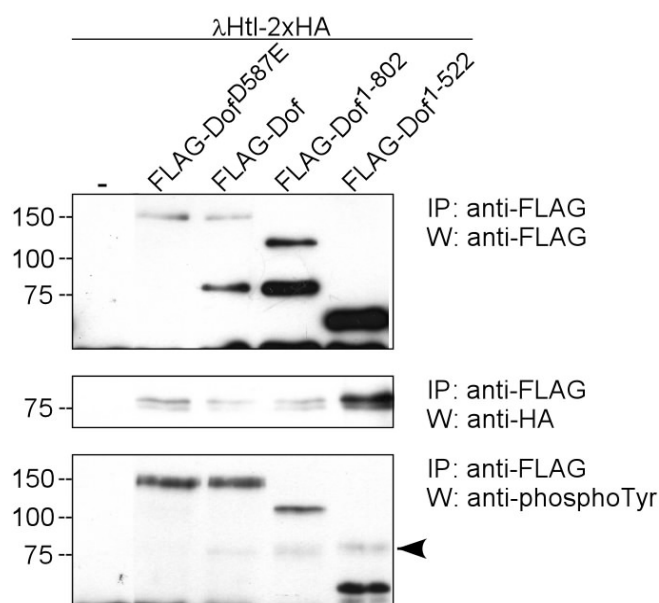


Figure 34. The effect of the point mutation D587E on the interaction of Dof with an activated FGF receptor. Western blots of immunoprecipitations (IP) from S2 cells expressing a constitutively active form of HA epitope-tagged Heartless (λ Htl-2xHA) and mutant Dof constructs bearing a FLAG epitope tag fused to their N-terminus. Protein complexes were immunoprecipitated from the lysates using antibody against FLAG, and the precipitated proteins were detected on Western blots using anti-FLAG anti-phospho-tyrosine or anti-HA antibodies. Arrowhead marks the the tyrosine phosphorylated band corresponding to the constitutively active form of Heartless.

To see if cleavage influences tyrosine phosphorylation of Dof, phosphorylation state of full length Dof and both, N- and C-terminal cleavage products were analysed. From earlier experiments we learned that the N-terminal cleavage product of Dof was less strongly phosphorylated than the

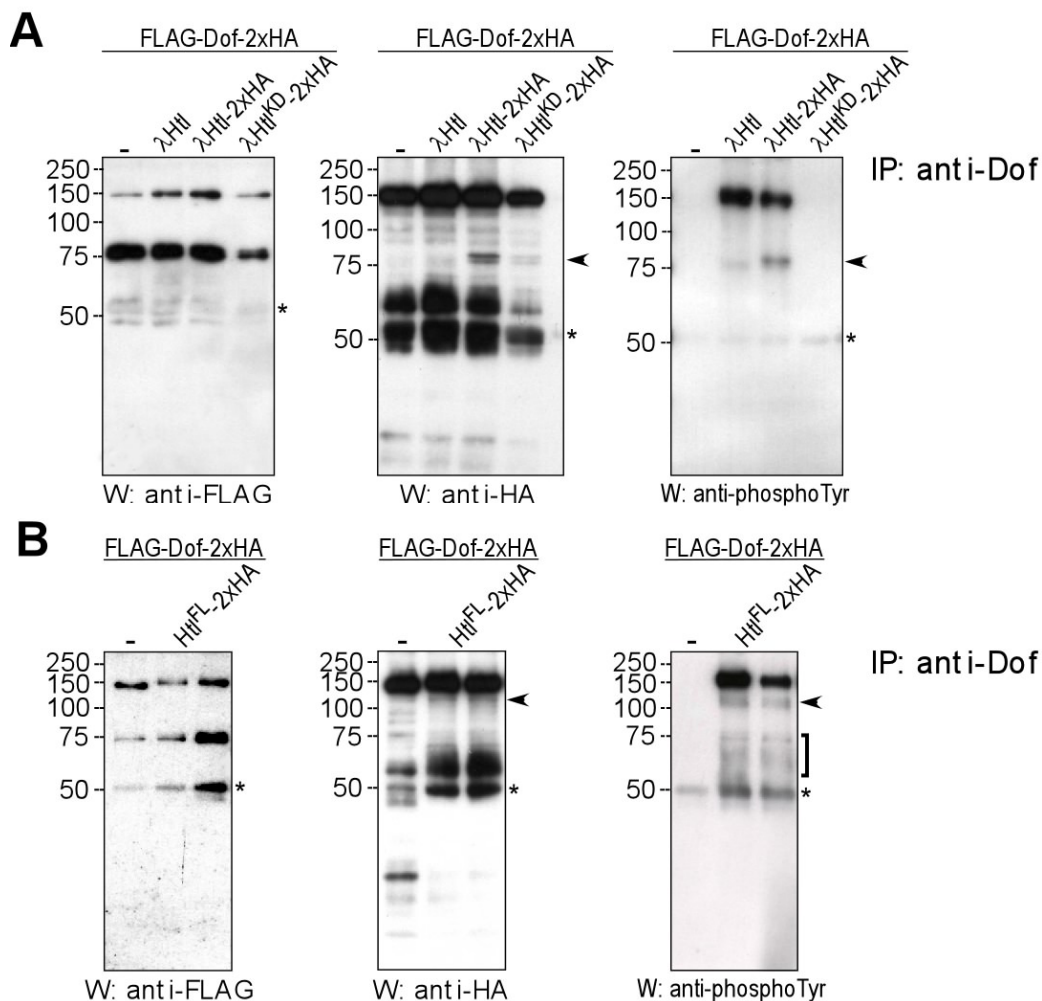


Figure 35. Analysis of the phosphorylation state of Dof cleavage products in the presence of an activated FGF receptor in S2 cells. Western blots of immunoprecipitations (IP) from lysates of S2 cells (A) expressing Dof bearing a FLAG tag on its N- and an HA tag on its C-terminus alone or in the presence of a constitutively active form of Heartless with or without an HA epitope tag (λ Htl-2xHA and λ Htl respectively) or a kinase dead version of the constitutively active form of Heartless (λ Htl^{KD}-2xHA); or (B) expressing Dof bearing a FLAG tag on its N-terminus and an HA tag on its C-terminus alone or in the presence of HA-tagged wild type Heartless (Htl^{FL}-2xHA). Protein complexes were immunoprecipitated from the lysates using antibody against Dof, and the precipitated proteins were detected on the Western blots using anti-FLAG, anti-HA or anti-phosphotyrosine antibodies. Asterisk mark the heavy chains of the Dof antibody used for IP. Arrowheads mark the position of the different Heartless proteins on the blots probed with antibody against HA or phospho-tyrosine residues. Note the different size of the protein bands corresponding to the constitutively active form of Heartless or to wild type Heartless. In panel B black line marks weak tyrosine phosphorylated protein bands corresponding the size of N- and C-terminal Dof fragments.

full length form (see also Figure 14 in Chapter 2.2). We also found that sites of tyrosine phosphorylation are more abundant C-terminal to the caspase cleavage site; which might explain this difference in the phosphorylation level. Alternatively, full length Dof might be a better substrate for phosphorylation. To test this, we took advantage of the Dof construct bearing different epitope tags fused the two ends of the molecule (FLAG-Dof-2xHA) and co-expressed it with activated Heartless in S2 cells. A polyclonal antibody directed against Dof efficiently precipitated N- and C-terminal fragments of Dof as well as the full length protein from cell lysates (Figure 35A). Full length Dof was efficiently tyrosine phosphorylated but there was no sign of phosphorylated cleavage products in the same immunoprecipitate. The protein band at about 80 kDa detected via the antibody directed against phospho-tyrosine corresponds to the activated FGF receptor that co-precipitated with Dof. To eliminate eventual masking of protein bands of the phosphorylated

cleavage products by overlapping receptor bands the experiment was repeated with wild type Heartless protein, which appears at higher molecular weight on protein gels (Figure 35B). Wild type Heartless was auto-activated under these expression conditions in S2 cells. It was as effective as constitutively active Heartless to interact with Dof, and full length Dof was also efficiently phosphorylated (see also Figure 14 in Chapter 2.2). However, consistent with the results above, we detected only very weak phosphorylated protein bands with corresponding sizes to the N- and C-terminal cleavage products. This shows that the weak phosphorylation level of the N-terminal cleavage product is not a consequence of the split-off of the phosphorylated tyrosine residues of full length Dof among the cleavage products. This rather indicates that caspase cleavage products of Dof might be preferentially in a non-phosphorylated state in S2 cells. In summary, this is the first indication for the existence of a regulatory crosstalk between caspase cleavage and phosphorylation of Dof in S2 cells. In future experiments, the use of phosphatase inhibitors in living cells might help to distinguish whether Dof is protected from cleavage in its tyrosine phosphorylated state or caspase cleavage products of Dof are immediate substrates of phosphatases.

2.5.5 Analysis of the *in vivo* function of Dof cleavage and caspase activity in FGF signalling

We do not have data whether Dof is cleaved *in vivo*. Thus, we tested genetically if the mutation of the caspase cleavage site influences *in vivo* function of Dof. Transgenic flies were generated bearing full length Dof with the same point mutation in the caspase cleavage site as described above. Cell culture experiments indicated that caspase cleavage site mutant Dof inhibits the cleavage of wild type Dof. To analyse this potential dominant effect *in vivo*, the mutant Dof^{D587E} transgene was overexpressed in the mesoderm and in the tracheae of wild type embryos. The formation of Eve-positive cells in the mesoderm and the development of the tracheal network were assayed. No alterations were observed in these developmental processes upon overexpression of the transgene (data not shown). Next, we tested the capability of the mutant molecule to rescue *dof* loss of function phenotypes in the same tissues. The caspase cleavage site mutant Dof transgene was as efficient as a wild type Dof construct to reconstitute Eve-positive cell clusters in the mesoderm or a fully developed tracheal network (data not shown), indicating that caspase cleavage might not be essential for Dof function *in vivo*. Since the correlation of the expression levels of transgenic Dof constructs to endogenous Dof protein levels is not known, dose dependent effects of caspase cleavage cannot be excluded in Dof function. Similarly cannot be excluded that caspase cleavage of Dof affects other Dof-dependent morphogenetic processes which were not assayed here.

An alternative way to test if caspase cleavage of Dof has physiological relevance is to analyse the requirement of caspase activity in developmental processes in which Dof is involved. Cleavage of Dof was sensitive to the caspase inhibitor p35 in S2 cells. Therefore we tested the effect of p35 on tracheal development by ectopically expressing UAS-p35 transgene with the tissue specific *btlGal4* driver. p35 had no apparent effect on the development of the tracheal network in embryos (data not shown). This indicates that caspase activity might not be essential for tracheal development in embryos. Alternatively, unlike in S2 cells, Dof cleavage might be under the direct control of the p35-insensitive caspase Dronc. It also cannot be excluded that the level and/or the timing of p35 expression might not be sufficient to inhibit caspase activity during the time window of embryonic development of this tissue.

Loss of function analysis of caspase genes might discriminate these possibilities. There are seven different caspases in *Drosophila* each encoded by a single gene but the only caspase with recorded mutant alleles is *dcp-1*. A lethal P insertion *l(2)k05606* located in the *dcp-1* gene was originally described as a *dcp-1* allele causing “fragile tracheae” phenotype among many other defects in homozygous mutant larvae (Song et al., 1997). Genetic analysis of the locus revealed that the P

insertion affects the function of the *pita* gene and not of *dcp-1*, which is inserted in the first intron of *pita*, since independent mutant alleles of *pita* caused similar phenotypes as the P element insertion ((Laundrie et al., 2003) and personal communication to K. McCall). Imprecise excision of this P element generated null alleles of *dcp-1* (Laundrie et al., 2003). These flies are homozygous viable and can be maintained as a stable stock indicating that *dcp-1* function is not essential in *Drosophila*. As expected these flies showed normal tracheal development during embryogenesis and a sensitised, heterozygous *dof* mutant genetic background did not alter this phenotype (data not shown).

Loss of function phenotype of *dronc* was analysed using the deficiency Df(3L)AC1. This chromosomal deletion removes a genomic region between the cytological breakpoints 67A2 and 67D11-13, which includes the *dronc* locus. Based on available complementation analysis and the genomic sequence the deleted region spans 133 annotated genes having *Rdl* as the first distal and *can* as the first proximal gene that are definitely not removed (www.flybase.org). Homozygous Df(3L)AC1 embryos showed significant defects in tracheal development (Figure 36). Dorsal trunks failed to form and visceral branches did not develop properly. Interestingly, dorsal branches appeared always normal and also the anterior and posterior lateral branches were only weakly affected with some fusion problems. The severity of the phenotype varied but at least half of the dorsal trunks failed to fuse or form and most of the visceral branches were misguided or not formed in the embryos. This phenotype indicates that the general motility of tracheal cells might be maintained but regional differentiation or migratory guiding information might be affected. No other overlapping deficiencies exist in this region which could narrow down the number of potential genes causing this phenotype, and genes of this region have not been directly shown to be involved in tracheal development. The genes *Gap1* and *shc* code for signalling molecules and are also located in the deleted region. *Gap1* is GTPase activating protein specific for Ras. *Shc* is an adaptor protein utilised by several RTKs. *Shc* was reported to be involved in *Drosophila* EGF signalling (Lai et al., 1995; Luschnig et al., 2000). EGF plays a role in the regional differentiation of tracheal cells and mutations in components of the cascade like *rhomboid*, *DER* and *spitz* cause specific loss of dorsal trunks and visceral branches during tracheal development, reminiscent to the phenotype of Df(3L)AC1 mutant embryos.

Thus, this result shows that the deficiency Df(3L)AC1 removes a gene which is essential for tracheal development but the identity of this gene is not known. *Shc* is a potential candidate for this function but further analysis of mutant *shc* alleles is required to clarify this as well as to reveal if loss of *dronc* function contributes to the phenotype.

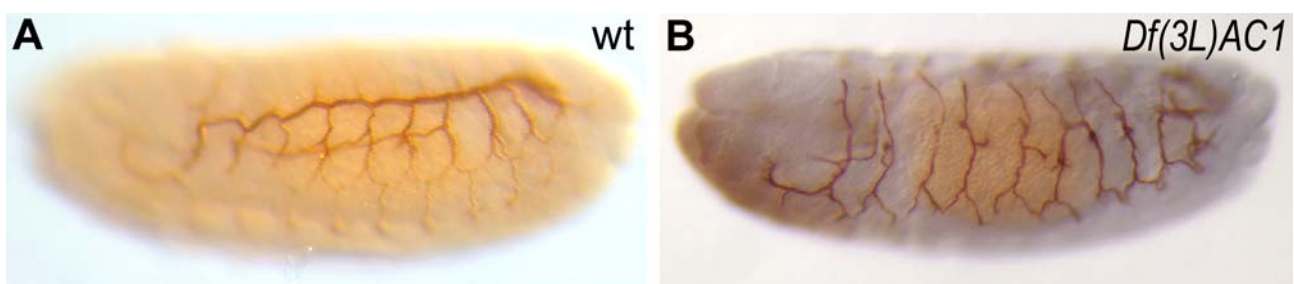


Figure 36. Analysis of tracheal development in embryos homozygous for the deficiency Df(3L)AC1. Stage 15 (A) wild type embryo or (B) an embryo homozygous for the deficiency Df(3L)AC1 stained with 2A12 antibody to visualise the lumen of the tracheal network. Note the gaps in the dorsal trunk and the missing or stalled visceral branches.

As an alternative approach to eliminate *Dronc* activity in the tracheae mutant forms of the molecule with dominant negative effects were expressed using *btlGal4*. Overexpression of the caspase dead version of full length *Dronc* or an N-terminal fragment bearing only the CARD domain of the molecule has been shown to inhibit *Dronc* activity efficiently in the developing *Drosophila* eye (Meier et al., 2000). Embryos expressing these constructs in their tracheae showed

no significant differences compared to wild type (data not shown). However, 2-3 % of the embryos showed aberrations in tracheal branching. Similarly, if constitutively active forms of Dronc were overexpressed in the tracheae branching defects were rarely observed but few embryos showed abnormalities in their tracheae (data not shown). Thus, it suggests that if Dronc has any role in tracheal development its activity might require a tight balancing. However, no significant ratio of affected embryos was detected, indicating that overexpression of different mutant Dronc proteins in the tracheae did not interfere with the normal embryonic developmental program of this tissue. It cannot be excluded that the concentration and timing of the expressed transgenes was not sufficient to influence endogenous processes.

In summary, the genetic analysis for the involvement of Dronc in tracheal development was not completed. Future work on loss of function analysis of the gene and *in situ* detection of Dronc activity by specific antibodies directed against activated forms of Dronc as well as genetic studies on components of the caspase activating cascade might help to reveal if Dronc is involved in the regulation of FGF signalling.

2.6 Indications for the translational control of Dof protein levels in S2 cells

In vivo analysis of the caspase cleavage site mutant form of Dof did not support a physiological role of caspase cleavage in the function of the molecule. One explanation might be that dose dependent effects of Dof processing are masked due to high levels of transgenic expressions. This would then indicate that regulation of Dof protein levels might be an important issue in development. This possibility seemed to be interesting to follow up from two reasons. First, significant difference was always observed in the expression levels of different Dof constructs transiently transfected in S2 cells. Secondly, a ribosomal protein, rpS10 was found to be a yeast two hybrid interactor of Dof (Battersby et al., 2003) and this interaction was confirmed in co-immunoprecipitation assay (see Figure 13C in Chapter 2.1). Two hints that Dof protein expression might be controlled in S2 cells and translation might be involved in this control.

The construct Dof[1-522] showed much higher protein levels in the cells than all the other Dof constructs (Figure 37). Thus, the expression level of the construct Dof[1-446] which had a further truncation of 76 amino acids was similar to the ones that contained the C-terminal part. This indicated that both the lack of the C-terminal part of Dof as well as the presence of the particular 76 amino acids might be required for higher protein levels. This difference was specific for Dof protein, since co-transfected constitutively active Heartless was expressed at comparable levels in all transfections.

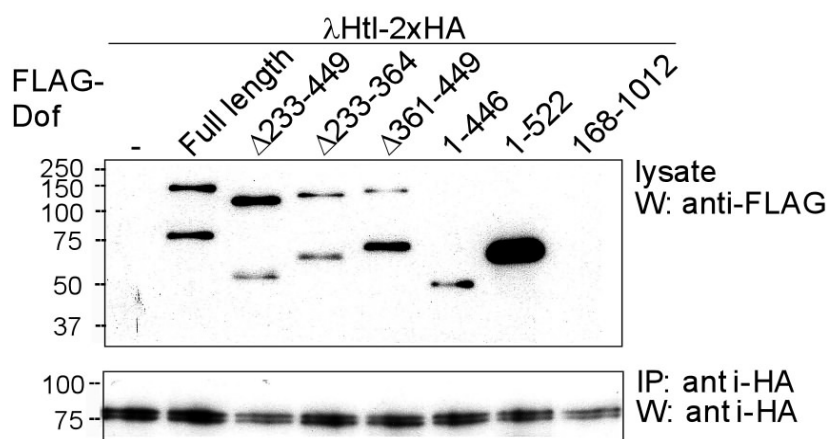


Figure 37. Expression level of different Dof constructs in S2 cells. Western blots of whole cell lysates and immunoprecipitation (IP) from S2 cells expressing mutant versions of Dof bearing N-terminal FLAG epitope tags and a constitutively active form of HA epitope-tagged Heartless (λ Htl-2xHA). This figure is an outlet of the experiment presented in Figure 16 in Chapter 2.2. For details refer to that.

The first question was from where this elevated protein level derives. Each of these Dof constructs was cloned into the same expression vector and transfected into S2 cells using equal amounts of DNAs. Protein expression was under the control of the copper inducible metallothionein promoter and induction was performed simultaneously in all cells. These argue that differences in transcription levels of the different Dof constructs should not be expected. To test this hypothesis directly dof mRNA levels of S2 cells transiently transfected with different Dof constructs were compared using quantitative real-time RT-PCR on total RNAs isolated from these cells. mRNA levels of FLAG-Dof[1-522], FLAG-Dof[1-446] and as controls of FLAG-Dof and FLAG-Dof^{D587E} were calculated (for details see Materials and Methods). Figure 38A shows the fold mRNA expression of these constructs relative to that of the wild type FLAG-Dof. FLAG-Dof[1-522] showed a slight increase in transcription levels compared to wild type Dof (ca. 1.3 fold) and FLAG-

Dof[1-446] mRNA levels were comparable to these values, albeit with high deviation depending on the approach reverse transcription was performed. FLAG-Dof^{D587E} produced lower amounts of mRNA (ca. 0.4-0.5 fold). Thus, the C-terminally truncated Dof constructs did not show significant difference in mRNA levels compared to each other or to the wild type Dof construct indicating that they are transcribed at comparable levels in S2 cells. The lower transcription levels of FLAG-Dof^{D587E} are not understood. It has to be remarked that FLAG-Dof^{D587E} was the only construct with a different polyadenylation signal (see Materials and Methods).

Despite of the similar mRNA levels the protein levels of the different Dof constructs showed significant differences on Western blot analysis of the cell lysates of the same transfections which were used for RNA isolation (Figure 38B). Equal amounts of cell lysates contained about ten times more FLAG-Dof[1-522] than FLAG-Dof[1-446] protein estimated from dilution series of the two proteins on Western blots (data not shown). Protein levels of the two full length constructs were comparable to that of FLAG-Dof[1-446], although in case of the wild type construct it was hard to estimate due to its cleavage in the cells.

