Plasmid Constructs

5’Flag-Dof.pNB40

To introduce a Flag epitope tag at the 5’ end of the Dof transcript II ORF a 2 step PCR proceedure was used. Initially one fragment was amplified from the cDNA 1f11 (Vincent et al., 1998) with the primers 5’GCTTCAGTAAGCCAGATGCTAC and 5’CGTCCTTGTAATCCATGGTCCCGGGGAATTCTTATTAATTCCAAAACCTCAGC ACGAC and another fragment was amplified with the primers 5’GACCATGGATTACAAGGACGATGACGATAAG and 5’CAATCTCCTCGCTGAAGGGACCATCG. These PCR fragments were mixed together and reamplified to generate a single product using the oligos 5’GCTTCAGTAAGCCAGATGCTAC and 5’CAATCTCCTCGCTGAAGGGACCATCG. The final PCR product was digested with PpuMI and BssHII and inserted into a corresponding 1f11 cDNA vector. The presence of a Flag epitope tag in the resulting clone, 5’Flag.Dof.pNB40, was confirmed by sequence analysis.

Not-5’Flag-Dof-Asc.pNB40

5’Flag.Dof.pNB40 was linearized with NotI, treated with Klenow and ligated to AscI linkers (NEB #1171). The linkered DNA was then digested with AscI and recircularized. Not-5’Flag-Dof-Asc.pNB40 was created from the resulting clone using linkers (NEB # 1126) to convert the PpuMI site to a NotI site.

Not-5’FLAG-5’Dof.pKS+

A 723bp NotI-EcoRV fragement from Not-5’Flag-Dof-Asc.pNB40 was inserted into the corresponding sites of pBluescript II pKS+.

Dof-3'Xba.pNB40

An Xba site was introduced at the 3'end of the Dof transcript II ORF by two PCR steps. The primers 5' ATCTACGGCAAGCTGACCAAGAG and 5'GATTATTACTTATCGTCATCGTCCTTGTAATCTGTAGTTTGGCGTTTCCTCCTC TGC were used to amplify a frament from the cDNA 1f11 (Vincent et al., 1998), and another fragment was amplified from this clone with the primers 5'GACGATGACGATAAGTAATAATCTAGATGAACTGCTAGGCCATCCCAACG and 5'CAAAGGCCCAAGGCACTCTTTCG. These fragments were then mixed together and used as the template for a second PCR with the primers 5'ATCTACGGCAAGCTGACCAAGAG and 5'CAAAGGCCCAAGGCACTCTTTCG. The resulting product was digested with AccI and SpeI and inserted into a AccI-SpeI vector derived from the cDNA 1f11.

5'Flag-Dof.pAlter

A 3577 bp fragment released by an EcoRI-SphI digest of 5'Flag-Dof.pNB40 was inserted into a corresponding pAlter vector.

5'Flag-Dof.pUAST

A 3.2Kb Eco RI–SpeI fragment derived from 5'Flag.Dof.pNB40 was inserted into an EcoRI–XbaI pUAST vector.

Dof-3'Xba.pUAST

Dof-3'Xba.pNB40 was linearized with AseI, treated with Klenow and then ligated to EcoRI linkers (NEB # 1018). An EcoRI and SpeI digest of the linkered DNA released a 3.2Kb fragment that was inserted into an EcoRI-XbaI pUAST vector.

Dof [1-802].pUAST

Dof-3'Xba.pUAST was linearized with XhoI, treated with Klenow and ligated to XbaI linkers (NEB # 1082). Following digestion of the linkered DNA with XbaI a 11629 bp fragment was isolated and recircularised to create Dof[1-802].pUAST.

5'Flag-Dof[1-674].pUAST

Site directed mutagenesis of ssDNA derived from 5'Flag-Dof.pAlter was performed using the oligo 5'AGATCCAATGCCTAATCTAGAGCGGCCGCGATTATGCACGA. A 540bp ClaI-EheI fragment containing the mutation was inserted into an identical 5'Flag-Dof.pNB40 vector. To generate 5'Flag-Dof[1-674].pUAST a 2067bp EcoRI-XbaI from the resulting clone was inserted into an EcoRI-XbaI pUAST vector.