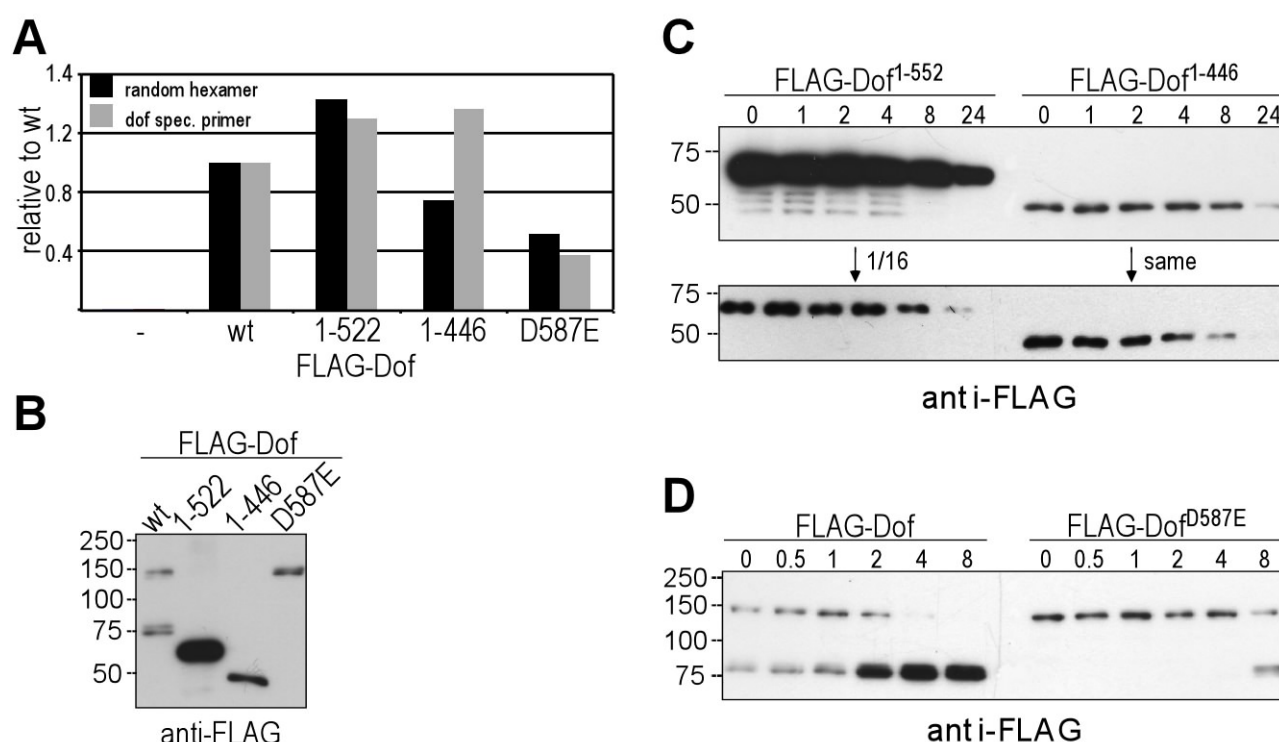


Figure 38. Analysis of the difference in expression levels of mutant Dof proteins in S2 cells. (A) Quantitative analysis of the amount of mutant Dof mRNAs transiently expressed in S2 cells by real-time RT-PCR. Bars on the diagram show the fold mRNA expression of the mutant Dof constructs marked below each bar relative to wild type Dof mRNA levels. Black and grey bars denote two experimental results on the same isolated RNAs using random hexamers or dof specific primers for reverse transcription respectively. (B) Western blot of whole cell lysates from S2 cells expressing mutant versions of Dof bearing N-terminal FLAG epitope tags, and from which RNA was isolated for real-time RT-PCR. Expression level of mutant Dof proteins was detected using antibody directed against the FLAG tag. (C-D) Western blot analysis of whole cell lysates from S2 cells expressing N-terminally FLAG-tagged wild type and mutant Dof proteins. Cells were lysed after different hours as labelled above each lane following the addition of cycloheximide. The amount of Dof proteins was monitored by probing the blots with antibody against the FLAG tag. (C) Note, the expression level of FLAG-Dof[1-522] at the time point cycloheximide was added (lane "0") was about sixteen times higher than that of FLAG-Dof[1-446]. (D) Note that whole cell lysate from S2 cells expressing FLAG-Dof^{D587E} also contained N-terminal fragments of Dof after eight hours of incubation with cycloheximide (lane "8").

In summary, elevated protein levels of the construct containing the first 522 amino acids of Dof was not combined with significantly increased mRNA levels compared to other Dof forms, indicating that the difference might be restricted to protein levels. However, further numbers of independent experiments might be optional to confirm these data statistically.

To test whether the accumulation of higher protein levels of FLAG-Dof[1-522] was due to higher stability of the protein the half life of different mutant Dof proteins was monitored in S2 cells after blocking *de novo* protein synthesis with 20 μ M cycloheximide. Metabolic labelling of the cells with [³⁵S]-methionine confirmed that this concentration of the drug was sufficient to block translational activity of S2 cells completely (data not shown). Both C-terminally truncated Dof proteins showed high stability, FLAG-Dof[1-446] appeared as stable as FLAG-Dof[1-522] (Figure 38C). The stability of full length wild type Dof could not be determined because it was processed in the cells (Figure 38D). However, the simultaneous increase in the level of the N-terminal cleavage product with the disappearance of the intact Dof form indicated that full length Dof was not degraded in the cells but only processed into the cleaved form. This is supported by the observation that the caspase site mutant Dof protein which was not cleaved appeared also stable in the cells. However, at eight hours after the addition of cycloheximide even the caspase site mutant Dof molecule was processed to some extent in the cells. This result suggests that the introduced point mutation at amino acid position 587 in Dof did not completely eliminated being a substrate for caspases. Translational block induces programmed cell death, which activates caspases in the cell. In consistence with this about 40 % of the cells were apoptotic after incubation with cycloheximide for eight hours compared to the 5-8 % at the time point when the drug was added (data not shown). Thus, the cleavage of Dof^{D587E} might be an issue of higher caspase concentrations but it can not be similarly excluded that proteases including caspases cleaved Dof^{D587E} which were not present in S2 cells under the conditions the effect of the point mutation was verified. This phenomenon was not analysed further.

In summary, no significant differences were detected in the stability of the two C-terminally truncated forms of Dof. They both showed also comparable mRNA levels in the cells suggesting that the elevated protein levels of the construct FLAG-Dof[1-522] might derive from an increased level of translation.

How can cause a 76 amino acid long stretch such a high difference in translation levels? Theoretically two mechanisms are possible: this region could play a regulatory role as RNA giving a secondary structure to the mRNA that can be more efficiently transcribed than the mRNA coding for the shorter Dof construct. Alternatively the 76 amino acid region could be responsible for a regulatory protein-protein/-RNA interaction which than through an unknown mechanism could increase the translation rate of the own mRNA. The ribosomal protein S10 (rpS10) belonged to the potential interacting partners of Dof identified in a yeast two hybrid screen and this interaction was confirmed in co-immunoprecipitation experiments with full length Dof in S2 cells (see Figure 13C in Chapter 2.1). Since protein translation is linked to ribosomes, rpS10 seemed to be a good candidate to contribute in the translational regulation of Dof.

The region in the Dof protein responsible for rpS10 binding was mapped by immunoprecipitation using different deletion constructs of Dof. The Dof construct lacking the last 200 amino acids as well as constructs with inner deletions which remove the DBB domain or/and the Ankyrin repeats interacted with rpS10 as efficiently as wild type Dof (Figure 39 and data not shown). The construct FLAG-Dof[1-522] co-immunoprecipitated also efficiently with the ribosomal protein whereas FLAG-Dof[1-446] failed to interact with rpS10 (Figure 39). Thus, the amino acid region 447-522 was identified as a binding site for the ribosomal protein S10 in Dof. Strikingly, this region is responsible for the increased translation level of the construct FLAG-Dof[1-522]. Hence, the capability of FLAG-Dof[1-522] to interact with rpS10 correlated with an

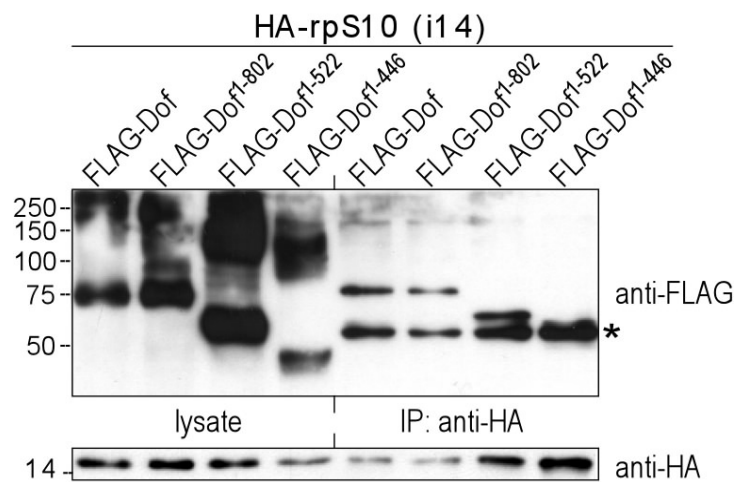


Figure 39. Interaction of the ribosomal protein S10 with mutant forms of Dof. Western blots of whole cell lysates and immunoprecipitations (IP) from S2 cells expressing HA epitope-tagged ribosomal protein S10 (rpS10, identical to the i-clone i14) and mutant versions of Dof bearing N-terminal FLAG epitope tags. Protein complexes were immunoprecipitated from the lysates using antibody against HA, and the precipitated proteins were detected on the Western blots using anti-FLAG or anti-HA antibodies. The asterisk marks the heavy chain of the HA antibody used for IP. Note that protein content of the lysate was improperly denatured before SDS-PAGE (“smear” lanes on the Western blot probed with anti-FLAG antibody).

elevated translation of the molecule, suggesting that rpS10 might contribute to the translational regulation of Dof.

To test if the presence of FLAG-Dof[1-522] in the cells could “inherit” translational upregulation to other dof transcripts it was co-expressed with wild type Dof or full length Dof bearing a point mutation in the caspase cleavage site in S2 cells. FLAG-Dof[1-522] did not influence the expression levels of the two other Dof constructs (data not shown). This indicates that the process utilised by FLAG-Dof[1-522] can not be generalised for Dof transcripts as such. Further analysis is required to see if translational regulation of FLAG-Dof[1-522] is relevant for wild type Dof and if yes what the minimal requirements are for the upregulation of wild type Dof protein levels. This might help to understand the mechanism and the potential physiological relevance of the translational regulation of Dof.

2.7 The role of Ras effectors in FGF signalling in *Drosophila*

FGF signalling provokes complex cellular responses involving cell migration and differentiation. Migration is based on the quick and dynamic remodelling of the cytoskeleton while differentiation is manifested in reprogrammed gene expression. How the different effectors are activated during FGF signalling in flies is not understood. Dof is essential in FGF signalling and has diverse interaction partners (Battersby et al., 2003; Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Thus, Dof might have the capacity to activate different molecules and serve as a branching point in FGF signal transduction. In addition, downstream signalling cascades might diverge at a different point; a possible point is Ras. Constitutively active Ras efficiently overcome defects of *dof* loss of function mutation if specifically overexpressed in the affected tissues, indicating that Ras might play a central role in transmitting the FGF signal towards downstream effectors (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Ras can activate several known effectors in a GTP-dependent manner. One known essential Ras effector pathway of FGF signalling is the activation of the Raf-Mek-MAPK cascade. However, activated Raf has less stronger capacity to rescue *dof* mutant phenotypes than Ras. This raises the question whether Ras effector pathways other than MAPK activation are utilised in FGF signalling. So far the only evidence for the involvement of Ras protein in FGF-dependent developmental processes during *Drosophila* embryogenesis comes from expression studies of constitutively active Ras (Gisselbrecht et al., 1996; Imam et al., 1999; Michelson et al., 1998; Michelson et al., 1998; Reichman-Fried et al., 1994; Vincent et al., 1998). This bypasses the need of activation by endogenous factors. Several Ras effectors can be activated also by the Ras related small GTPase Rap1. In endogenous situations these two GTPases are regulated independently but in overexpression studies they might take over functions of each other. It raises the other question, to what extent Ras is the endogenous transmitter of the FGF signal during *Drosophila* embryogenesis.

At present there are six known GTP-dependent Ras effectors: Raf, the MAPK inhibitors IMP and RIN, PI3K, the Ral specific guanine nucleotide exchange factor RalGDS and AF6/Canoe (see Introduction). Ras proteins containing point mutations in the Ras effector loop were tested for binding affinity towards the three best studied effectors Raf, PI3K and RalGDS in mammalian cell culture systems (Rodriguez-Viciana et al., 1997; White et al., 1995). Amino acid position 35 in Ras proved to be essential for PI3K and RalGDS but not for Raf binding, since mutation of it led to specific Raf interaction but loss of PI3K and RalGDS interaction. Mutation of amino acid 37 generated specific binding for RalGDS whereas mutation at amino acid position 40 resulted in exclusive PI3K binding of Ras. IMP showed binding only with the effector loop mutant Ras1V12,G37 (Matheny et al., 2004). There are no data how these point mutations affect the binding of other effectors to Ras. Mammalian Ras1 constructs bearing the described point mutations in the effector loop in the background of a point mutation at amino acid position 12 (V12), which results in constitutive GTP binding and therefore constitutive activity are available in transgenic flies. Several studies verified the specific and distinguishable activities of these constructs (Bergmann et al., 1998; Halfar et al., 2001; Karim and Rubin, 1998; Mirey et al., 2003; Therrien et al., 1999). In the original mammalian cell culture studies the RasS35 effector loop mutant showed a weakened (ca. 30%) affinity to Raf compared with the wild type effector loop form (Rodriguez-Viciana et al., 1997), whereas in *Drosophila* systems they had comparable activity (Karim and Rubin, 1998).

Ras itself as well as components of the different downstream effector pathways including Raf, Mek, MAPK, PI3K, DRal and the two fly homologues of RalGDS Rgl1 and Rgl2 are maternally deposited in *Drosophila* eggs. Effects of zygotic mutations in their respective genes might show little or no effect because of the high maternal contribution. Germline clone analysis could overcome this problem. However, since these effectors play general roles in many different

signalling events germline clone analysis might be suitable to study early developmental processes but becomes less interpretable in the time course of development with the accumulation of secondary defects. Therefore to obtain insight into their loss of function and gain of function effects in FGF-dependent processes we analysed the consequences of tissue specific expression of dominant negative and constitutively active versions of the different molecules. We also took advantage of the available effector loop mutant Ras alleles. Mesoderm differentiation and tracheal development were chosen as two FGF dependent processes to study the signalling pathway downstream of *dof*.

FGF signalling has a biphasic role during mesoderm formation (Michelson et al., 1998). First, it is essential in the initial contact formation with the ectoderm. This early event is ras independent (Wilson, 2004). However, expression of constitutively active Ras (UAS-RasV12) with the mesoderm specific driver *twiGal4* can improve dorsal migration of mesodermal cells in *dof* mutant embryos to some extent (Michelson et al., 1998; Vincent et al., 1998) indicating that subsequent migration processes might use Ras-dependent molecular mechanisms. Nevertheless some cells of the collapsed mesodermal tube can eventually reach the competence zone for dorsal mesoderm differentiation even without active migration (Schumacher et al., 2004; Smallhorn et al., 2004). Subsequently, FGF signalling is involved in the cell fate specification of a subset of dorsal mesodermal cells which is manifested in the specific expression of Even-skipped (Michelson et al., 1998). The formation of pericardial progenitors depends on FGF signalling-induced MAPK activation, whereas DA1 muscle progenitors get a dual input from FGF and EGF signalling to the common Ras-MAPK cascade (reviewed in (Michelson et al., 1998)). Progenitors are singled out from the initial competence cluster of equivalent cells by Notch mediated lateral inhibition that antagonises with the Ras/MAPK cascade (Carmena et al., 2002).

2.7.1 The capacity of effector loop mutant Ras transgenes to rescue *dof* mutant phenotypes

Rescue of mesoderm development

Expression of RasV12 in the mesoderm of *dof* mutant embryos rescues the formation of Eve-positive clusters in the mesoderm and Even-skipped is expressed within the whole competence cluster - similarly as in wild type embryos expressing RasV12 ((Michelson et al., 1998) and Figure 40C). This shows that constitutive high levels of Ras activity in the cluster overcome the antagonising effects of Notch signalling in all cells and thus, progenitors fail to single out from the cluster. Furthermore, it shows that wild type cells and *dof* mutant mesodermal cells in the “dorsal fate” competence zone behaved similarly upon overexpression of constitutively active Ras. *dof* mutant embryos expressing RasV12 showed a nearly wild type number and an overall coordinated appearance of Eve-clusters. This effect could be due to the simultaneous rescue of migration and “dorsal” FGF signalling. However we do not have direct evidence that *dof* mutant mesodermal cells were not able to contact ectodermal regions with Dpp levels sufficient for dorsal fate induction in a regular manner.

The constitutively active effector loop mutant Ras constructs, RasV12,S35 and RasV12,G37 both showed a strong rescue of Eve-positive cells in *dof* mutant embryos (Figure 40D,E). RasV12,G37 was comparable in its rescue capacity with RasV12 in reconstituting the number of hemisegments with Eve-positive cells (summarised in Figure 44). On the other hand, the size of rescued Eve-positive clusters appeared smaller with the effector loop mutant Ras constructs than with RasV12. Elevated expression levels of these alleles through the combination of a maternal Gal4 driver with *twiGal4* increased the size of Eve-positive clusters comparable to the size observed with RasV12

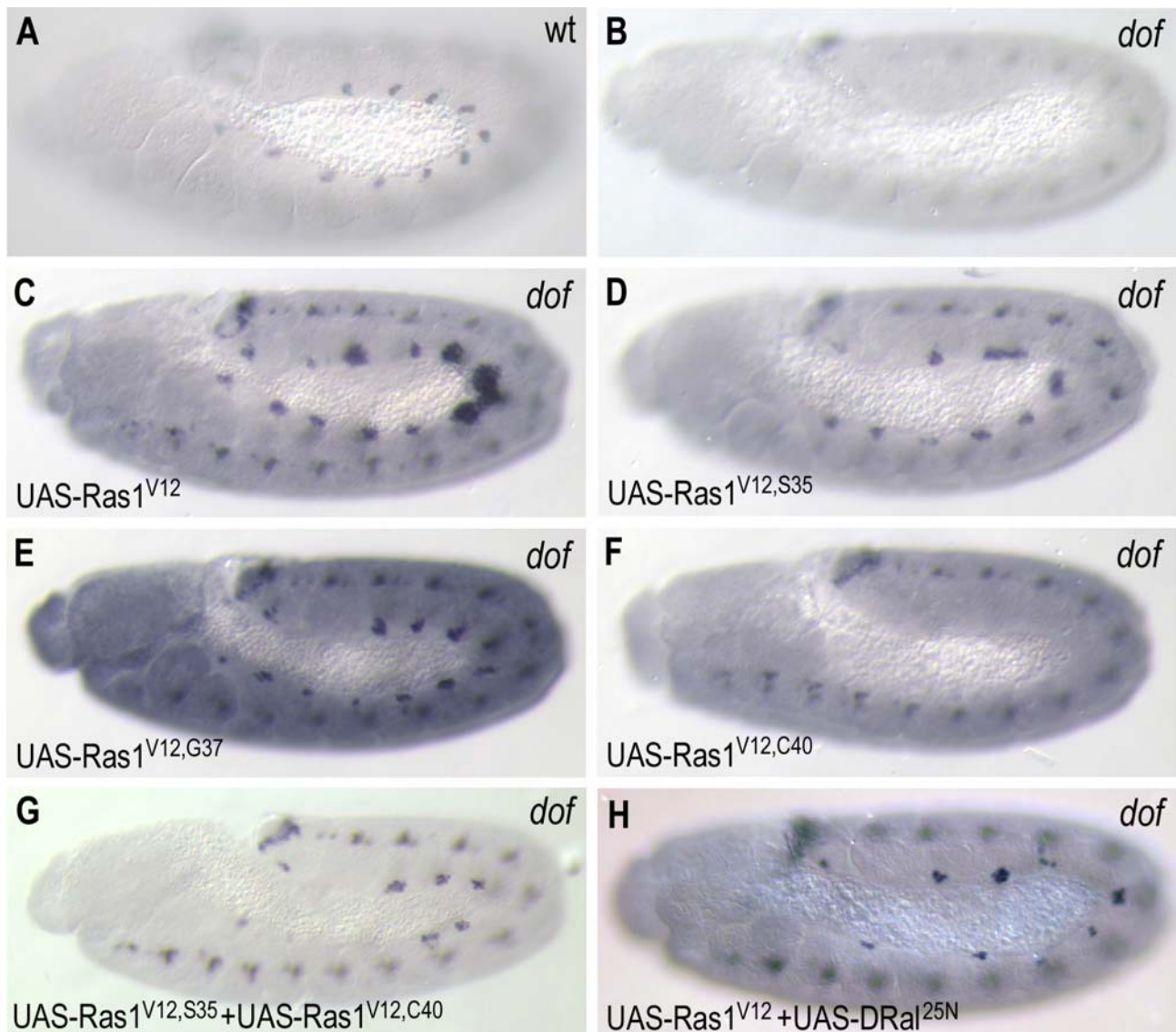


Figure 40. Capability of different mutant Ras proteins to rescue *dof* mutant phenotype in the mesoderm. Stage 11 embryos expressing different mutant UAS-Ras constructs under the control of the *twistGal4* driver are stained with Even-skipped antibody to visualise Eve-positive pericardial progenitor cells. (A) wildtype embryo, (B-H) *dof* mutant embryos (B) without or (C-H) with ectopic expression of (C) Ras1^{V12}, (D) Ras1^{V12,S35}, (E) Ras1^{V12,G37} and (F) Ras1^{C40} and with combined expression of (G) Ras1^{S35} and Ras1^{C40} or (H) Ras1^{V12} and DRal^{25N}. Note the variation in the size as well as in the number of Eve-positive clusters between the different genotypes. Anterior to the left.

(data not shown), indicating a dose dependent behaviour. This could be due to a lower activity of these alleles compared to RasV12 or due to different expression levels of the transgenes. In consistence with their similar rescue capacity, both mutant Ras molecules induced comparable levels of activated MAPK in the Eve-positive clusters (data not shown). RasV12,G37 was reported to have some residual Raf interaction capacity (ca. 10% of RasV12,S35) (Rodriguez-Viciano et al., 1997). An interesting question is whether this residual Raf binding capacity of RasV12,G37 is sufficient to activate MAPK and rescue Eve-positive cells as efficiently as RasV12,S35, which has about ten times higher affinity for Raf. It points out the possibility that these point mutations differentially affect a Ras induced, Raf independent regulation of MAPK activity. It would be consistent with the fact that the Ras effector IMP which regulates MAPK activity at the level of Mek activation can specifically interact with RasV12,G37 but not with RasV12,S35 (Matheny et al., 2004).

The residual Raf binding capacity of RasV12,G37 did not allow to discriminate whether the RalGDS binding capacity of RasV12,G37 had an input on the rescue of Eve-positive clusters. To test the contribution of Ral GTPase in Ras dependent effects on mesoderm development constitutively active Ras was expressed in combination with the dominant negative form of *Drosophila* Ral (DRal25N) under the control of *twiGal4* in *dof* mutant embryos. This simultaneous expression caused a less reliable rescue with high variability in the number, size and position of Eve-positive cells compared to the one of RasV12 alone (Figure 40H, and summarised in Figure 44). Interestingly, expression of DRal25N via *twiGal4* in wild type embryos had no effect on the formation of Eve-clusters (data not shown, summarised in Table 4). Dominant negative constructs need a certain latency period to accumulate high concentrations to be able to interfere with endogenous components. In the co-expression situation constitutively active Ras accumulated with the same timing as the antagonising DRal25N in the cells. It might be that in the wild type situation activated Ras starts to accumulate prior the expression of DRal25N and the sensitive period is already passed by the time of high dominant negative DRal concentrations. A relevant example is the *twiGal4* driven expression of the dominant negative form of Heartless, the mesoderm specific FGFR in *Drosophila* (DNHtl). It does not affect mesoderm migration, cells are able to reach dorsal most positions in the presence of DNHtl, but it affects Eve-positive cluster formation, indicating that sufficient inhibitory levels of the dominant negative molecule accumulated only by the second phase of FGF signalling (Michelson et al., 1998). This suggests that DRal might be involved in the first, migratory phase of mesoderm development.

A clearly distinct phenotype was observed by the expression of RasV12,C40 in *dof* mutant embryos, which failed to rescue Eve-positive cells in the mesoderm (Figure 40F, summarised in Figure 44). This effector loop mutant represents a “PI3K specific Ras” molecule with no detectable affinity towards Raf (Rodriguez-Viciano et al., 1997). Consistent with this no MAPK activation was observed in the mesoderm (data not shown). This is in accordance with the fact that MAPK activity is required to induce Eve expression (Carmena et al., 1998). To distinguish the possibilities of PI3K activity not being required or being involved but alone not being sufficient for mesoderm development, RasV12,C40 was expressed together with RasV12,S35 in *dof* mutant embryos. In the first case no improvement in Eve-cluster formation was expected whereas the second case would have a synergistic effect on the rescue of Eve-positive cells. The simultaneous expression of the two mutant Ras constructs led to a reduction in the number of Eve-positive clusters compared to the experiment where RasV12,S35 was expressed alone (Figure 40G, summarised in Figure 44), indicating that PI3K activity might be not required for the formation of Eve-clusters. Furthermore RasV12,C40 seems to have a dominant negative effect perhaps through its inability to bind Raf by probably diluting out the local concentration of Ras-Raf complexes at the plasma membrane or titrating out other Ras interacting factors.

Rescue of tracheal development

The same mutant Ras transgenes were tested for their capacity to rescue *dof* mutant phenotypes in the tracheae. Ras constructs were expressed by *btlGal4* in *dof* mutant embryos and their rescue capacity was quantified by counting the number of reconstituted dorsal trunk fusions (summarised in Figure 44). Tracheal network was visualised via the lumen specific antibody 2A12, therefore in our assay, successful fusion meant continuous lumen formation between adjacent dorsal trunks. The expression of constitutively active Ras partially rescued *dof* mutant tracheal defects ((Imam et al., 1999; Vincent et al., 1998) and Figure 41C). It appeared very potent to reconstitute dorsal trunk junctions but several missing or misdirected branches could be observed. RasV12,S35 provided a significantly weaker rescue (Figure 41D). In further contrast to the mesodermal data RasV12,G37 behaved very differently from RasV12,S35 in the tracheal system. It rescued migration in the sense

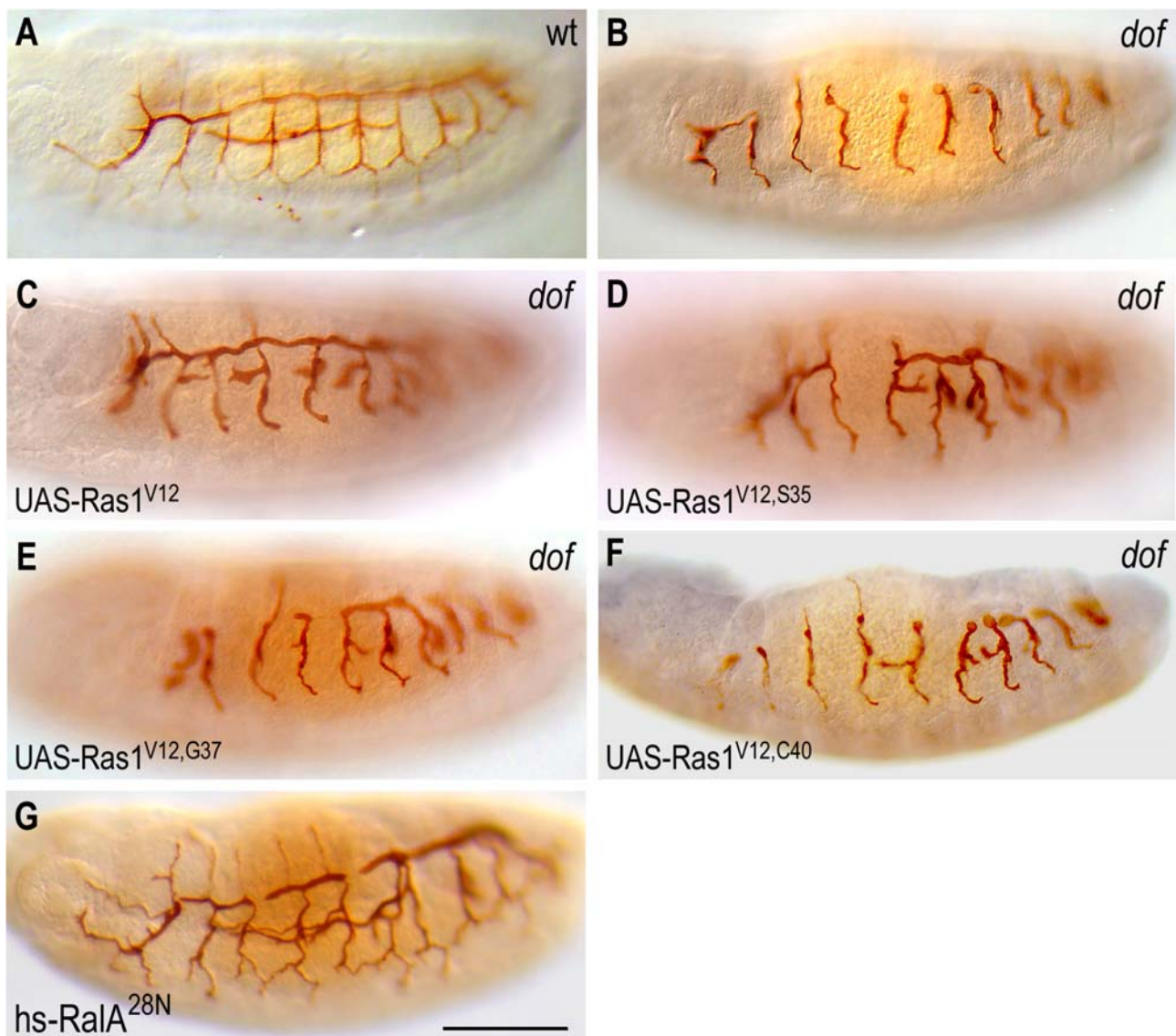


Figure 41. Capability of different mutant Ras proteins to rescue *dof* mutant phenotype in the trachea. Stage 15 embryos expressing different mutant UAS-Ras constructs under the control of the *btlGal4* driver are stained with 2A12 antibody to visualise the lumen of the tracheal network. (A) wildtype embryo, (B-F) *dof* mutant embryos (B) without or (C-F) with ectopic expression of (C) Ras1^{V12}, (D) Ras1^{V12,S35}, (E) Ras1^{V12,G37} and (F) Ras1^{C40}. Anterior to the left. Scale bar represents 100 μ m.

that branches grew out from the tracheal trunk but dorsal trunk fusion impaired remarkably and showed a wide variation in the embryos (Figure 41E, summarised in Figure 44). This weak rescue might be explainable with the residual Raf binding activity of RasV12,G37. To test independently whether Ral GTPase activity contributes to tracheal development, dominant negative DRal was overexpressed by *btlGal4*. No tracheal defects could be observed even at increased expression levels by using two copies of *btlGal4* (data not shown). As discussed above certain driver lines turn on gene expression early enough to enable manipulations with active molecules in tissue specific manner but too late to show effects with dominant negative constructs in the same developmental process due to the required lag/accumulation phase of this type of molecules. To circumvent this potential problem we took advantage of an available heat shock inducible dominant negative mammalian RalA construct (hs-RalA28N). Heat shock induced expression of RalA28N in wild type embryos prior to tracheal pit formation interfered with later tracheal development (Figure 41G). Control embryos subjected to the same heat shock conditions did not show tracheal fusion defects

(data not shown). Nevertheless it cannot be excluded that RalA28N acted on the tracheae in a non-autonomous manner or its effect was only a secondary consequence of defects in unrelated developmental processes. In summary, we have no direct evidence that the RasV12,G37 induced tracheal branching in *dof* mutant embryos is a consequence of RalGDS activation.

The effector loop mutant RasV12,C40 showed identical behaviour in the tracheae as observed in the mesoderm. It could not rescue any aspects of tracheal branching or fusion (Figure 41F). This further indicates that Raf binding capacity of Ras might be essential to induce tracheal branching.

2.7.2 Capacity of known downstream effectors of Ras to rescue *dof* mutant phenotypes

Analysis of effector loop mutant Ras constructs indicated that the Raf binding affinity of Ras is essential for its rescue capacity in *dof* mutant embryos. However these experiments also suggested a role of Ras for Raf-independent MAPK regulation and for Ral GTPase activation. To test to what extent Raf activation can overcome *dof* mutant phenotype in the developing mesoderm and tracheae, a constitutively active Raf transgene (*actRaf*) was expressed under the control of *twiGal4* and *btlGal4* respectively. Surprisingly, constitutively active Raf was completely unable to rescue *Eve*-positive cells in the mesoderm (Figure 42A).

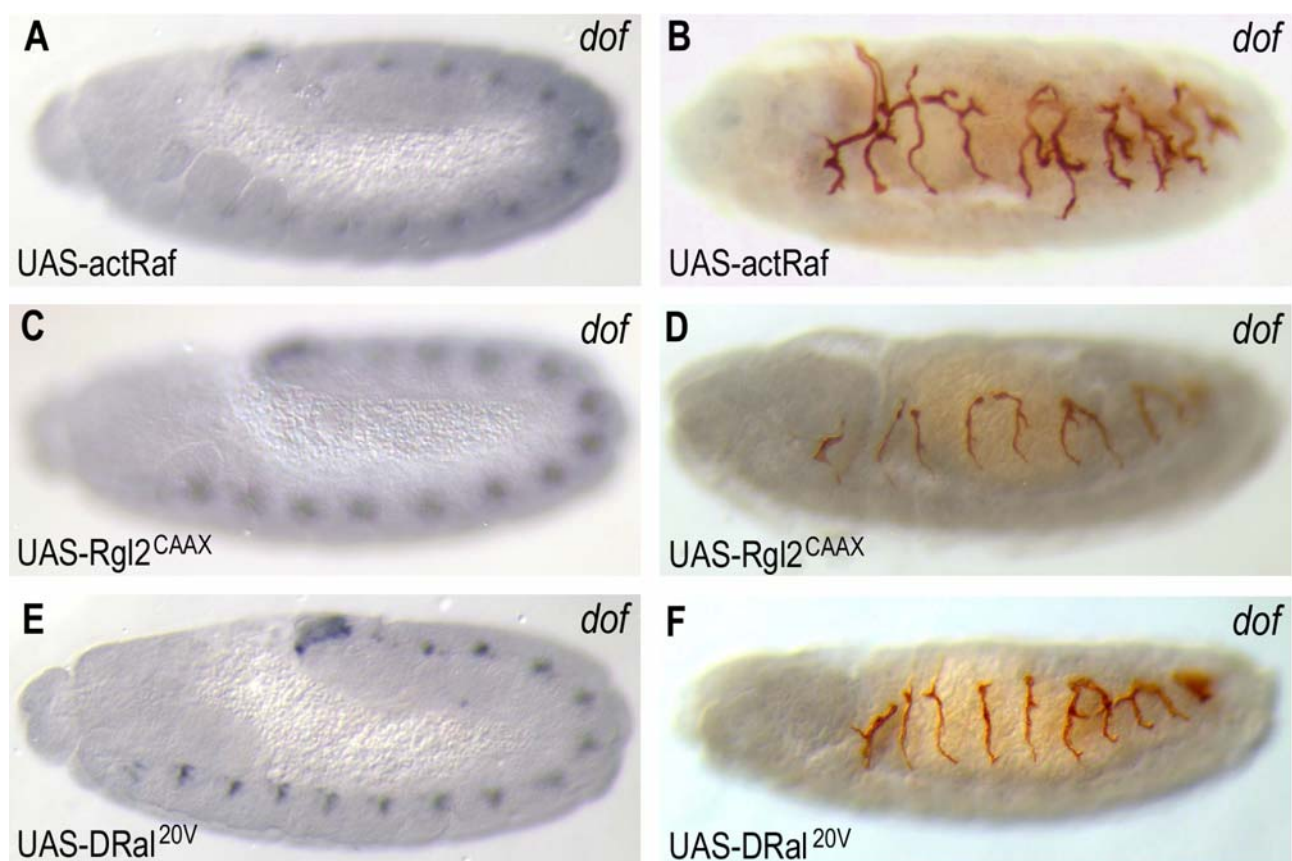


Figure 42. Constitutively active forms of different Ras effectors have weak if any capacity to rescue *dof* mutant phenotypes. *dof* mutant embryos expressing (A,B) UAS-actRaf, (C,D) UAS-Rgl2^{CAAX} or (E,F) UAS-DRal^{20V} (A,C,E) in the mesoderm under the control of *twiGal4* or (B,D,F) in the tracheae driven by *btlGal4*. (A,C,E) Stage 11 embryos are stained for Even-skipped to visualise *Eve*-positive cell clusters in the mesoderm. Note the lack of signal in the mesoderm; the signal out of focus derives from *Eve*-positive cells of the CNS. (B,D,F) Stage 15 embryos are stained with the trachea lumen specific 2A12 antibody. Anterior to the left.

To distinguish between the different possibilities why actRaf failed to induce dorsal mesodermal fate we monitored the presence of activated MAPK in the mesoderm during the period of Eve-positive cell fate determination. Activated MAPK accumulated in cells at positions of the Eve-positive competence clusters (Figure 43D-F). Thus, mesodermal cells must have reached dorsal positions and constitutively active Raf was able to activate MAPK. There are three different explanations why actRaf was not able to turn on Even-skipped expression in dorsal mesodermal cells: (i) activated MAPK levels were not sufficient to induce Eve expression, (ii) Ras might have Raf independent regulatory role on MAPK activation or (iii) Ras dependent regulation of additional factors might be required to turn on Even-skipped expression. Future work is required to discriminate these possibilities.

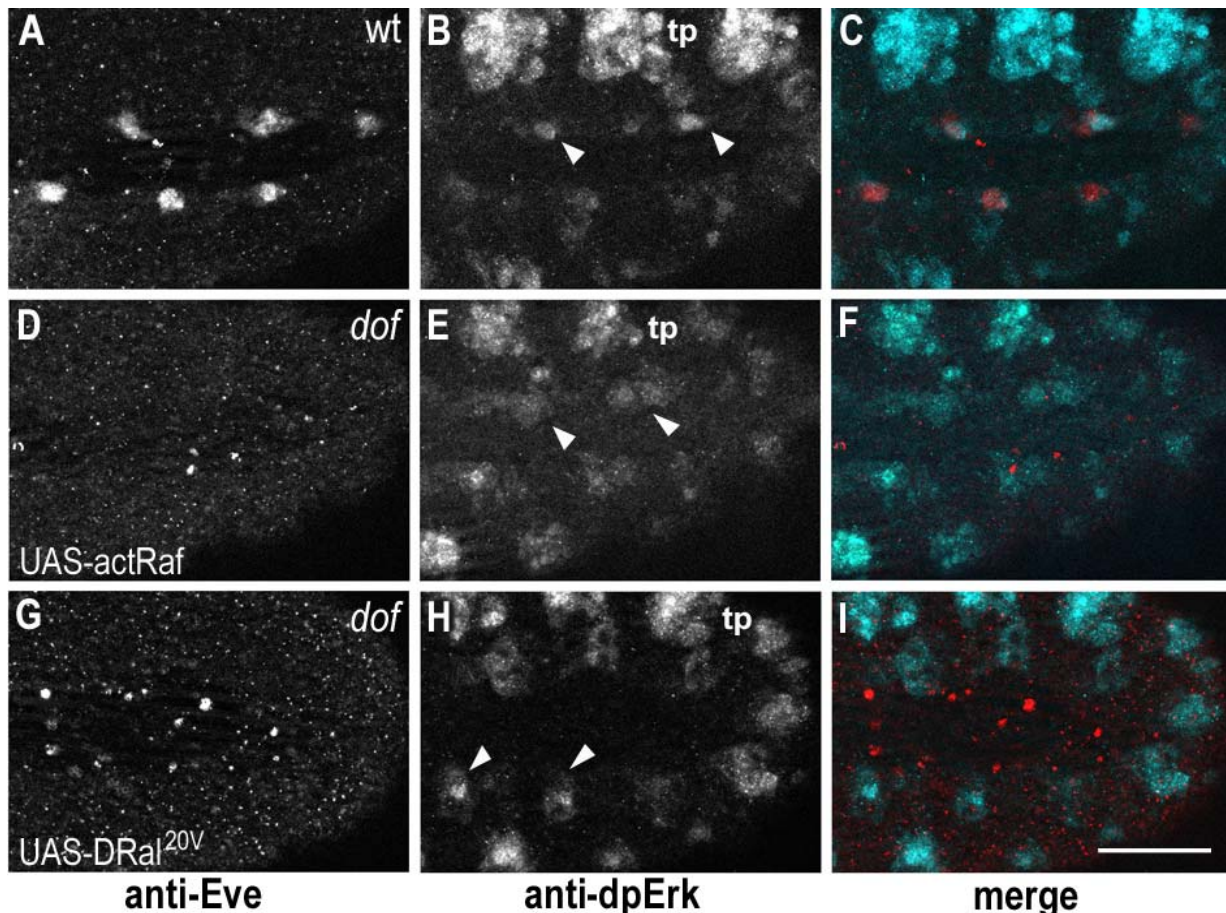


Figure 43. Constitutively active Raf and DRal are able to induce MAPK activation in the mesoderm. (A-I) Single optical sections showing the posterior one third of stage 11 embryos stained for (A,D,G) Even-skipped and for (B,E,H) phosphorylated MAPK (dpErk). (C,F,I) shows an overlay of the two signals (Eve red, dpErk blue). In (A-C) wild type embryos Even-skipped colocalises with dpErk accumulation. (D-I) *dof* mutant embryos expressing (D-F) UAS-actRaf or (G-I) UAS-DRal20V under the control of *twiGal4* show dpErk accumulation but no Even-skipped expression in the mesoderm. Arrowheads indicate examples of dpErk positive cells in the mesoderm; tp marks trachea pits with high accumulation of dpErk induced by EGF signalling. Dorsal up, anterior to the left. Scale bar 50 μ m.