Dof[1-522].pUAST and Dof[1-446].pUAST

For each of these constructs a stop codon and a XbaI site were introduced into 5'Flag-Dof.pAlter by ssDNA site directed mutagenesis with the oligos 5'ACCGAGGACTAAAATCTAGAGGCTTCCGTGGCCGA and 5'CACTACTTCAAACTAGTGTAATCTAGAGGCGAGAGCTG respectively . A SfiI-XhoI fragment from each of the clones, containing the mutations, was introduced into a Sfi-XhoI vector derived from Dof-3'Xba.pUAST. To generate Dof[1-522].pUAST and Dof[1-446].pUAST the resulting pUAST clones were cut with XbaI and fragments of10787bp and 10563bp, respectively,were religated.

Dof[1-277].pUAST

A 843bp EcoRI-BglII fragment from Dof[1-802].pUAST was inserted into an EcoRI-BglII 5'Flag-Dof.pAlter vector to remove the 5' Flag epitope tag. An XbaI site was generated in the resulting clone by digestion with BglII, treatment with Klenow and ligation to XbaI linkers (NEB # 1082). Subsequently, the linkered DNA was digested with XbaI and reclosed. Dof[1-277].pUAST was created from this clone by transferring a 851bp EcoRI-Xba fragment, encoding amino acids 1-277 of Dof, into an EcoRI-XbaI pUAST vector.

Dof[89-1012].pUAST, Dof [168-1012].pUAST and Dof [227-1012].pUAST

To introduce a methionine at positions 89, 168 and 227 of Dof the oligos 5’GATGGTCCCATGGGCGAGGAGAT, 5’TGCGCGACAATGCCATGGTGCAGATCAG and 5’TGGGCCAAACCATGGTGGTTGCT were used for ssDNA mutagenesis of 5’Flag-Dof.pAlter. The introduction of an NcoI site in each case was confirmed by DNA sequence analysis. Dof[89-1012].pNB40 and Dof[168-1012].pNB40 were generated by transfering 275bp and 38bp NcoI-EcoRV fragments from the 5’Flag-Dof.pAlter mutants to a corresponding 5’Flag-Dof.pNB40 vector. Dof[227-1012].pNB40 was produced by introduction of a 134bp NcoI-Bsu36I fragment from the 5’Flag-Dof.pAlter mutant into a NcoI-Bsu36I 5’Flag-Dof.pNB40 vector. The pUAST derivatives were produced from the pNB40 constructs Dof[89-1012], Dof[168-1012] and Dof[227-1012] by introducing 2961bp, 2724bp and 2547bp EcoRI-SpeI fragments, respectively, into an EcoRI-XbaI pUAST vector.

5’Flag-Dof[277-1012].pUAST

An 11434 bp fragment from a Bgl II digest of 5’Flag-Dof.pUAST was religated to generate 5’Flag-Dof[277-1012].pUAST.

Dof[89-802].pUAST and Dof[168-802].pUAST

A 1367bp EagI-SphI fragment from Dof[89-1012].pUAST and a 1130bp EagI-SphI fragment from Dof[168-1012].pUAST were inserted into a EagI-SphI Dof[1-802].pUAST vector, respectively.

5’Flag-Dof[Δ361-449].pUAST

To create 5’Flag-Dof[Δ361-449].pAlter ssDNA mutagenesis of 5’Flag-Dof.pAlter was carried out with the oligo 5’GAGCGTCAGCCACTAGTGACTCCGCCAGGCGA. A

1340bp Bsu36I-XhoI fragment of the mutant, containing the deletion, was subsequently ligated to a Bsu36I-XhoI vector generated from 5'Flag-Dof.pUAST.

5'Flag-Dof[Δ233-364].pUAST

Site directed mutagenesis of ssDNA generated from 5'Flag-Dof.pAlter was carried out with the oligo 5'TCTGCTGGCAGAACTAGTCTTCACCCGGCTGG to create 5'Flag-Dof[Δ233-364].pAlter. A 320bp EcoRV-SfiI fragment of this construct was inserted into an EcoRV-SfiI vector derived from 5'Flag-Dof.pNB40. Subsequently,a 2066bp EcoRI-XhoI fragment of the resulting clone, 5'Flag-Dof[Δ233-364].pNB40, was inserted into a EcoRI-XhoI 5'Flag-Dof.pUAST vector.

5'Flag-Dof[Δ233-449].pUAST

The construct 5'Flag-Dof[Δ233-449].pAlter was created by the ligation of a 3502bp AflIII-SpeI fragment derived from 5'Flag-Dof[Δ233-364].pAlter to a 5081bp fragment from an AflIII-SpeI digest of 5'Flag-Dof[Δ361-449]. A 1232bp EcoRV-XhoI fragment of 5'Flag-Dof[Δ233-449].pAlter was then ligated with an EcoRV-XhoI 5'Flag-Dof.pNB40 vector. 5'Flag-Dof[Δ233-449].pUAST was produced by the ligation of a 1810bp EcoRI-XhoI fragment from the resulting pNB40 clone to an EcoRI-XhoI 5'Flag-Dof.pUAST vector.