In the tracheae actRaf behaved differently. It was able to rescue tracheal cell branching in *dof* mutant embryos, albeit at a much weaker level than RasV12 or RasV12,S35 ((Vincent et al., 1998), Figure 42B and summarised in Figure 44). Tracheal branches had an uncoordinated appearance, more than half of the dorsal trunks failed to fuse and formation of ectopic secondary branches could be observed. On the other hand the same Raf construct was reported to be able to turn on the MAPK target gene DSRF (*Drosophila Serum Response Factor*) in the tracheae of *dof* mutant embryos with similar efficiency as RasV12, indicating that it induced MAPK activation and subsequent

transcriptional activation efficiently (Vincent et al., 1998). Thus, the higher capacity of the “Raf specific” effector loop mutant RasV12,S35 to rescue tracheal migration might be independent of its MAPK activating function and other downstream effectors might be responsible for it. However, we cannot exclude differences in the strength and expression level of the different transgenic constructs.

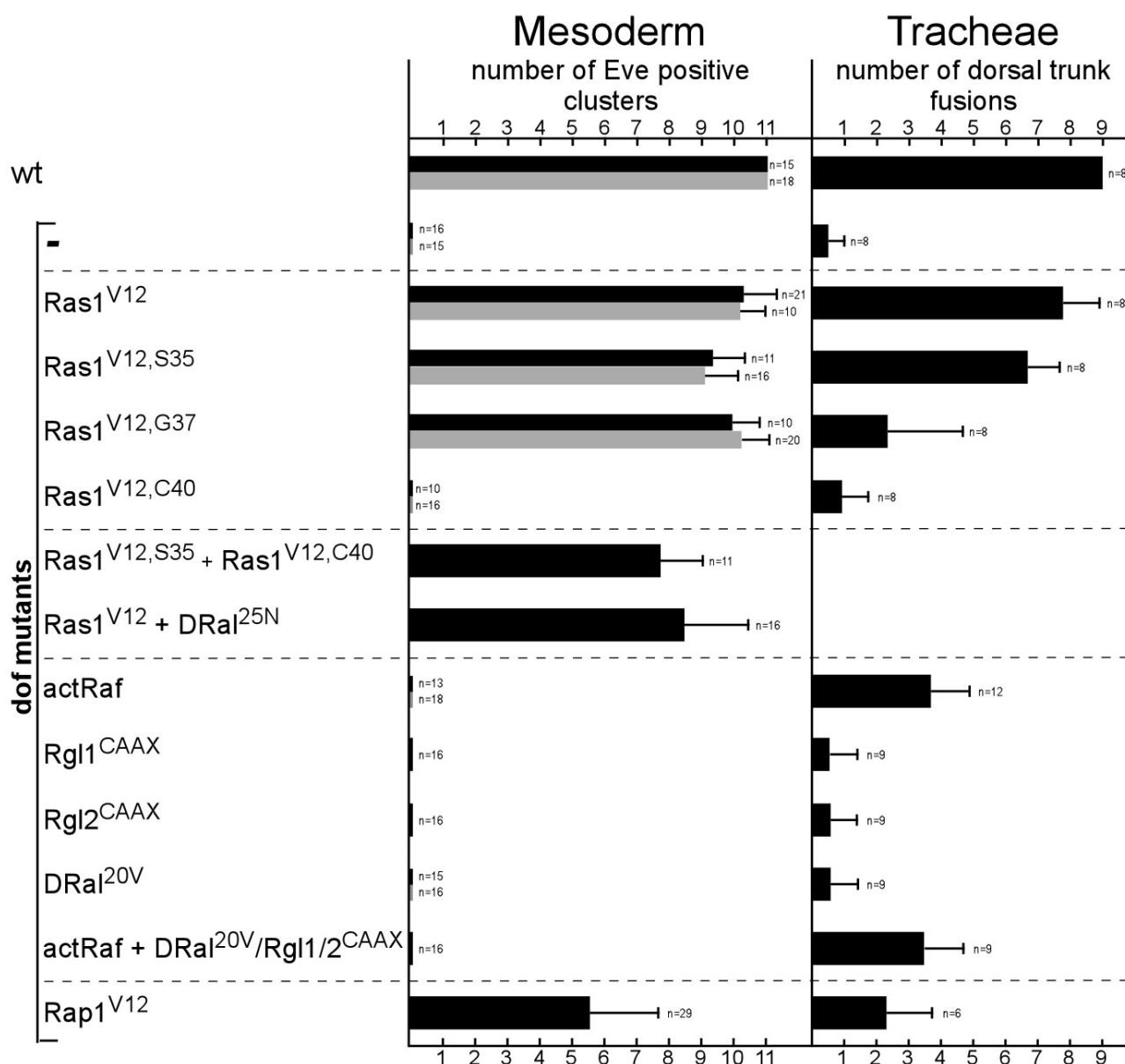


Figure 44. Capacity of mutant forms of different signalling molecules to rescue *dof* mutant phenotypes in the embryo. Mutant signalling molecules tested in *in vivo* assays are listed on the left. All represent UAS transgenic constructs. See text for details of the mutations. To achieve a comparison for the ability of the transgenes to rescue mesoderm development the number of Eve-positive clusters formed on one lateral side of stage 11 embryos was determined following *twiGal4* (black bars) or both *twiGal4* and a maternal *Gal4* (grey bars) driven expression of the constructs. To assess the ability of a transgene to rescue tracheal development, transgene expression was driven by *btlGal4* followed by counting the junctions formed by the dorsal trunk on one lateral side of stage 15 embryos. The diagrams show the average number and standard deviation of the counted structures. Number of embryos analysed in each experiment is depicted on the diagram. Both lateral sides of each embryo were counted and taken as independent values.

Next, components of the RalGDS effector cascade were analysed. *Drosophila* has a single RalGDS family RalGEF homologue encoded by the *rgl* gene (Mirey et al., 2003). It has two isoforms, Rgl1 and Rgl2. To achieve constitutively active Rgl molecules, a CAAX lipid modifications signal was introduced into the protein (Mirey et al., 2003). Rgl1CAAX, Rgl2CAAX or the constitutively active form of *Drosophila* Ral GTPase (DRal20V) were expressed in *dof* mutant embryos under the control of *twiGal4* or *btlGal4*. The observed phenotype was identical in all cases: complete failure to rescue Eve-positive clusters or tracheal branching (Figure 42C-F and data not shown). Surprisingly, constitutively active Rgl1, Rgl2 and Ral were able to induce activated MAPK accumulation at positions of Eve-positive competence clusters in the mesoderm (data not shown and Figure 42G-I). Thus, the “RalGDS specific” RasV12,G37 might not (exclusively) rely on its residual Raf binding capacity to activate MAPK but might do it in a Ral dependent manner. This might explain the observed comparable efficiency of RasV12,S35 and RasV12,G37 to accumulate phosphorylated MAPK and to rescue the formation of Eve-positive cells. It is not understood at present what mechanism might lie behind Ral-mediated MAPK activation.

Cell culture studies showed that Raf and RalGDS have a synergistic effect in cell transformation and differentiation (Okazaki, 1997). Effector loop mutant Ras proteins might still utilise cooperative action of Raf and Ral due to their residual binding properties, which might explain their rescue capacity and the lack of rescue potential of the single effectors. To test if simultaneous action of Raf and Ral is sufficient for efficient rescue of *dof* mutant phenotypes, constitutively active Raf was expressed together with Rgl1CAAX, Rgl2CAAX or DRal20V in the mesoderm and in the tracheae of *dof* mutant embryos. These embryos showed indistinguishable phenotypes from the one obtained by the expression of actRaf alone; thus, no Eve-positive clusters in the mesoderm and weak branching capacity of tracheal cells (data not shown). This indicates the requirement of additional Ras function(s) for efficient signal transmission during these processes. This Ras function could have a direct role in the spatio-temporal coordination of Raf and Ral activity or could be independent of these effectors.

2.7.3 Dominant effects of constitutively active Ras effectors

Constitutively active Ras induces Eve-positive clusters with supernumerary cells in the mesoderm and ectopic secondary branches in the tracheae upon ectopic expression in the corresponding tissue (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). We analysed this effect with the different effector loop mutant forms of Ras. In these overexpression studies mutant Ras proteins reflected the activity they showed in the rescue experiments. RasV12,S35 and RasV12,G37 were able to form larger Eve-clusters in the mesoderm as well as secondary branches at ectopic positions in the tracheae, whereas overexpression of RasV12,C40 did not influence these developmental processes at a detectable level (data not shown and Figure 45A). This indicates that the phenotypes caused by RasV12 overexpression depend on Raf and RalGDS but not on PI3K mediated processes. To confirm this, constitutively active forms of Raf, Rgl1, Rgl2 and DRal were overexpressed by *twiGal4* and *btlGal4*. Both, Raf and the three RalGDS effector pathway components formed ectopic secondary branches in the tracheae upon overexpression (data not shown and Figure 45B-D). Their appearance was variable ranging from small “papilloma-like” protrusions of the lumen to multiple-branch structures. The different rescue capability of constitutively active Raf and Rgl/DRal suggests that the formation of these secondary branches is mediated through different mechanisms. ActRaf was able to induce secondary branching even in *dof* mutant embryos indicating that activation of the MAPK pathway might be sufficient to induce secondary branches. DRal20V on the other hand was able to act on secondary branch formation

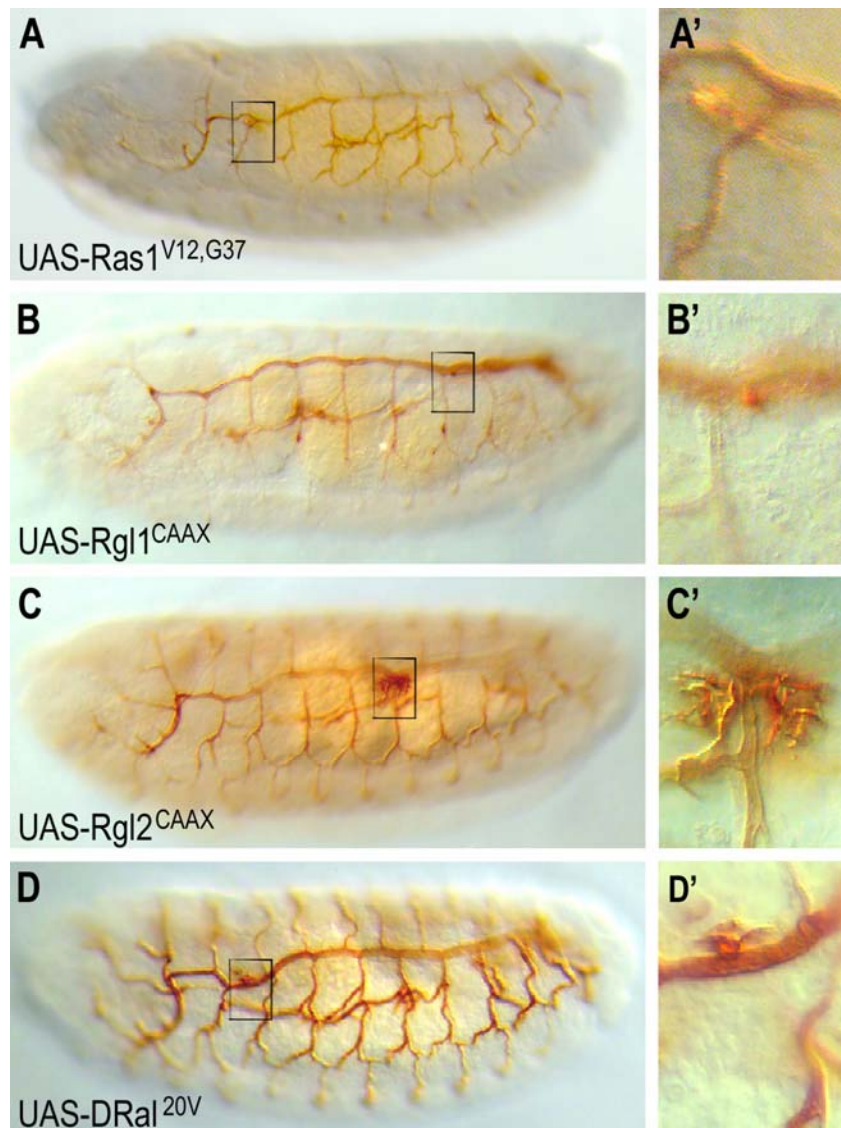


Figure 45. Ectopic secondary branches are formed by the overexpression of constitutively active signalling molecules in the trachea. Stage 15 embryos expressing (A) UAS-Ras1^{V12,G37}, (B) UAS-Rgl1^{CAAX}, (C) UAS-Rgl2^{CAAX} or (D) UAS-DRal^{20V} under the control of *btlGal4* are stained with 2A12 antibody to visualise the lumen of the trachea network. A', B', C' and D' are magnifications of areas marked with black boxes on A, B, C and D respectively. Anterior to the left.

only in wild type embryos but failed to induce any branching in *dof* mutants suggesting that its activity might require endogenous FGF signalling activity. The observation, that simultaneous expression of actRaf and constitutively active Rgl/DRal in *dof* mutant embryos failed to improve rescued levels of tracheal branching obtained by actRaf expression alone, indicates that the FGF dependent additional activity required for DRal action is not Raf dependent and so probably different from the MAPK cascade. The partial capacity of Ras^{V12,G37} to induce branching in the tracheae of *dof* mutant embryos suggests that this required additional activity might be Ras activation dependent. However as mentioned earlier, we cannot exclude the possibility that the tracheal branching induced by Ras^{V12,G37} is primarily due to its residual Raf binding capacity.

In contrast to their effect in the tracheae, no detectable alteration was observed in Eve-cluster formation by the ectopic expression of RalGDS effector pathway components in the mesoderm (data not shown). Constitutively active Raf was not tested in this assay (data summarised in Table 4). The fact that MAPK activating capacity of Ras^{V12,G37} in the presumptive Eve-positive competence cluster manifested in ectopic Even-skipped expression - but that of

DRal20V/RglCAAX did not - suggests that additional Ras dependent functions are required for Even-skipped expression.

2.7.4 Ras and/or Rap? – searching for the *in vivo* players in FGF signalling

To reveal the *in vivo* function of Ras in FGF signalling we tried to mimic Ras loss of function situations in the mesoderm and the tracheae. Mutational analysis was not the tool of choice because of reasons discussed above (high maternal contribution, broad developmental function from early on). Two strategies were applied to decrease Ras activity in a tissue specific manner: (i) expression of dominant negative forms of *Drosophila* or mammalian Ras proteins or (ii) expression of the Ras specific GTPase activating protein Gap1 under the control of *twiGal4* and *bt1Gal4*. Although all three transgenic constructs were efficiently used in different studies (Alvarez et al., 2003; Duchek and Rorth, 2001; Lee et al., 1996; Powe et al., 1999), no comparative analysis was available about the strength of these transgenes to inhibit Ras activity. Therefore we first tested them in the developing *Drosophila* eyes using the strong *GMRGal4* as well as the weaker, R7 cell specific *SevenlessGal4* driver line (Figure 46). All three constructs caused a rough eye phenotype, albeit with very different severity. *Ras85DN17*, the dominant negative form of *Drosophila* Ras and *Gap1* had weaker effect. *Ras1N17*, the dominant negative form of mammalian *Ras1* appeared as far the most efficient construct to inhibit Ras activity. The same ranking was obtained in the Ras inhibiting capacity of the three transgenes with both driver lines (*Ras1N17*>>*Gap1*=*Ras85DN17*), and the range of severity in the phenotype correlated with the strength of the drivers.

Next, the effects of these constructs were analysed in the mesoderm. No detectable alteration in the appearance of *Eve*-positive cells was observed when *Ras85DN17* or *Gap1* were overexpressed.

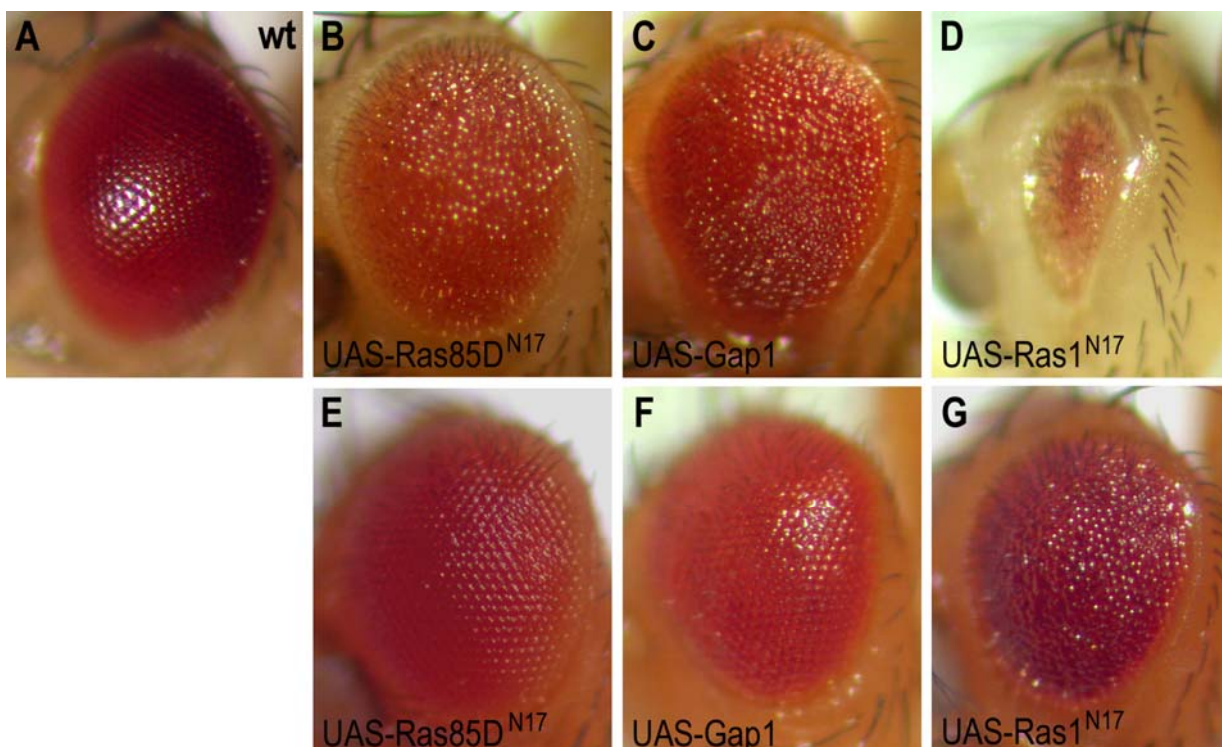


Figure 46. Comparative analysis of different transgenes in their strength to inhibit Ras activity in the eye. Whole-eye views of (A) wild type fly and flies expressing (B,E) UAS-Ras85DN17, (C,F) UAS-Gap1 or (D,G) UAS-Ras1N17 under the control of (B,C,D) *GMRGal4* or (E,F,G) *SevGal4*. Note the differences in the roughening and size of the eyes. Anterior to the left.

Ras1N17 expression by *twiGal4* led to the loss of Eve-positive DA1 muscle progenitors with an incomplete penetrance but did not influence the formation of Eve-positive pericardial progenitor cells (EPCs). The read-out of this at later developmental stages was the lack of DA1 muscle fibers in some hemisegments without the loss of EPCs (Figure 47B). It was consistent with the finding of other studies, in which the same transgenic combination induced a strong decrease in Eve-positive DA1s (greater than 50%), but rarely a loss of Eve-pericardial cells (James B. Skeath personal communication). In contrast to this, DN Heartless was able to block both DA1 progenitor and EPC formation in a non-selective fashion (Carmena et al., 1998; Michelson et al., 1998). DA1 muscle progenitor formation requires the dual input of FGF and EGF signals whereas EPCs rely only on FGF signalling. The phenotype we observed was reminiscent of those caused by ectopic expression of DN-EGFR or by loss of function mutations of components of the EGF signalling cascade (Buff et al., 1998; Carmena et al., 1998). However, it is possible that EPC formation is also Ras dependent but that it is not sensitive to this level of Ras1N17.

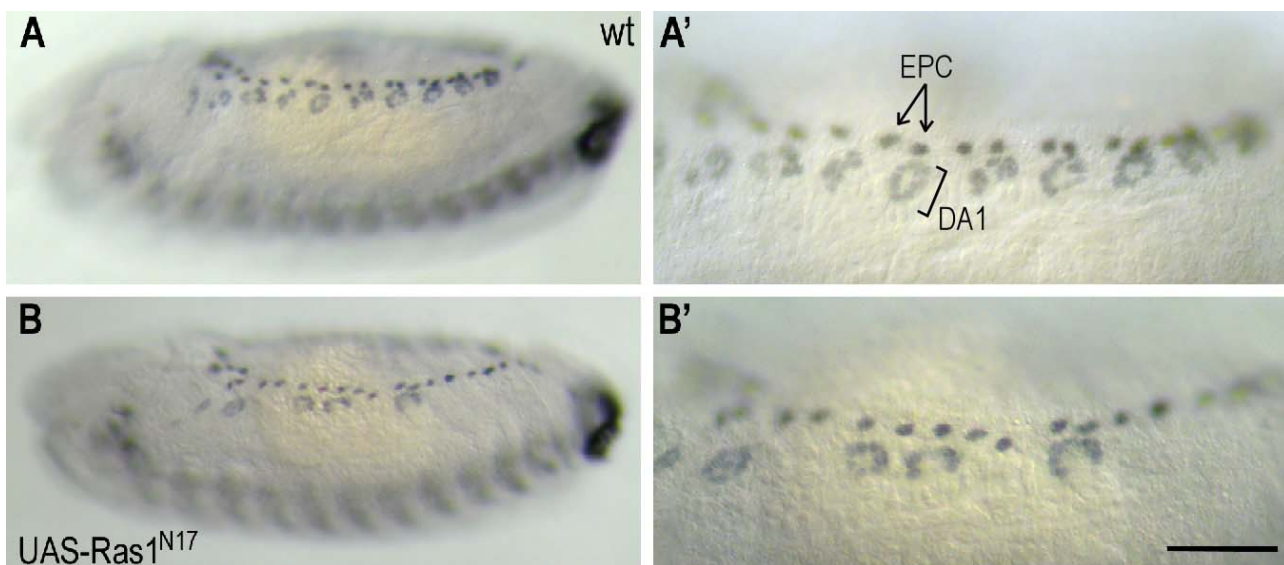


Figure 47. Ectopic expression of dominant negative Ras1 causes loss of DA1 muscles in the mesoderm. Stage 14 embryos are stained with Even-skipped antibody to visualise Eve-positive cells in the mesoderm. (A) wild type embryo contains two EPCs and a multinuclear DA1 muscle fibre in each hemisegment at this stage (magnified on A'). (B) Embryo expressing UAS-Ras1N17 under the control of *twiGal4* loses DA1 muscle fibres in some hemisegments. Note the unaltered number of EPCs (magnified on B'). Anterior to the left. EPC, Eve-positive pericardial cell; DA1, dorsal acute 1. Scale bar 50 μ m for A' and B'.

In tracheal expression studies using *btlGal4* the three transgenic constructs behaved identically, in that they had no effect on any aspects of tracheal network formation detectable with the lumen specific 2A12 antibody (data not shown). The use of two copies of *btlGal4* did not alter the observed phenotype. EGF signalling is involved also in tracheal development; it initiates the invagination of tracheal pits and plays a role in the regional differentiation of tracheal branches (Llimargas and Casanova, 1999; Wappner et al., 1997). EGF signals through Ras; thus, it was surprising that we failed to detect any defects in tracheal morphogenesis upon expression of dominant negative Ras. Our earlier experiments with dominant negative DRal as well as other studies on embryonic tracheae with dominant negative forms of Rho-family GTPases indicated that tracheal development might be insensitive to the level of dominant negative molecules which accumulate with the *btlGal4* driver line (Wolf et al., 2002). There are no studies on the tracheae using dominant negative forms of the EGF receptor or the FGF receptor *Breathless* under the control of the *btlGal4* driver. These would be important controls to test whether our observations are specific for Ras or EGF and FGF signalling events are insensitive in general to the levels and timing of dominant negative factors accumulated by *btlGal4* during the time window of our assay.

The only indication for the efficient expression of Ras1N17 by *btlGal4* was that it caused lethality at late larval/pupal stages (summarised in Table 4). Even assuming that the observation is specific for Ras it could not be excluded completely that this is only an issue of critical concentrations of the dominant negative construct. However, one possible explanation might be that other factors are involved in the transmission of the signal from the FGF (and EGF in the tracheae) receptors towards downstream effectors. The small Ras-related GTPase Rap1 is able to activate several known Ras effectors.

To test the potential involvement of Rap1 in tracheal development, a dominant negative form of Rap1 (Rap1N17) was expressed under the control of *btlGal4*. This approach was chosen based on the same consideration as in the case of Ras. In contrast to Ras1N17, Rap1N17 expression caused significant defects in the formation of the tracheae (Figure 48A,B). Remarkably, this was the only dominant negative construct in our assays that caused tracheal defects upon expression with *btlGal4*. The phenotype was variable but general in the sense that it affected all the different branches of the tracheal network. The formation and proper directionality of visceral branches was frequently affected, lateral branches were missing which often led to defects in lateral trunk fusion. Dorsal branches stalled occasionally or were misled and in the most severe cases several gaps were observed in the dorsal trunk, indicating a role of Rap1 in tracheal development.

To test if Rap1 might be sufficient to transmit FGF signal during tracheal development, a constitutively active version of the protein (Rap1V12) was expressed with *btlGal4* in *dof* mutant embryos. It had a weak, but significant capacity to induce tracheal branching and to induce the

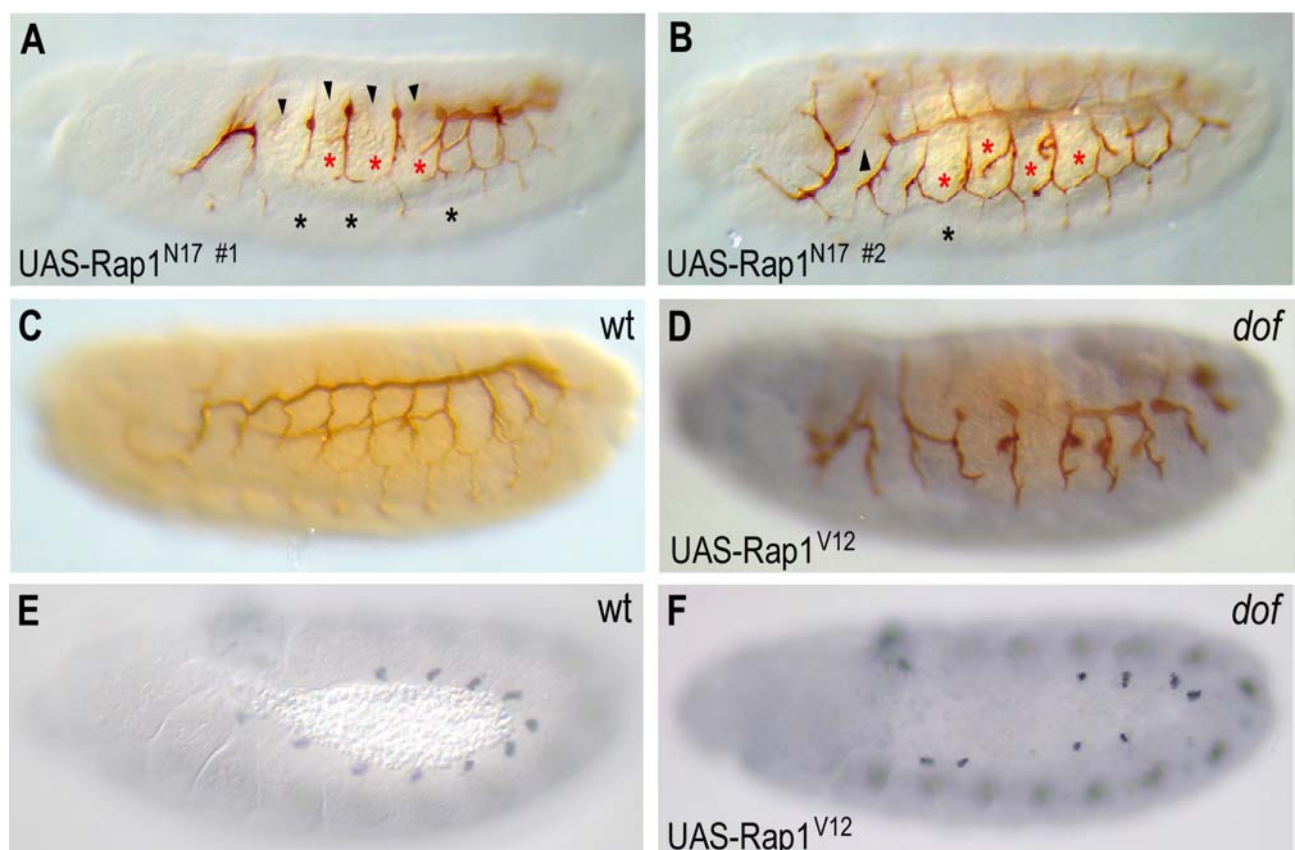


Figure 48. Effects of mutant forms of Rap1 in *in vivo* assays. (A-D) stage 15 embryos stained with 2A12 antibody to visualise the lumen of the tracheal network. (E,F) stage 11 embryos stained with Even-skipped antibody to visualise Eve-positive clusters in the mesoderm. (A,B) embryos expressing the UAS-Rap1N17 transgene (A and B two independent insertion lines) under the control of *btlGal4*. (C,E) control wildtype embryos. (D,F) *dof* mutant embryos expressing UAS-Rap1V12 driven by (D) *btlGal4* or (F) *twiGal4*. In A and B arrowheads, black asterisks and red asterisks indicate gaps in the dorsal trunk, missing ganglionic branches and missing or misled visceral branches respectively. Anterior to the left.

formation of dorsal trunk fusion occasionally (Figure 48D). Thus, constitutively active Rap1 was not able to efficiently take over the function of constitutively active Ras in the tracheae indicating that they might have differences in affinity towards downstream effectors. We have to remark the possibility for differences in the expression levels of the two transgenes. All these data suggest that both Ras and Rap1 might have *in vivo* functions in tracheal development probably acting in parallel. In mesodermal expression constitutively active Rap1 could partially circumvent the *dof* mutant phenotype, showed higher activity than in the tracheae but remained less efficient compared to constitutively active Ras (Figure 48F, summarised in Figure 44). Further loss of function studies are required to confirm the role of Rap1 in FGF dependent developmental processes in the mesoderm.

Table 4. Effects of tissue specific expression of different mutant signalling molecules on the development of *Drosophila*

	Tissue morphology		Survival rate	
	Mesoderm Effect on Eve positive clusters twiGal4	Tracheae Effect on tracheal network formation btlGal4	flies emerged x 100 flies expected	
			twiGal4	btlGal4
UAS-Ras^{V12}	bigger clusters	ectopic secondary branches	0%	0%
UAS-Ras^{V12,S35}	bigger clusters	ectopic secondary branches	3%	0%
UAS-Ras^{V12,G37}	bigger clusters	ectopic secondary branches	104%	16%
UAS-Ras^{V12,C40}	no effect	no effect	93%	93%
UAS-actRaf	n.d.	ectopic secondary branches	0%	0%
UAS-Rgl1^{CAAX}	no effect	ectopic secondary branches	1%	0%
UAS-Rgl2^{CAAX}	no effect	ectopic secondary branches	2%	0%
UAS-DRal^{20V}	no effect	ectopic secondary branches	1%	11%
UAS-Rap1^{V12}	n.d.	n.d.	34%	n.d.
UAS-Ras85D^{N17}	no effect	no effect	95%	123%
UAS-Gap1	no effect	no effect	95%	38%
UAS-Ras1^{N17}	missing DA1 muscle progenitors, no effect on pericardial cells	no effect	0%	1%
UAS-DRal^{25N}	no effect	no effect	107%	0%
hs-RalA^{28N}	n.d.	missing lateral and dorsal branches, fusion defects number of dorsal trunk fusions: 6.25±1.48	n.d.	n.d.
UAS-Rap1^{N17}	n.d.	missing lateral, dorsal and visceral branches fusion defects	99% 101%	25% 34%

Mutant signalling molecules tested in *in vivo* assays are listed on the left. They represent UAS transgenes with the exception of RalA28N, which is under the control of a heat shock promoter. Transgenes with described constitutive activity (top) are separated with a dashed line from transgenes which showed dominant negative effects in other studies (bottom). UAS transgenes were expressed under the control of *twiGal4* and *btlGal4* followed by descriptive analysis of Eve-positive clusters and tracheal network formation respectively. To test how UAS transgenes effect vitality by tissue specific expression flies heterozygous / hemizygous either for the UAS transgene or for the driver line were crossed to each other. Thus, the number of emerged flies with and without transgenic expression was expected to be equal. Survival rate was calculated by dividing the number of emerged flies with transgene expression (=flies emerged) through the number of emerged flies without transgene expression (=flies expected). To follow chromosome segregation balancer chromosomes or sex determination were used. The way of calculation could not take attention about the effects of balancer chromosomes or transgene function independent effects of transgenic chromosomes (some UAS transgene insertions are homozygous lethal) on vitality.

3. Discussion

The FGF signal transducer molecule Dof is essential in all known aspects of FGF signalling in *Drosophila*. It has been shown to act upstream of the conserved Ras-MAPK pathway and has been proposed to serve as an adaptor molecule transmitting the signal from the FGF receptor to the MAPK cascade and probably to other effectors. Dof does not share sequence similarity with factors involved in FGF signalling in vertebrates. Its vertebrate homologues BCAP and BANK are involved in B-cell receptor signalling which indicates that the ancestor gene lost its role in FGF signalling but gained an adaptor function in a novel signalling cascade during vertebrate evolution. However, the downstream effector cascades utilised in FGF signalling are conserved from worms to humans. How and to what extent Dof can activate these conserved pathways and what different roles it might have compared to its vertebrate functional relatives is not known. In this project Dof mediated interactions were characterised especially the interaction of Dof with the FGF receptor to obtain an insight into the mechanism of Dof function. Furthermore, potential post-translational modifications of the protein were analysed with respect on their physiological relevance in the function of Dof. In the last part of this work the role of downstream effector pathways of the FGF signalling cascade was investigated.

3.1 Interaction of Dof with yeast two hybrid interactors

Selected candidates of an earlier yeast two hybrid screen using Dof as bait were analysed for their interaction with Dof in *Drosophila* S2 cells by immunoprecipitation assay. The selection was based first of all on structural and functional characteristics of the yeast interactors and did not considered classical criteria used at the verification of yeast two hybrid interactions, e.g. different growth behaviours under different selection conditions, the reading frame of the identified clones or the number of identified clones representing the same interactor. In fact, immunoprecipitation data showed, that these criteria are not necessary valid for discrimination of interactions. Interactors identified as single clones growing only under less stringent selection conditions (e.g. i188 and i25 coding for CG11275 and *gustavus* respectively) or the interactor i14 (coding the ribosomal protein S10), the clones of which were out of frame with the Gal4 activation domain sequence appeared as strong interactors in our immunoprecipitation assay, indicating that they represented equally potent interaction candidates. In general, the yeast two hybrid screen appeared as a reliable pre-selection for proteins that were able to form a complex with Dof in *Drosophila* cell culture assay.

Dof interacted in the immunoprecipitation assay with itself, with the FGF receptor Heartless, with the SUMO conjugating enzyme Ubc9 and with the ribosomal protein S10. In this work the functional relevance of these interactions was investigated in more detail and will be discussed latter. The immunoprecipitation experiments also confirmed that Dof might bind and directly transduce the FGF signal to potential regulators of actin cytoskeleton (i19 - CDEP) and membrane trafficking (i52 - GAP69C). It also might be involved in the establishment and maintenance of epithelial integrity (i8 - sinuous). The signal dependent recruitment of the regulatory subunit of the protein phosphatase PP2A was reported to regulate the activation of the MAPK cascade (Ory et al., 2003). Thus, the interaction of Dof with a regulatory subunit of the protein phosphatase PP2A (i234) indicates a potential function in the Ras-MAPK signal transduction cascade. The clone i188 coding the predicted gene CG11275 interacted also efficiently with Dof in S2 cells. It is a cytoplasmic BTB domain containing protein without known function. BTB domain proteins are substrate-specific adaptors in an SCF-like modular ubiquitine ligase complex (Pintard et al., 2004). Interesting question of further studies might be if CG11275 might play a role in the degradation of Dof.

Surprisingly, Dof showed weak interaction also with the nuclear protein Bap60 (i133) in a way that might conclude that Bap60 was able to interact only with a C-terminal fragment of Dof (see Chapter 2.1 for details). How does it fit to the yeast data? First, it is not known if Dof is processed in yeast cells. Even so, a C-terminal fragment of Dof would not give positive interaction results, since the Gal4 DNA binding domain, which was N-terminally fused to Dof, would have been cleaved off from the molecule. Nevertheless, yeast interaction data bypass the effects of different compartmentalisation behaviour of molecules by targeting both fusion proteins to the nucleus. It might be possible, that in the *Drosophila* S2 cell culture assay Bap60 did not bind full length FLAG-Dof, because the two molecules were not in the same cellular compartment, and only the C-terminal fragment of FLAG-Dof entered the nucleus, whereas in yeast the C-terminal interacting site was available in the full length form of Dof. Bap60 grouped to Dof interactors, the binding site of which mapped either to the N-terminus or to a region within or in the vicinity of the coiled coil motif (Battersby et al., 2003). Although it is pure speculation, our immunoprecipitation data with Bap60 would be the first hint, that C-terminal fragment of FLAG-Dof has distinguishable features from the full length molecule. Further experiments are required including mapping of the interaction domain for Bap60 binding in Dof, confirming immunoprecipitation with C-terminally tagged Dof constructs and high resolution localisation studies of Dof with different N- and C-terminal epitope tags in cells in the presence/absence of Bap60 to analyse this hypothesis.

These interaction results indicate that Dof is able to interact with molecules representing diverse cellular functions and so give a broad field of interesting processes for further investigation.

3.2 Conclusions from subcellular localisations of Dof

Cellular localisation studies were performed with some of the yeast interactors. Beside the fact that Dof showed different levels of co-localisation with them, an interesting observation was that the co-expression of different factors influenced the cellular distribution of Dof, ranging from membrane enrichment to diffuse appearance (see Figure 11). In addition, localisation studies in *Drosophila* embryos ectopically expressing mutant Dof proteins showed that loss of protein domains alters subcellular localisation (Wilson et al., 2004). These indicate that Dof might have interacting partners located in different cellular compartments and the loose of interacting domains for the one partner might allow interaction with other partners. These data derived from non-signalling cells in which not all the components are necessarily present that determine Dof localisation. Nevertheless, these data indicate that Dof might recruit differentially localised molecules to cortical regions of the cytoplasm. Alternatively, Dof itself might have a very dynamic re-localisation during signalling which would require dynamic changes in binding affinity towards the different interacting partners. Two ways how this could be achieved are conformational changes and post-translational modifications of the molecule. Interaction behaviour of a panel of yeast interactors with different Dof deletion construct indicated that Dof might exist in different conformations. Based on the self-interaction capacity of the different deletion constructs a “closed” and an “open” structure has been proposed in which intramolecular interaction between the N-terminal DBB domain and the C-terminal part of the molecule provide the force for a “closed” conformation (Battersby et al., 2003). The results of this project show that Dof indeed utilises different modifications potentiating the molecule to dynamic interactions.

3.3 Interaction of Dof with the FGF receptor

In consistence with earlier yeast data Dof interacted with the FGF receptor Heartless in S2 cells. Dof was able to bind a constitutively active as well as the kinase dead form of the receptor. Thus, the interaction was independent from the phosphorylation state of Heartless, indicating that Dof might bind to FGF receptors in a constitutive, signalling-independent manner. However, our data cannot exclude the potential role of receptor dimerisation in this interaction. The interaction domain for FGF receptor binding is mapped to the DBB domain which is in agreement with yeast two hybrid data. The mutation of a single lysine at amino acid position 297 in the DBB domain eliminates interaction of Dof with the FGF receptor in yeast. This was also confirmed in our immunoprecipitation assay. Transgenic Dof constructs lacking amino acids 233-364 or bearing the point mutation K297R do not have the capacity to rescue the *dof* mutant phenotype in flies pointing out the essential function of the DBB domain and the pivotal role of lysine 297 within this region ((Wilson et al., 2004) and R. Wilson personal communication). Our immunoprecipitation results highly suggest that this region and lysine 297 within this region are required for efficient FGF receptor binding. However, DBB domain might play a role also in other critical interactions during FGF signalling.

Similarly to yeast data, the presence of the C-terminal 200 amino acids in Dof weakened the interaction of Dof with Heartless, indicating an inhibitory function of the C-terminal part. This suggests that an “open” conformation of Dof might be the active form during signalling.

On the side of all these confirming results a general remarkable difference was detected between yeast interaction and immunoprecipitation data. In S2 cells each Dof construct showed some interaction with different forms of Heartless although this was strongly reduced in the constructs lacking the DBB domain, like Dof^{Δ233-364}. What might be the difference in the interaction of Dof with Heartless in yeast in contrast to that in S2 cells resulting the residual binding of Dof^{Δ233-364} to the FGF receptor in S2 cells? The cell culture Heartless construct contains additional 12 amino acids of the juxtamembrane region, which are not present in the yeast constructs. However in yeast, the smaller receptor fragment lacking in addition the N-terminal lobe of the kinase domain showed a weak interaction at lower restriction selection conditions with Dof constructs lacking parts of the DBB domain but the larger receptor fragment did not bind to this constructs at all. Results of the S2 cell interaction assay resembled that of the small and not of the large receptor fragment of the yeast assay indicating that the 12 amino acids in the juxtamembrane region of Heartless might be irrelevant in the interaction with Dof. An additional difference, that the residual Dof-interaction of Heartless might cause, is the dimeric versus monomeric state of the receptor molecules in the two assays. Dimerisation of FGF receptors leads to conformational changes (Mohammadi et al., 1996). Therefore, the weak interaction might be a reflection of a difference in binding affinity of Dof for the activated and non-activated receptor. Remaining parts of the DBB domain might be sufficient for residual binding to the activated receptor dimer. Petit et al. reported that different Dof deletion mutant versions retained the ability to form a complex with activated Breathless in S2 cells, but constructs lacking the entire region of amino acids 168-364 were not tested in their study neither (Petit et al., 2004). Alternatively, receptor dimerisation might generate novel interaction surfaces for Dof domains independent from the DBB domain. Concluded from our deletion studies a DBB independent interaction domain might span between amino acids 446 and 802. Interestingly there are tyrosine residues within this stretch that are phosphorylated in the presence of an activated FGF receptor (see Chapter 2.3). However, C-terminal parts of the Dof protein lacking the first 485 or 600 amino acids are not able to interact with an activated FGF receptor in *Drosophila* S2 cells (Petit et al., 2004) indicating that the amino acid region 485-802 is not utilised for the interaction. Amino acids 446-485 were not studied independently from the DBB domain for receptor interaction.

3.4 The role of lysine 297 in the interaction of Dof with the FGF receptor

The lysine at position 297 in the DBB domain is essential for efficient interaction of Dof with the receptor both in yeast and in S2 cells. Importantly, this point mutation did not alter the interaction of Dof with any other candidates of the yeast two hybrid screen which showed DBB domain dependent binding (Battersby, 2001). This indicates that the mutation did not cause structural distortion in the molecule and that the FGF receptors require a different interaction surface in the DBB domain than other binding partners. This lysine is located within a consensus SUMO attachment site. The mutation of this residue destroys the site for sumoylation since the lysine side chain is the site of SUMO attachment. This gives rise to two possibilities. First, lysine 297 might be required as an essential residue in the interaction surface of Dof towards the FGF receptor. Secondly, the lysine might be required as a site of SUMO attachment and thus, SUMO modification might be essential for the interaction of Dof with the FGF receptor. Considering that the consensus site of sumoylation and the mechanism of sumoylation are evolutionarily conserved the probability of Dof sumoylation in yeast might be possible. In future work, one way to distinguish these two possibilities might be to analyse FGF receptor binding capacity of Dof in cells in which the sumoylation machinery is affected. RNA interference against essential components of this cascade, like SUMO or Ubc9 might be an efficient tool to generate such a situation.

All Dof homologues share the conserved DBB domain. However, lysine 297 is conserved only in the closest known homologue of Dof, in the *Anopheles* Dof molecule, in which it is similarly part of a consensus sumoylation site. Vertebrate relatives of Dof lost lysine at this position and neither have consensus sumoylation sites in other parts of their sequence. This indicates that a potential sumoylation of the DBB domain is not conserved and that the DBB domain might have other essential functions beside receptor binding.

3.5 Sumoylation of Dof

Dof interacts with components of the sumoylation machinery in yeast (Battersby et al., 2003). In this work immunoprecipitation experiments confirmed the interaction of Dof with the SUMO conjugating enzyme Ubc9.

Dof is sumoylated in S2 cells upon co-expressing Ubc9 and SUMO. However, the interpretation of this result is not that simple. Dof is processed in S2 cells. In our sumoylation assay only the N-terminal Dof fragment becomes SUMO modified but not the full length molecule. The N-terminal fragment of Dof contains three consensus sumoylation sites including the one with the lysine at position 297 in the DBB domain. It is an important question of further studies which of these sites are targets for sumoylation.

Why is only the N-terminal fragment sumoylated in the S2 cell assay? Yeast two hybrid data indicate that Dof is able to form a “closed” conformation by intramolecular interaction of the N-terminal DBB domain with the C-terminal part of the molecule (Battersby et al., 2003). This conformation might inhibit SUMO attachment to the N-terminal part. Cleaving off the C-terminus might liberate the N-terminal fragment for SUMO conjugation. Important point is to clarify if the full length Dof molecule can be a substrate of sumoylation. For this, further experiments might be optional to try to increase the proportion of sumoylated Dof in S2 cells. The depletion of Ulp1 expression levels might be a successful tool, since Ulp1 RNAi increases the sumoylation of certain substrates in *Drosophila* S2 cells (Bhaskar et al., 2002; Smith et al., 2004). It might be an interesting point to test if Dof interacting partners that bind to the DBB domain and thereby might “attack” the “closed” conformation of Dof could promote sumoylation of full length Dof.

It has been shown for some proteins that binding interactions with other macromolecules enhanced their sumoylation (Chen, 2003; Hardeland, 2002). Co-expression of the FGF receptor as a

potential sumoylation-dependent interaction partner of Dof might therefore promote/stabilise SUMO attachment of Dof.

If sumoylation of Dof is essential for interaction with the FGF receptor it might need the reconsideration of the signalling dependence of this interaction. The example of the cAMP stimulation-dependent sumoylation of Mek1 in *Dictyostelium* shows, that cytosolic sumoylation might be a very temporary event and tightly regulated (Sobko et al., 2002). Thus, an interesting point of further investigation is if sumoylation of Dof is regulated by FGF signalling *in vivo*.

Our genetic studies on Ubc9 and SUMO for their involvement in mesoderm and tracheal development during embryogenesis were not conclusive. Zygotic mutants of the genes do not show mesoderm or tracheal specific defects which might be due to a high maternal contribution of the genes. Germ line clone analysis indicated in consistence with earlier findings that they might be required during oogenesis. Alternative genetic approaches like clonal analysis might help to reveal if these genes play a role in FGF signalling. However, sorting out the genetics of the Ubc9 gene is the most important prerequisite of any further work.

3.6 Phosphorylation sites in Dof

It has been shown that Dof is phosphorylated in the presence of an activated FGF receptor (Wilson et al., 2004). In this work gel filtration experiments showed that Dof is present in a complex with the FGF receptor preferentially in a phosphorylated form and non-phosphorylated form of Dof do not co-fractionates with the receptor. This indicates that Dof might become phosphorylated in complex with the FGF receptor probably the FGF receptor itself.

The sites of tyrosine phosphorylation were analysed in Dof. Phosphorylation sites were mapped by mutational analysis to the consensus tyrosine motifs for PI3K and Corkscrew binding at amino acid positions 486 and 515 respectively. Tyrosine at position 515 has been shown to be required for the binding of Corkscrew in an activated receptor-dependent manner (Petit et al., 2004), supporting that tyrosine 515 might be real target of phosphorylation. In addition, three tyrosine residues were identified as phosphorylation targets at positions 592, 613 and 629, which do not belong to known conserved tyrosine motifs. Their sequence environment is conserved in *Anopheles* Dof. Amino acid insertions between the conserved sequence patches in *Anopheles* indicate that the three tyrosine sites might represent three independent moduls in Dof. Two of these motifs are very similar and the same sequence motif is present two more times in the C-terminus of both *Drosophila* and *Anopheles* Dof. Protein database search with the consensus sequence motif generated from these eight related sites shows that this motif is present in other phosphorylated signalling molecules also in vertebrates. The *Drosophila* adaptor Disabled and the planar polarity protein Dishevelled were also found among the molecules bearing this motif. Both become tyrosine phosphorylated upon receptor tyrosine kinase signalling however, the phosphorylation sites of them are not fully characterised (Inobe et al., 1999; Le and Simon, 1998). Interestingly, the Dof homologue human BANK protein also shares this motif. It might be an interesting point whether this tyrosine motif represents a conserved consensus phosphorylation site. No less interesting is the question whether the phosphorylation of this site is utilised for protein-protein interaction and if so, which signalling molecules might interact with this motif.

The C-terminal part of Dof is also phosphorylated. It contains several Grb2 consensus binding sites; the novel motif is present two times, once overlapping with a consensus site for RasGAP or Crk binding. The identification which of these tyrosines is phosphorylated might provide further insight into signalling dependent interactions of Dof.

3.7 Functional relevance of the identified phosphorylation sites

Detailed structural-functional analysis of Dof was performed in two independent works (Petit et al., 2004; Wilson et al., 2004). Findings of the two works are summarised here briefly. Both have shown that the first 522 amino acids of Dof have significant biological activity. This Dof form is able to activate MAPK and induce tracheal migration. The mutation of the tyrosine of the consensus Corkscrew binding site in this Dof construct results a dead molecule, indicating that the binding of Corkscrew might be essential for Dof to activate MAPK. Mutation of the PI3K binding site has no effect on the function of this construct. However, introducing the point mutation of the Corkscrew binding site in full length Dof or introducing an internal small deletion which removes the PI3K and Corkscrew sites has little effect on the activity of the molecule. On the other hand, if the point mutation of the Corkscrew site is present in a Dof construct lacking the last 200 amino acids this form shows a strong reduction in its capacity to rescue tracheal branching and its activity in the mesoderm appears even more affected. Nevertheless, it still shows some activity. The C-terminal part of Dof contains multiple Grb2 binding sites, which might be also utilised in Dof to activate the MAPK cascade. The lost importance of the Corkscrew binding site in the full length molecule might indicate a redundancy in the use of the Grb2 and Corkscrew interactions by Dof to activate the MAPK cascade. This is further supported by the strongly reduced activity of Dof upon the additional deletion of the potential Grb2 binding sites. The fact that the mutation of the Corkscrew binding site in a Dof molecule containing the first 600 amino acids is sufficient to eliminate the activity of the molecule completely but the same mutation in the context of the first 802 amino acids (along with two additional tyrosine point mutations in the PI3K and N-terminal Grb2 consensus binding sites) results a molecule with a weak but significant activity points out a role of the region between amino acids 600 and 802 in Dof. This region contains the coiled coil domain but the deletion of this domain does not alter the activity of full length Dof. However, it has not been tested in combination with other mutations. Interestingly, this region contains three tyrosines identified as phosphorylation targets. Interesting question is for further analysis if the phosphorylation of these tyrosines is responsible for the remaining activity of the described mutant Dof construct. An important question is not addressed so far in these studies, namely whether this mutant construct maintains some capacity to activate MAPK or the residual activity of the molecule is MAPK independent. This might help narrow down the function of the 600-802 region of Dof and thus probably the function of the phosphorylated tyrosine residues.

These structural-functional studies also point out that these three tyrosines are not absolutely essential in the molecule, since the first 522 amino acids are the minimal requirement for biological activity. This might indicate a redundant function or other functions which might gain importance in the context of the full length molecule.

3.8 Phosphorylation as a landmark of full length Dof

During the investigation of the consequences of Dof cleavage in S2 cells we found striking differences in the phosphorylation state of Dof and its cleavage derivatives. Whilst the full length form of Dof is efficiently phosphorylated in the presence of an activated receptor both the N- and C-terminal fragments of Dof show only very weak if any phosphorylation. This raises the possibility that phosphorylation might be a landmark of signalling activity of Dof and prevents the cleavage of Dof by caspases. Alternatively, Dof cleavage products might be an immediate substrate of phosphatases which might allow generating a new subpopulation of Dof in signalling cells with distinct interaction properties. Further analysis using phosphatase inhibitors might help to distinguish these two possibilities. This is the first indication for a potential crossregulation of the phosphorylation state and the caspase-mediated cleavage of Dof.

Interestingly, the one identified phosphorylation site in Dof is mapped to the tyrosine residue at position 592 which is only 5 amino acids apart from the caspase cleavage site in Dof (amino acid position 587) and thus, the phosphorylation of this site might have an allosteric inhibitory effect on the cleavage of the molecule. However, no significant differences can be observed in the accumulation of cleavage products of Dof in the presence and absence of an activated FGF receptor in S2 cells, which would support such a function of this phosphorylation.

3.9 Dof is a substrate for caspases in S2 cells – and in flies?

Dof is cleaved in S2 cells. This project revealed that the cleavage is caspase dependent. Dronc activity is essential for this cleavage. However, the sensitivity of the cleavage against the caspase inhibitor protein p35, which cannot inhibit Dronc, indicates that Dronc might act indirectly on Dof by activating downstream effector caspases. A potential candidate might be Dcp-1, since dcp-1 RNA interference reduced Dof processing in S2 cells. Mutational analysis indicates that the consensus caspase cleavage site at position 584-587 in Dof might be utilised for cleavage. It might be an important point to analyse if Dof is processed *in vivo*.

What might be the *in vivo* relevance of caspase cleavage of Dof? Signalling molecules often serve as survival signals by maintaining the signalling capacity of the cells. Signalling is an inhibitor of apoptosis (Gorski and Marra, 2002; Meier et al., 2000). Thus, cleavage of Dof might be a potent tool to downregulate FGF signalling. Dof function has been investigated in early mesoderm development and in tracheal morphogenesis. Apoptosis has not been described as essential part of the developmental program of these tissues. However, recent findings indicate that apoptosis might be involved in the proper shaping of the developing tracheae (A. Bilstein, personal communication) and it was also reported that upon Rac hyperactivation cells delaminate from the tracheal epithelium and undergo apoptosis (Chihara et al., 2003). Thus, Dof cleavage might be essential to allow apoptosis induction in tracheal cells. However, a Dof construct bearing only the first 522 amino acids has significant biological activity, indicating that a potential cleavage of Dof at position 587 might not be efficient to downregulate Dof activity. Growing body of evidence emerges that regulated caspase activity is also required in non-apoptotic developmental processes (Geisbrecht and Montell, 2004; Huh et al., 2004; Huh et al., 2004), although molecular targets of these caspase activities are not uncovered yet. In *in vivo* functional analysis the caspase cleavage site-mutant Dof transgene had comparable capacity to wild type Dof transgenes to rescue mesoderm and tracheal development, indicating that processing of Dof might not be required for Dof function. These observations on Dof function together would fit neither to an apoptotic nor to a non-apoptotic role of Dof cleavage. It cannot be excluded that Dof cleavage has a function which is masked due to the overexpression of the transgenes or that cleavage is required in other developmental processes which were not analysed in this work. Further genetic analysis of caspases and regulators of caspase activation as well as monitoring *in situ* caspase activity also in other dof-dependent developmental processes might help to circumvent this problem and allow an insight into the physiological role of Dof cleavage. Nevertheless, in the next chapter a potential role of caspase cleavage in the translational control of Dof is discussed. This might fit to our observations in the sense that such a function could not have been revealed by the functional studies performed so far.

3.10 Potential translational regulation of Dof and its relationship to caspase cleavage

The ribosomal protein S10 was found as an out-of-frame interactor of Dof in a yeast two hybrid screen. In this work immunoprecipitation experiments verified it as a real Dof interactor. The

interaction site for rpS10 is mapped to the region between amino acids 446 and 522. Strikingly, in Dof deletion mutants which lack C-terminal parts of the molecule the presence of this region correlates with a strong increase of Dof protein levels in S2 cells. This difference in protein levels is neither due to different transcription levels nor due to different stability of the mutant Dof proteins, since both mRNA abundance and protein half life are comparable in case of these constructs. This indicates that the presence of the rpS10 binding site in these constructs correlates with a higher translational rate of the construct. This might indicate a potential contribution of rpS10 in the translational regulation of Dof.

What might be the reason that wild type Dof and other mutant Dof constructs bearing the rpS10 binding sites but also containing C-terminal parts of the molecule do not show such an elevated protein levels? One possible explanation might be that the C-terminal half of Dof has an inhibitory effect on rpS10 binding. This might indicate a potential role of caspase cleavage in liberating the N-terminal part of the molecule for the interaction with the ribosomal protein. Thus, full length Dof might have to be processed first to be able to interact with rpS10 which might explain lower translation levels compared to the deletion construct with “free” rpS10 binding site on its C-terminal end. Based on this hypothesis a decrease in Dof protein levels would be expected upon the inhibition of caspase activity in the cells. In caspase RNA interference and DIAP1 overexpression experiments we have indications for such an effect but further comparative quantitative analysis is required to test the significance of this phenomenon. The analysis of the caspase site mutant Dof construct does not support a difference in protein levels compared to wild type Dof. However, potential differences in mRNA and protein levels derived from different vectorial background cannot be excluded.

This 70 amino acid region contains also the PI3K and Corkscrew binding sites which are targets for phosphorylation. An additional interesting question might be whether and how phosphorylation influences the interaction of Dof with rpS10. Further experimental work is required to narrow down the palette of potential interconnections of phosphorylation, caspase cleavage and translational regulation of Dof.

What mechanism might be responsible for such a translational regulation? Several studies indicate that ribosomal proteins can have additional functions in the cell besides acting as structural components of the ribosome (Bhat et al., 2004). The ribosomal protein L13a acts as a transcript-specific translational repressor of ceruloplasmin upon a regulated release from the 60S ribosomal subunit, proposing a model in which the ribosome functions not only as protein synthesis machine, but also as a depot for regulatory proteins that modulate translation (Mazumder et al., 2003). For transcript-specific translational regulation some specific recognition motif is necessary within the mRNA which distinguishes it from other transcripts. L13a for instance binds to a specific motif in the 3' UTR region of ceruloplasmin. However, in the Dof expression constructs only the protein coding sequence is present. Certainly, the identified interaction between rpS10 and Dof cannot be sufficient to explain transcription-specific translational regulation of Dof. Thus, interesting further questions are if Dof itself or rpS10 are able to bind to the coding part of dof mRNA or to other mRNA-binding proteins. No less interesting question is if Dof binds rpS10 as part of the ribosome or a ribosomal release of S10 is required for the interaction. An additional point is to the complexity that the *Drosophila* genom encodes two rpS10 genes. The identified yeast interactor clones represented only the one gene, rpS10b. Although the two gene products are highly similar it might be an interesting question if both S10 proteins can interact with Dof.

Not much known about rpS10, which would indicate a function in transcript-specific translational regulation. It is unique for eukaryots, considered as one of the four core components of the S40 ribosomal subunit (Malygin et al., 2000) and involved in the formation of the binding surface for the translation initiation factor eIF-3 (Westermann and Nygard, 1983). Besides these structural studies rpS10 was reported to be transcriptionally upregulated in certain diseases and cancer cells (Frigerio et al., 1995; Maak et al., 2001) and its transcription is differentially regulated

during different developmental stages in *Dictyostelium* (Tapparo et al., 1998). The only indication for a potential additional role of rpS10 might be that it interacts with human securin/PTTG. Human rpS10 binds to the C-terminal 70 amino acids of securin/PTTG (Pei, 1999). Interestingly, an interaction of securin with the human homologue of the DnaJ chaperon mapped to the same amino acid residues in the molecule. Although no functional relevance is known of these interactions it might be an interesting question to address whether this shared interaction region mutually excludes simultaneous binding of the two interactors. This would help to reveal whether the accessibility of an rpS10 binding surface in securin might be regulated by chaperons. On the analogy of this we could imagine a hypothetical model in which C-terminal parts of Dof might inhibit rpS10 interaction and accessibility of this binding site might be regulated by the caspase-mediated cleavage of the molecule.

3.11 The role of different Ras effector pathways in FGF signalling

The Ras-MAPK pathway is essential in FGF-dependent developmental processes. However, it cannot be responsible for the diverse cellular responses. It is essential to reprogram gene expression patterns which determine cell fates but rapid and dynamic cytoskeletal rearrangements which give rise to changes in cell shape, cell adhesion and cell motility might require other FGF-dependent effectors. Multiple factors have been described which contribute to these processes, like E-cadherin and Rac in cell adhesion regulation or Rho and Cdc42 and their regulators in filopodia formation and migration (Chihara et al., 2003; Lee and Kolodziej, 2002; Schumacher et al., 2004; Smallhorn et al., 2004; Wolf et al., 2002). Some of them act in parallel to FGF signalling and others are regulated in an FGF-dependent manner, but how FGF receptor transmits the signal towards these effectors is not known. Ras has several known effectors which might serve a link to these processes; thus, we took an approach and investigated which other Ras-induced pathways might be utilised. Constitutively active effector loop mutant Ras proteins, constitutively active forms of components of the Raf and Ral effector pathways were tested for their capacity alone or in different combinations to rescue *dof* mutant phenotypes or to cause any dominant effects in mesoderm and tracheal development. In addition, dominant negative forms of the same signalling molecules were tested for their effect on these processes.

3.11.1 Mesodermal studies of Ras effectors

Studies on the formation of Eve-positive clusters in the mesoderm are a read-out of independent action of FGF signalling: dorsal migration of mesodermal cells and cell fate differentiation. This work and earlier studies showed that mesodermal cells of *dof* mutant embryos reached dorsal regions and were able to express high levels of activated MAPK upon the expression of constitutively active forms of Ras, Raf or Ral (Vincent et al., 1998; Wilson, 2004). An important issue of further analysis is whether these constructs play an active role in the “migratory” phase of FGF signalling or their effect is restricted to cell differentiation. Analysis of *pebble* mutant embryos that affect mesoderm migration in an FGF signal-independent manner and thus, do not interfere with FGF-dependent cell differentiation showed that some cells of the collapsing epithelial tube occasionally can reach dorsal positions and differentiate into Eve-positive cell clusters; albeit in a very uncoordinated fashion. Since the expression of the listed transgenes led to a coordinated appearance of dorsal mesodermal clusters marked by Eve or activated MAPK, it raises two possibilities. Either loss of function mutation of *dof* leads to a much less severe migration defect than that of *pebble* or these transgenes have a positive input on dorsal migration of the mesoderm. Markers of dorsal mesodermal cell fate, the expression of which depends only on Wingless and Dpp

signalling and thus, are a sole read-out of the efficiency of mesoderm migration might be a useful tool to discriminate these possibilities. Since this is not clarified yet, from here on I will focus on the aspects of FGF-dependent cell fate differentiation in the mesoderm.

Although all three constitutively active transgenic constructs, Ras, Raf and Ral can activate MAPK in the presumptive Eve-cluster, only activated Ras is able to turn on Even-skipped expression. Not even the simultaneous expression of activated Raf and Ral can result Eve-positive cells, indicating that additional Ras-dependent factor(s) is required to turn on Eve. This factor might act either on the level of MAPK activation in a Raf-independent manner or on the regulation of MAPK dependent gene activation or on a crossregulatory level of FGF and Dpp, Wg or Notch signalling. Constitutively active Ras effector loop mutants, which are able to activate MAPK (RasV12,S35 and RasV12,G37, the “Raf- and Ral-specific” Ras forms respectively) can also induce Eve expression in the mesoderm, indicating that the binding of the additional factor is not affected by these point mutations. Thus, the requirement of PI3K can be excluded. The failure of RasV12,C40, the “PI3K specific” Ras effector loop mutant, to turn on Eve expression might be either due to the fact that this unknown factor cannot bind to this Ras form or it might require basal level of MAPK activity for its action. The identity of this factor is not known at present. It cannot also be excluded completely that the observed differences are due to differences in the strength of the transgenes to activate MAPK. Finally, expression of dominant negative Ral construct indicated that Ral might play a role in early phases of mesoderm development. Considering the regulatory role of this GTPase in filopodia formation it might be an interesting question to re-address, after the unclear aspects of mesoderm spreading in *dof* mutants (see above) had been resolved.

3.11.2 Studies of Ras effectors in the tracheae

The same set of mutant signalling molecules behaved differently in the tracheae of *dof* mutant embryos compared to the mesoderm. However, the assay was also different, since we concentrated on the capability of the constructs to induce tracheal branching and thus, cell migration.

Constitutively active Ras is effective to induce migration and “Raf specific” RasV12,S35 does so too, although with a remarkably less efficiency. It cannot be excluded that the two alleles have different affinity for Raf binding as reported in mammalian studies (Rodriguez-Viciano et al., 1997), however same transgenic insertions were found to have comparable activity to each other in flies (Therrien et al., 1999). In contrast to the mesoderm, the “Ral specific” RasV12,G37 has a strongly reduced capacity to induce tracheal branching. In further contrast to the mesoderm, activated Raf has also the capacity to induce branching, albeit in a very reduced and uncoordinated fashion. The fact that activated components of the Ral effector pathway cannot rescue tracheal branching in *dof* mutant embryos and they do not synergise with activated Raf in the induction of tracheal migration indicates that the branching activity of RasV12,G37 might not be due to its Ral activating capacity but maybe due to a residual Raf binding. This suggests also that the moderate rescue capacity of RasV12,S35 compared to RasV12 might not be due to the lack of Ral activating capacity. Nevertheless, activated components of the Ral pathway are able to induce ectopic branching upon overexpression in wild type tracheae, indicating that Ral might be involved in tracheal migration but it might rely on the activity of an FGF-dependent factor independent from Raf and maybe activated by Ras. To this latter statement it is a critical point of further analysis to test if RasV12G37 has the capacity to activate MAPK in the tracheae, which would be an indication for residual Raf binding. It might be an interesting object of further studies to test if Ral activity is able to induce filopodia formation in tracheal cells defective in FGF signalling and the lack of branching is only because these extensions are abortive and cells fail to migrate out or if the capacity of activated Ral to induce cell outgrowth relies obligatorily on an unknown FGF-dependent factor.

The analysis of the “PI3K specific” effector loop mutant RasV12C40 showed that PI3K activation is not sufficient to induce tracheal branching. Interestingly, effector loop mutants RasV12,S35 and RasV12,G37 which lost the capacity of PI3K binding showed both a significantly reduced capacity to induce tracheal migration. Thus, it is an interesting point to test if PI3K activity might contribute to effective migration. It has to be noted, that zygotic mutants for different subunits of PI3K do not show tracheal defects (R.Wilson, personal communication). However, the high maternal contribution of the gene might be sufficient to overcome zygotic requirement of the gene.

3.11.3 Implications for a role of Rap1

An unexpected result of our genetic analysis on the role of Ras in FGF signalling is that dominant negative form of the molecule fails to affect FGF signalling in the mesoderm and in the tracheae. It significantly inhibits EGF dependent processes in the mesoderm (formation of DA1 muscles), which validates the functionality of the construct. However, this might be only an issue of differences in the threshold levels of MAPK activity to induce cellular differentiation. The possibility of different thresholds might derive from the fact that DA1 progenitors require a dual input of EGF and FGF signalling for differentiation which might result higher Ras activation levels whereas EPCs require only FGF dependent Ras activation which might provide only lower activated Ras levels.

In the tracheae dominant negative Ras had neither effects on FGF nor on EGF signalling. An important question might be to distinguish whether this phenomenon is specific for Ras or other dominant negative molecules as DN-FGF and -EGF receptors might also fail to interfere with endogenous signalling, indicating that levels of the tissue specific expression might not be sufficient.

Based on this non-conclusive results and on the fact that so far only overexpression studies with the constitutively active protein indicated an essential role for Ras in FGF signalling in flies we addressed the question to what extent Ras might be the endogenous transducer of the FGF signal. Emerging number of studies indicate that the closely related Ras family GTPase Rap1 acts through the similar set of effector pathways as Ras. The potential role of Rap1 was tested by analysing the effect of dominant negative and constitutively active forms of the protein on mesoderm and tracheal development in wild type and *dof* mutant embryos respectively. One conclusion of our results is that Ras might have essential functions since activated Rap1 shows significant differences to Ras in its capacity to rescue FGF-dependent developmental processes; first of all it has in the tracheae a weak activity. In addition, our results indicate that Rap1 might be involved in both developmental processes although maybe to different extent. It might be an object of further studies what effectors are utilised by Rap1 and how Ras and Rap1 might interact to concert downstream events of FGF signalling.

As a last interesting remark, Dof contains also a consensus binding site for the adaptor Crk which can specifically activate Rap1 through its binding to the Rap1GEF C3G. This site is located in the C-terminal part of the molecule where also the consensus Grb2 binding sites are present, interaction sites for potential Ras activation. However, the C-terminal tail of Dof is not essential; thus, these binding sites are essential neither. As discussed above, Corkscrew might be involved to transduce the signal from Dof to Ras. Interesting subject of future projects might be to reveal what mechanisms might be used to activate Rap1 in FGF signalling.

4. Materials and Methods

DNA constructs

Expression vectors

The metallothionein inducible pRmHA-3 vector (Bunch et al., 1988) (kindly provided by M. Reth) was used for protein expression in *Drosophila* S2 cells with the exception of the construct pAC-HisSUMO^{GG} (a gift from F. Schweisguth). The pmetHA vector was generated by introducing an oligonucleotide cassette coding for the HA epitope tag with the DNA sequence AATTCGACCATGGCTTACCCATACGATGTTCCAGATTACGCTAGCCCATGGTAC between the EcoRI and KpnI sites of the pRmHA-3 vector. The pRmHA-3-RI vector was a derivative of pRmHA with a unique XbaI site in the multi-cloning region by destroying the second XbaI site in the vector (kindly provided by R. Wilson). For transgenic expression in flies cDNAs were cloned into the pUAST vector (Brand and Perrimon, 1993).

Dof constructs for S2 cell expression

Mutant dof constructs pRm-FLAGDof[168-1012], pRm-FLAGDof[1-446], pRm-FLAGDof[1-522], pRm-FLAGDof[1-802], pRm-FLAGDofΔ233-449, pRm-FLAGDofΔ361-449, pRm-FLAGDofΔ233-364, pRm-FLAGDof^{P13K}, pRm-FLAGDof^{CSW}, pRm-FLAGDof^{P13K,CSW}, pRm-FLAGDof^{drk}, pRm-FLAGDof^{drk,P13K,CSW}, pRm-FLAGDof[1-802]^{P13K}, pRm-FLAGDof[1-802]^{CSW}, pRm-FLAGDof[1-802]^{P13K,CSW}, pRm-FLAGDof[1-802]^{drk}, pRm-FLAGDof[1-802]^{drk,P13K,CSW}, pAlterFLAGDof, pAlterFLAGDof^{P13K}, pAlterFLAGDof^{CSW} and pAlterFLAGDof^{P13K,CSW} were generated as described (Wilson et al., 2004) and kindly provided by R. Wilson.

pRm-FLAGDof and pRm-HADof were generated by inserting the *EcoRI/SpeI* fragments of epitope-tagged Dof constructs 5'FLAG-Dof.pNB40 and 5'HA-Dof.pNB40 (provided by R. Wilson) into the *EcoRI/XbaI* partially digested pRmHA-3 vector.

pRm-FLAG:Dof:2xHA was generated by inserting the *NcoI/XbaI* dof fragment of RW471 (provided by R. Wilson) between the same sites of the pRmHA-3-RI vector.

pRm-FLAGDof^{K297R} was generated by cloning the *EcoRV/SacI* dof fragment of pAlterFLAGDof^{SUMO K.O.#3} (A288 in (Battersby, 2001) and provided by A. Battersby) into pRm-FLAG:Dof:2xHA digested by *EcoRV/SacI*.

pRm-FLAGDof[1-802]Δ233-449, pRm-FLAGDof[1-802]Δ361-449, pRm-FLAGDof[1-802]Δ233-364 and pRm-FLAGDof[1-802]K297R were constructed by inserting the *EcoRV/SacI* dof fragment from pRm-FLAGDofΔ233-449, pRm-FLAGDofΔ361-449, pRm-FLAGDofΔ233-364 and pRm-FLAGDofK297R into the *EcoRV/SacI* digested pRm-FLAGDof[1-802] vector respectively.

Mutant dof constructs bearing point mutations of different amino acid residues were generated by *in vitro* mutagenesis applying the pAlter system (Promega), with the exception that the *Escherichia coli* strains JM109 and ES455 were used. The presence of the desired mutation was confirmed by DNA sequence analysis. The following mutagenesis primers were used:

STOPXbaI: ACCGAGGACTAAAATCTAGAGGCTTCCGTGG
 592F: GCCACTCTTTCAGACCGACAAATTCAG
 613F: TGGGCAATGACTTTGTGCTGCAGCCCTC
 629F: TGATGGCAACTTTTTGTTCCAGCCATCG
 654F: AGACAGCCCGGATTTGGAACACTGAAGC
 726F: CGACGCGAGTTCGAGCGGATCCAGGAGC
 D587E: GACGAAGTGAAGCGGCGCCACTC

pRm-FLAGDof[1-522]^{PI3K}, pRm-FLAGDof[1-522]^{csw} and pRm-FLAGDof[1-522]^{PI3K,csw} were generated by mutagenesing pAlterFLAGDof^{PI3K}, pAlterFLAGDof^{csw} and pAlterFLAGDof^{PI3K,csw} with the STOPXbaI primer followed by *EcoRV/XbaI* digestion and ligation of the 1.0 kb fragments into the pRm-FLAGDof[1-522] vector cut with the same enzymes.

pRm-FLAGDof[1-522]^{drk} and pRm-FLAGDof[1-522]^{drk,csw} were created by the ligation of the 0.6 kb *EcoRI/EcoRV* fragment of pRm-FLAGDof^{drk} with the *EcoRI/EcoRV* digested pRm-FLAGDof[1-522] and pRm-FLAGDof[1-522]^{csw} vectors respectively.

pRm-FLAGDof[1-522]^{drk,PI3K} was generated by the one-step ligation of the 0.6 kb *EcoRI/EcoRV* fragment from pRm-FLAGDof^{drk} and the 1.0 kb *EcoRV/XbaI* fragment from pAlterFLAGDof^{PI3K,STOPXbaI} into the *EcoRI/XbaI* digested pRmHA-3-RI vector. pRm-FLAGDof[1-522]^{drk,PI3K,csw} was cloned the same way with the exception that the 1.0 kb *EcoRV/XbaI* fragment of the pAlterFLAGDof^{PI3K,csw,STOPXbaI} construct was used.

pRmFLAGDof[1-802] constructs with pointmutations 592F; 613F; 629F; 654F,726F and 592F,613F,629F were generated by mutagenesing pAlterFLAGDof with the appropriate primers followed by *SfiI/SacI* digestion and ligation each of the 2.3 kb fragments into the pRm-FLAGDof[1-802] vector digested with *SfiI/SacI*.

pRmFLAGDof[1-802]^{PI3K,csw} constructs with mutations 592F; 613F; 629F; 592F,613F; 592F,629F; 613F,629F and 592F,613F,629F were generated by the mutagenesis of pAlterFLAGDof^{PI3K,csw} with the appropriate primers followed by *StuI/SacI* digestion and ligation each of the 2.3 kb fragments into the pRm-FLAGDof[1-802] vector digested with *StuI/SacI*.

pRmFLAGDof^{PI3K,csw,592F,613F,629F} was generated by the insertion of the 1.5 kb *StuI/SacI* fragment of pAlterFLAGDof^{PI3K,csw,592F,613F,629F} into the pRm-FLAGDof vector digested with the same enzymes.

pRm-FLAGDof^{D587E} was generated by *in vitro* mutagenesis of pAlterFLAGDof with the mutagenesis primer D587E followed by *EcoRV/XhoI* digestion and ligation of the 1.8 kb fragment into the *EcoRI/XhoI* cut pRm-FLAGDof vector.

pUAS-FLAGDof^{D587E}

A 2.4 kb *EcoRI/XhoI* fragment was isolated from the pAlterFLAGDof^{D587E} plasmid and inserted into the pUAS-FLAGDof vector (obtained from R. Wilson) digested with the same enzymes.

***i*-clones for S2 cell expression**

Dof yeast two hybrid interacting clones i6, i8^{in frame} (A308), i14, i19, i25, i51, i77, i133, i163, i184, i188, i234 and i249 were described previously (Battersby, 2001) and generously provided by A. Battersby. To put the *i*-clone i14 in the same reading frame as the upstream Gal4 activating domain in the pAct2 vector a one nucleotide frame shift was generated by linearising the plasmid with *BamHI* followed by relegation after filling up the 5' overhangs by T4 DNA polymerase. The derived form was called i14^{in frame}.

Clones i6, i8^{in frame}, i14^{in frame}, i19, i25, i51, i77, i133, i163, i184, i188 and i234 were cut from the pAct2 vector with *NheI/XhoI* and inserted into the *NheI/SalI* digested pmetHA vector. To achieve a two nucleotide frameshift i249 was cloned into pmetHA by inserting the *SmaI/XhoI* i249 fragment into the *SmaI/SalI* vector.

pmetHA-CG11275 was generated by PCR amplification of CG11275 from the cDNA clone RH28808 with the primers ACGGTACCAAACCATGAGCATCTCGGCGGTGG and CGGGTACCAAGTGGCTACACCAGGTTGGC having each a *KpnI* site in the overhang sequence. After *KpnI* digestion of the PCR product it was inserted into the *KpnI* linearised pmetHA vector. Sequence fidelity was confirmed by DNA sequence analysis.

Heartless constructs for S2 cell expression

pRm-Htl^{FL}-2xHA was generated as following: RW433 contained the sequence coding for the cytoplasmic part of Htl fused to a double HA epitope tag on its C-terminus (kindly provided by R. Wilson). The htl cDNA clone pSP73 (kindly provided by A. Michelson) was digested with *Bgl*II/*Bst*XI and the 1.65 kb fragment was inserted into the vector RW433 cut with *Bgl*II/*Bst*XI. The 2.3 kb *Eco*RI/*Xba*I fragment of the derived construct was ligated into the pRmHA-3-RI vector digested with the same enzymes.

pRm-λHtl-2xHA and pRm-λHtl^{K.D.}-2xHA were generated as described (Wilson et al., 2004; Wilson, 2004) and provided by R. Wilson.

Cell culture, transient transfection, immunoprecipitation and Western blot analysis

Stable transfectant *Drosophila* S2 cell lines expressing N-terminally FLAG-tagged Dof under the control of the actin 5C promoter (actin::FLAG-Dof) alone or together with a constitutively active form of Breathless or Heartless under the control of a heat shock inducible promoter (hs::λ-Btl and hs::λ-Htl-2xHA respectively) were generated and cultured as described previously (Wilson et al., 2004).

Drosophila S2 cells were cultured and transiently transfected as described (Rolli et al., 2002). 10⁶ cells were used for a transfection. The expression of the pRmHA-3 vector derived constructs was induced by the addition of CuSO₄ to a final concentration of 1 mM. After 24 hours the cells were harvested, washed once in ice-cold phosphate buffered saline and lysed in 0.5 ml PLC-TX0.5 buffer (10% (v/v) glycerol, 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% (v/v) Triton X-100, 1.5 mM MgCl₂, 1mM EGTA, 200 μM Na₃VO₄, 10 mM NaF supplemented with Protease inhibitor Cocktail (Sigma)) for 45 minutes followed by centrifugation at 22,160g for 20 minutes at 4 °C. For SDS-PAGE, supernatants were boiled in 1 x SDS-PAGE loading buffer and volumes corresponding to 7.5 μl of the lysate were loaded into the lanes. For immunoprecipitation, 90 μl of lysates were incubated overnight at 4 °C with anti-FLAG M5 (Sigma), anti-HA 12CA5 (Boehringer Mannheim) or anti-Dof (Vincent et al., 1998) antibodies. Protein G sepharose beads (Amersham Pharmacia), that had been incubated in 5% (w/v) BSA in PLC-TX0.5 were added to the samples and incubated together for a further two hours at 4 °C followed by three washes, five minutes per wash, in 0.5 ml of PLC-TX0.5. Beads were boiled in 30 μl 2 x SDS-PAGE loading buffer and the supernatants were fractionated by SDS-PAGE (10% (w/v) polyacrylamide gel) loading 10 μl of the sample into one lane followed by Western blotting according to standard methods (Hybond ECL nitrocellulose membrane and ECL detection kit from Amersham Pharmacia) using anti-FLAG M5 (1:4000), anti-HA 3F10 (1:3000) (Boehringer Mannheim), anti-His₆ (1:500) (Roche), anti-phosphoTyrosine 4G10 (1:4) (courtesy of F. Sprenger) and anti-SUMO (1:1000) (kindly provided by F. Schweisguth) primary antibodies and appropriate horse radish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.)

Size exclusion chromatography

10 ml confluent culture of stable transfectant S2 cells expressing actin::FLAG-Dof, actin::FLAG-Dof and hs::λ-Btl or actin::FLAG-Dof and hs::λ-Htl-2xHA were incubated at 37 °C for 30 minutes to induce expression of the proteins under the control of a heat shock promoter and allowed to recover for 4 hours at 22 °C before lysis. Cells were harvested and lysed in 150 μl PLC buffer (10% (v/v) glycerol, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 1.5 mM MgCl₂, 1mM EGTA, 200 μM Na₃VO₄, 10 mM NaF supplemented with Protease inhibitor Cocktail (Sigma)) for 30 minutes followed by centrifugation at 22,160g for 15 minutes at 4 °C.

Supernatant was taken and re-centrifugated at 45,000rpm for 30 minutes at 4 °C. ÄKTAexplorer chromatography system was used with a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) for size exclusion chromatography of the lysate. Column was equilibrated with PLC buffer containing only 0.05% (v/v) Triton X-100 and calibrated with standards: thyroglobulin, 669kDa; apoferritin, 440 kDa and alcohol-dehydrogenase, 150 kDa. 100 µl of prepared lysate was applied to the column and was eluted with the same buffer as used for equilibration at a flow rate of 0.2 ml/min. 0.5 ml fractions were collected and proteins were precipitated by the addition of 100 µl of 72% trichloric acid. Pellet was washed twice in ice cold acetone and re-dissolved in 25 µl 2x SDS-PAGE loading buffer. Samples were boiled and separated by SDS-PAGE. The position of FLAG-Dof, λ-Htl-2xHA and λ-Btl in the elution profile was determined by Western blot analysis using antibodies against FLAG, HA and phospho-tyrosine residues respectively.

RNAi in *Drosophila* S2 cells

dsRNAs were prepared as described (Worby, 2001). Nucleotides 152-937 of the dcp-1 ORF, 84-763 of the drice ORF and 1-765 of the dronc ORF were PCR amplified with sequence specific primers each containing overhangs with T7 RNA polymerase binding site (TTAATACGACTCACTATAGGGAGA) using the plasmid constructs pacFLAG-Dcp-1(C-A), pacFLAG-Drice(C-A) and pMT-V5/HisDronc (kindly provided by P. Meier) respectively.

Primer sequences following the T7 RNA polymerase binding site:

dcp-1_5': -GCCCTTGCCGGCCAACAAGTTTG
dcp-1_3': -AGCGCAGTATGCGCGTCAGCATGGA
drice_5': -TGCCCTGGGCTCCGTGGGATCCG
drice_3': -ATCCAGGAACCGTCGAGTAGGCG
dronc_5': -ATGCAGCCGCCGGAGCTCGAGATTGG
dronc_3': -CGAGGAGGTCACCATTGTCAGAAG

For in vitro transcription MEGAscript T7 kit (Ambion) was used. 15 µg of dsRNA was added to 2×10^6 cells in 1ml serum free *Drosophila* S2 media (Gibco) after removing culture media supplemented with 5% FCS and washing the cells once with serum free *Drosophila* S2 media. Cells were incubated for 1 hour, medium was removed and transient transfection was directly applied to the cells as described above. Cells were harvested 72 hours after dsRNA treatment. Control mock RNAi was performed the same way without adding dsRNA to the solution.

Caspase inhibition in transiently transfected *Drosophila* S2 cells

Cells were transiently transfected as described above. 2 hours prior harvest cells were incubated with the drug Z-VAD dissolved in DMSO (Sigma) in a final concentration of 20 µM or 50 µM. As a control same volume of DMSO was added to the cells. Cells were harvested and lysed in PLC-TX0.5 buffer. Alternatively Z-VAD was added to the lyses buffer in a final concentration of 20 µM. pIE1-4-p35 (kindly provided by G. Jones) construct was transiently transfected to S2 cells in equal (300ng), in double or in four times higher amount than the co-transfected pRm-FLAGDof.

Metabolic [³⁵S]-methionin radioactive labelling of *Drosophila* S2 cells

Cycloheximide was added to 0.5 ml cultures of *Drosophila* S2 cells (10^6 cells/ml) in a final concentration of 0, 5, 10, 20, 50 and 100 mM. After 1 hour incubation time 15 µCi [³⁵S]-methionin was supplied to each sample and incubation was continued for further 4 hours. Cells were harvested

and directly boiled in 2 x SDS-PAGE loading buffer. SDS-PAGE was carried out and autoradiogram of vacuum dried polyacrylamide gel was developed.

Dof protein stability analysis in *Drosophila* S2 cells

S2 cells were transiently transfected with different mutant Dof constructs. At time point of induction cells were split into six parts each of a volume of 0.5 ml in 24-well plates. 24 hours after induction cycloheximide (CHX) was added to each well in a final concentration of 20 mM. Cells of individual wells were harvested and lysed in 83 µl PLC-TX0.5 buffer at time points 0, 1, 2, 4, 8 and 24 hours after CHX addition. SDS-PAGE of whole cell lysates was performed followed by Western blot analysis.

Real-time RT-PCR

Equal amount (300ng) of different mutant dof DNA constructs were transiently co-transfected with 300ng of pDeGFP in *Drosophila* S2 cells as described above. Cells were harvested and split into 3 parts. The first part containing 500 µl of the 3 ml cell suspension was lysed in 83 µl PLC-TX0.5 buffer and Dof protein level of whole cell lysates was analysed on Western blot. In parallel, RNeasy Mini kit (Qiagen) was used to prepare total RNA from the two other parts containing equal volume (1250 µl each) of cell suspension in two independent reactions according the manufacturer's instructions. Samples were homogenised by passing the lysate 5 times through a 20-gauge needle fitted to an RNase-free syringe. RNA was eluted from the column two times with 40 µl RNase-free water. After an RNase-free DNase I (Roche) treatment for 30 minutes at 37 °C RNA was precipitated with LiCl and redissolved in 30 µl RNase-free water. Reverse transcription was carried out using ThermoScript reverse transcriptase kit (Invitrogen) according the manufacturer's instructions following the determination of the RNA concentration. 1 µg of total RNA of each sample was reverse transcribed in a 20 µl reaction using 50ng random hexamer primers and in a separate reaction 10 pmol dof specific primer with the sequence TGCACAGGCGATTCAGGCCC. For negative control reverse transcription reaction was carried out without the addition of the enzyme. Random hexamer primers were first elongated in a 10 minutes incubation step at 25 °C followed by 45 minutes incubation at 50 °C. Reverse transcription with dof specific primers was carried out directly at 50 °C for 45minutes. 2 µl of each reaction was used for real-time PCR.

Quantitative, real-time PCR was performed on a Light Cycler I machine supported by the LightCycler software version 3.0 (Roche). PCR was carried out using the QuantiTect™ SYBR® Green PCR kit (Qiagen) according the manufacturer's instruction with the following primers:

dof amplification RTdof_5' AGGTGCAGATCAGCAATATCC
RTdof_3' ATCCCTCGGTGCAGCTAGAG
GFP amplification RTGFP_5' GCTTGCCGGTGGTGCAGATG
RTGFP_3' CTCGTCCATGCCGAGAGTG

dof primers amplified a 140 bp long product, nucleotides 504-644 of the dof coding sequence. GFP primers amplified nucleotides 598-710 of the eGFP coding sequence (112 bp long product). The PCR program contained an initial 15 minutes activation step at 94 °C followed by 55 cycle of 15 sec at 94 °C, 30 sec at 50 °C and 20 sec at 72 °C with an acquisition at the temperature of the extension. Melting curve was analysed between 65 and 95 °C. Real-time PCR with GFP primers was carried out on each random-hexamer reverse transcription reaction and on negative controls. Standard curve was generated using 2×10^3 – 2×10^9 copies of the pDeGFP plasmid. dof real-time PCR was done on all reverse transcription reactions. Standard curves were generated using 2×10^5 –

2×10^9 copies of the different mutant Dof DNA constructs used for transient transfection. The C_t value of each dof and GFP reaction was determined followed by the calculation of the amount (copy number) of the templates in the samples using the standard curves. The obtained dof amounts were corrected with the difference in length of the transfected dof constructs relative to the full length dof construct, since the equal weights used in transfection did not correspond to equal copy numbers of the different dof plasmids due to the different length of the dof insertions. The ratio of the resulted dof amount and GFP amount in each sample was determined. Results are presented as a fold expression of the different mutant dof RNAs relative to full length dof RNA.

Immunocytochemistry

Drosophila S2 cells were transiently transfected with different constructs by the standard procedure. pDeGFP construct (kindly provided by M. Reth) was co-transfected to identify transfected cells by GFP autofluorescence. 24 hours following transfection cells were induced for protein expression and from this point onwards cultured on cover slips. After additional 24 hours culture media were removed, cells were washed once with phosphate buffered saline (PBS), fixed with 4% formaldehyde diluted in PBS for 10 minutes on cover slips and permeabilised with 0.5% Triton X-100 for 30 seconds. Following several wash steps and blocking with 1x PBS supplemented with 1% (w/v) BSA and 0.1% (v/v) Tween 20 standard procedure of immunostaining was carried out using rabbit anti-Dof (1:200) (Vincent et al., 1998) and anti-HA 3F10 (1:6000) (Boehringer Mannheim) primary antibodies and anti-rabbit and anti-rat secondary antibodies coupled with Alexa568 and Alexa647 fluorochromes (Molecular Probes) respectively.

Drosophila strains and genetics

UAS-DofD587E transgenic flies were generated by the standard procedure of P element mediated germ line transformation. UAS-Ras1^{V12}/CyO_{wg}lacZ; dof,e/TM3_{elav}lacZ, UAS-Raf^{*},dof,e/TM3_{elav}lacZ, twiGal4,dof,e/TM3_{elav}lacZ and btlGal4; dof,e/TM3_{elav}lacZ flies were kindly provided by R. Wilson. UAS-Ras1^{V12,S35}, UAS-Ras1^{V12,G37} and UAS-Ras1^{V12,C40} flies were provided by A. Rubin whereas UAS-Rgl1^{CAAX} and UAS-Rgl2^{CAAX} by J. Camonis. UAS-DRal^{wt}, UAS-DRal^{20V} and UAS-DRal^{25N} transgenic flies were provided by H. Okano. UAS-Rap1^{wt}, UAS-Rap1^{V12} and UAS-Rap1^{N17} were obtained from U. Gaul. SevGal4,UAS-Gap1/TM3 flies were provided by M. Mlodzik. *rala* mutant flies with the P element insertions PL056, PG069 and PG089 were kindly provided by A. Vincent. FRT40A lwr⁴⁻³/CyO and FRT40A semi¹⁰⁷/CyO flies were kindly provided by S. Tanda. *pita*³ and *dcp-1*^{Prev-1} flies were obtained from K. McCall. UAS-droncCARDonly, UAS-ΔN-dronc, UAS-pro-dronc and UAS-pro-dronc(C>A) flies were generously provided by P. Meier. l(2)k01211, lwr⁰⁵⁴⁸⁶ and lwr⁰²⁸⁵⁸ lines are part of the Berkely *Drosophila* Genome Project (BDGP; (Spradling et al., 1999; Spradling et al., 1995)). hs-RalA28N (BL4843), UAS-RalA^{72L} (BL4844), UAS-Ras85D^{N17} (BL4845), UAS-Ras1^{N17} (BL4846), l(2)04993 (BL11378), Df(3L)AC1 (BL997), UAS-p35 (BL6298, BL5072 and BL5073) and P{PZ}Dcp-1⁰²¹³² (BL11179) flies were obtained from the Bloomington stock center under the stock numbers remarked in brackets. btlGal4 flies (Shiga, 1996) were used for overexpression studies in the tracheae and twiGal4 flies (multiple insertions on the first chromosome, constructed by N. Brown) for mesodermal overexpression.

To remove maternal contribution of a gene the autosomal FLP-DFS technique was used (Chou and Perrimon, 1996). To combine l(2)04993 with the FRT40A site standard genetic recombination and selection techniques were used (Theodosiou and Xu, 1998).

For rescue experiments *dof^f* allele (Vincent et al., 1998) was combined with UAS-transgenic strains and crossed to the appropriate driver lines. *dof* mutant embryos were identified by using balancer chromosomes carrying P[ry+, *elav-lacZ*] insertions. If necessary standard genetic recombination was used and flies carrying P[w+, UAS-transgene] insertions were selected and tested for complementation with the *dof^f* allele. In case of the recombination of two P[w+, UAS-transgene] insertions different eye colours were screened. Changes in eye colour were confirmed by the de-recombination of the two insertions.

To test how UAS transgenes effect vitality by tissue specific expression flies heterozygous / hemizygous either for the UAS transgene or for the driver line were crossed to each other. Survival rate was calculated by dividing the number of emerged flies with transgenic expression (=flies emerged) through the number of emerged flies without transgenic expression (=flies expected). To follow chromosome segregation balancer chromosomes (CyO; TM3,sb; TM6B) or sex determination were used.

Immunohistochemistry

Standard procedures were followed to collect embryos, which were fixed over 30 minutes at 37°C using a phosphate buffered saline with 3.7% (v/v) formaldehyde. Immunohistochemistry was carried out according standard protocols. Antibodies directed against Eve (1:5000) (courtesy of M. Frasch), 2A12 (1:20) (kindly provided by N. Patel) and beta-Galactosidase (1:1500) (Sigma G4644) were used to detect the appropriate proteins *in situ* by using the coupled peroxidase system (Vectastain ABC kit, Vector Labs). Two colour stainings were accomplished in sequential steps, with the addition of Ni and Co in the second detection reaction to produce a dark precipitate. Embryos were sorted and embedded in Araldite as described (Leptin and Grunewald, 1990). Photographs were taken on a Zeiss Axioplan microscope using a Kontron ProgRes 3008 digital camera, and the images were processed using Adobe Photoshop 7.0.

Antibodies directed against Eve (1:5000) and diphospho-MAPK (1:400) (SigmaM8159) were used to detect the proteins *in situ* by using secondary antibodies coupled to fluorochromes Alexa568 and Alexa488 (1:500) (Molecular Probes). Embryos were embedded into Vectashield mounting medium (Vector Labs) and photographs were taken on a Zeiss Axioplan2 Imaging microscope with Apotome system using AxioVision v.4.2. Images were converted and processed using Adobe Photoshop 7.0.

Eggshell preparation

Eggs were collected from apple juice agar plates fixed in glycerol : acetic acid (1:4) for 1 hour at 60 °C and embedded into Hoyers medium (30 g gum arabicum and 200 g chloralhydrate in 50 ml water subsequently mixed with 20 g glycerol) mixed with lactic acid (1:1) and incubated under cover slips overnight at 60 °C.

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Abstract

The FGF signal transducer molecule Dof has been shown to act upstream of the Ras-MAPK pathway. It is unknown how Dof transmits the signal from the activated FGF receptor to the MAPK cascade. Several approaches were taken to analyse this mechanism.

1. One way to resolve this question is to analyse Dof mediated interactions. In this work interaction of Dof with selected candidates of a yeast two hybrid screen was analysed biochemically. Most but not all of the tested candidates were able to form a complex with Dof in *Drosophila* S2 cells. The predicted capability of Dof for self-association was validated by showing that the molecule was able to form dimers/oligomers in *Drosophila* S2 cells. Co-immunoprecipitation studies of Dof with the FGF receptor Heartless showed that the DBB domain – an essential region of the molecule needed for dimerisation and shared with BCAP and BANK – is required for efficient receptor binding. A consensus sumoylation site is located within the DBB domain of Dof. Mutation of this site impaired binding to the FGF receptor Heartless. Dof interacted with the SUMO conjugating enzyme Ubc9 and was sumoylated in *Drosophila* S2 cells suggesting that SUMO modification of the molecule might regulate interaction with the FGF receptor.

2. As a second approach phosphorylation of Dof was analysed that might influence the activity of the protein in signal transmission. Dof becomes phosphorylated in the presence of an activated FGF receptor at the conserved tyrosine motifs for PI3K and Csw binding. In addition, we identified a novel tyrosine motif as a phosphorylation target which appears four times in Dof. Strikingly, it is also present in BANK and some other signalling molecules. The C-terminal part of Dof is also phosphorylated and might be involved in conformational regulation of the protein, since the removal of the last 200 amino acids improved the binding affinity of Dof to Heartless.

3. Dof is cleaved in *Drosophila* S2 cells, which might give an additional level in the regulation of signalling. This cleavage is caspase dependent, regulated by Dronc and requires an intact caspase cleavage site in Dof. However, a Dof transgene mutant for the caspase cleavage site rescued efficiently *dof* mutant phenotypes indicating that this site is not essential for Dof functions assayed in this work. Mutant Dof constructs with C-terminal truncations of different lengths that removed the caspase cleavage site varied in their expression levels despite their similar stability in S2 cells. These differences were not seen in mRNA abundance but only in protein levels and higher expression levels correlated with the presence of a region in Dof identified as a binding site for the ribosomal protein S10. Full length Dof showed expression levels comparable to that of the mutant construct lacking the ribosomal protein binding site. Thus, this is the first indication that Dof cleavage products might have additional roles in the regulation of Dof function.

4. One essential function of Dof is to activate the Ras-MAPK pathway in vivo. To find out if only the MAPK signalling panel is utilised by Ras activity the role of the three Ras effectors Raf, RalGDS and PI3K were determined in FGF signalling during *Drosophila* embryogenesis. This analysis showed that both the Raf and RalGDS effector pathways might have a function in FGF signalling but that additional Ras activity is required either to regulate these cascades or to turn on other effector pathways. Our results indicate that PI3K is not needed for FGF signal transduction. In addition to Ras, Rap1 might be also involved in FGF signalling downstream or in parallel to Dof. How Rap1 is activated and to what extent the two closely related Ras family GTPases share downstream effectors is not understood at present.

Zusammenfassung

Es wurde gezeigt, dass Dof, ein mögliches Adapter-Molekül, im FGF-Signalweg überhalb der Ras-MAPK Signalkaskade agiert. Es ist aber unbekannt wie Dof das Signal vom aktivierten FGF-Rezeptor zu der MAPK-Kaskade weiterleitet. In dieser Arbeit wurden verschiedene Strategien angewendet, um diesen Mechanismus zu analysieren.

1. Eine Möglichkeit diese Frage zu adressieren ist die Analyse der molekularen Interaktionen von Dof mit anderen Proteinen. In dieser Arbeit wurden die Interaktionen von Dof mit ausgewählten Kandidaten, die in einem Hefe-2-Hybrid Screen gefunden wurden, biochemisch untersucht. Die meisten dieser Kandidaten können in *Drosophila* S2 Zellen Komplexe mit Dof bilden. Die vorausgesagte Fähigkeit von Dof, Komplexe mit sich selbst einzugehen, wurde in S2 Zellen nachgewiesen. Ko-Immunoprecipitations-Untersuchungen mit Dof und dem FGF-Rezeptor Heartless zeigten, dass die DBB Domäne von Dof –eine essentielle Region, die nötig für Dimerisation ist und auch in den Proteinen BCAP und BANK vorhanden ist– notwendig für eine effiziente Bindung an den Rezeptor ist. Innerhalb der DBB Domäne von Dof liegt eine konservierte SUMOlations Stelle. Mutationen dieser Stelle verhindern die Bindung von Dof an Heartless. Dof interagiert mit Ubc9, einem SUMO konjugierenden Enzym, und wird in S2 Zellen SUMOliert. Eine SUMOlation von Dof könnte also die Interaktion mit dem Rezeptor regulieren.

2. Die Phosphorylierung von Dof könnte die Aktivität des Proteins bei der Signalübertragung beeinflussen. Dof wird an den konservierten Tyrosin-Motiven für die Bindung von PI3K und Csw phosphoryliert wenn ein aktivierter Rezeptor vorhanden ist. Zusätzlich haben wir ein neues Tyrosin-Motif gefunden, welches viermal in Dof vorhanden ist und auch in BANK und anderen Signal-Molekülen präsent ist. Der C-Terminus wird ebenfalls phosphoryliert und könnte bei der Regulation der Konformation notwendig sein, da die Deletierung der letzten 200 Aminosäuren am C-Terminus die Bindung von Dof an Heartless verbessert.

3. Dof wird in S2 Zellen kaspaseabhängig gespalten. Dies könnte eine weitere Stufe der Regulation des Signalweges sein. Diese Spaltung wird durch Dronc reguliert und benötigt eine intakte Kaspasen-Spaltungsstelle in Dof. Ein Dof-Transgen, in dem diese Stelle mutiert ist, rettet jedoch effizient den *dof* Phänotyp in *Drosophila*. Dies deutet darauf hin, dass diese Stelle nicht essentiell für die Funktion von Dof ist, die in dieser Arbeit analysiert wurde. Konstrukte, bei denen der C-Terminus in verschiedenen Längen, aber immer mit der Kaspasen-Spaltungsstelle, deletiert wurde, unterscheiden sich in ihrer Expressionsstärke, obwohl sie ähnlich stabil in S2 Zellen sind. Diese Unterschiede konnten nur auf Protein-Ebene, aber nicht auf mRNA-Ebene gesehen werden und die stärkere Expression korrelierte mit der Anwesenheit einer Bindungsstelle für das ribosomale Protein S10 in der Proteinsequenz von Dof. Die Expressionsstärke des kompletten Dof-Konstruktes entspricht den Deletions-Konstrukten, in denen diese Bindungsstelle fehlt. Dies ist der erste Hinweis, dass Spaltungsprodukte von Dof zusätzliche Funktionen bei der Regulation von Dof haben.

4. Die Aktivierung des Ras-MAPK Signalweges ist eine essentielle Funktion von Dof *in vivo*. Es wurde untersucht ob die Ras-Aktivität nur die MAPK-Kaskade aktiviert. Hierzu wurde die Rolle der drei Ras Effektoren Raf, RalGDS und PI3K innerhalb des FGF-Signalweges während der Embryogenese von *Drosophila* untersucht. Die Raf und RalGDS Signalwege könnten eine Funktion in der FGF-Signalkaskade haben, aber zusätzliche Ras-Aktivität ist notwendig um diese oder andere Effektor-Signalwege zu aktivieren oder regulieren. PI3K hingegen wird für den FGF-Signalweg nicht benötigt. Zusätzlich zu Ras könnte auch Rap1 in den FGF-Signalweg involviert sein - abwärts oder parallel zu Dof. Wie Rap1 aktiviert wird und in wieweit diese beiden nah verwandten GTPasen der Ras Familie ihre Signalwege teilen ist zurzeit nicht bekannt.

Abbreviations

bp	base pairs
BSA	Bovine Serum Albumin
CHX	cycloheximide
cDNA	complementary DNA
Da	Dalton
dsRNA	double stranded RNA
EGTA	Ethylene Tetracetic Acid
FGF	fibroblast growth factor
g	gram
GFP	green fluorescence protein
GLC	germ line clones
His	histidine
k	kilo
l	liter
lacZ	beta-galactosidase
M	mol per liter
m	milli
μ	micro
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTK	protein tyrosine kinase
RNAi	RNA interference
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcription PCR
SDS	sodium-dodecyl-sulphate
Z-VAD	N-Benzylloxycarbonyl-Valine-Alanine-Asparagine
³⁵S	isotope of sulphur with atomic weight of 35

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Teilpublikationen:

1. Wilson R, Battersby A, Csiszar A, Vogelsang E, Leptin M. (2004) A functional domain of Dof that is required for fibroblast growth factor signaling. *Mol Cell Biol.* 24(6):2263-76.
2. Battersby A, Csiszar A, Leptin M, Wilson R. (2003) Isolation of proteins that interact with the signal transduction molecule Dof and identification of a functional domain conserved between Dof and vertebrate BCAP. *J Mol Biol.* 329(3):479-93.

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