5'Flag-Dof[233-364>BCAP].pUAST

This clone was derived from a partial BCAP cDNA isolated by Genome Systems (details provided upon request). A 390bp fragment was amplified using the primers 5'CAATCTAGACGAGAAGGTATCAACAG and 5'TCTGACTAGTTCGTCTTCTTCCAGT, then digested with XbaI and SpeI and cloned into a XbaI-SpeI pBluescript II vector. The XbaI-SpeI insert of this clone was subsequently ligated into the SpeI site of 5'Flag-Dof[Δ233-364].pAlter. A 2441bp EcoRI-XhoI fragment derived from the resulting clone was transferred to a corresponding 5'Flag-Dof.pUAST vector.

BCAP.pUAST:

A 2758 bp NotI-partial SpeI fragment of the mouse BCAP cDNA clone KG1221 (Okada et al., 2000) was inserted into a NotI-XbaI pUAST vector.

SH4-Dof.pUAST:

The Nco-BglII fragment encoding the FLAG epitope tag of Not-5'FLAG-5'Dof.pKS+ was replaced with a fragment encoding the SH4 motif of human Src which was created by annealing the oligos 5'CATGGGCAGCTCCAAGAGCAAGCCCAAGGACC CCAGCCAGCGCCGTCGCAA and 5'GATCTTGCGACGGCGCTGGCTGGGGT CCTTGGGCTTGCTCTTGGAGCTGCC. A 590bp NcoI-EcoRV fragment of the resulting clone was isolated and ligated to a 5'Flag-Dof.pNB40 NcoI-EcoRV vector. To create SH4-Dof.pUAST a 3277bp fragment from 5'SH4-Dof.pNB40 was ligated to a EcoRI-XbaI pUAST vector.

5'SH4-Dof[1-802].pUAST:

A 1682bp EagI-SphI fragment from SH4-Dof.pUAST was ligated to a EagI-SphI vector prepared from Dof[1-802].pUAST.

5'Torso^{4021}-Dof.pUAST:

A fragment encoding the extracellular portion of Torso^{4021} was amplified from pRM-Torso^{4021}(Sprenger et al., 1993) using the oligos 5'AGAGACCATGGTTATTTTCTATGCGAAGT and 5'TCTCTAGATCTTTCGTCTGCAGAACGTC. After digestion of the PCR product with Nco and BglII a 3-way ligation was carried out with a 812bp BglII-Bsu36I fragment from 5'Flag-Dof.pNB40 and a 5479bp Bsu36I-NcoI fragment from Not-5'Flag-Dof-Asc.pNB40 to generate 5'Torso4021-Dof.pNB40. The pUAST construct was derived from a partial NotI digest of pUAST-λbtl (Lee et al., 1996), which was filled in using Klenow, ligated to AscI (NEB #1171) linkers, and then digested with AscI and reclosed. To produce 5'Torso4021-Dof.pUAST a Not-Asc pUAST vector, which was prepared from a clone

containing an AscI site at the 3'end of λ-btl (pUAST-λbtl.3'Asc), was ligated to a 5092bp Not-AscI fragment from 5'Torso4021-Dof.pNB40.

5'Torso^{4021}-Dof[1-802].pUAST:

A 3063bp Sfi-SphI fragment from 5'Torso^{4021}-Dof.pUAST was ligated to a corresponding Dof[1-802].pUAST vector.

5'Flag-Dof-95F.pUAST and 5'Flag-Dof[1-802]-95F.pUAST

To create 5'Flag-Dof-95F.pAlter mutagenesis was carried out with ssDNA derived from 5'Flag-Dof.pAlter using the oligo 5'GATTGCCCACAAGTTTCAGAACACAGC. To produce 5'Flag-Dof-95F.pUAST an EcoRI-SpeI fragment from this construct was inserted into an EcoRI-XbaI pUAST vector. 5'Flag-Dof[1-802]-95F.pUAST was created from a Xho I digest of 5'Flag-Dof-95F.pAlter that was treated with Klenow and ligated to Xba I linkers (NEB # 1082). Digestion of the linkered DNA with XbaI and EcoRI generated a 2455 bp fragment that was inserted into an EcoRI-XbaI pUAST vector.

5'Flag-Dof-486F.pUAST, 5'Flag-Dof-515F.pUAST and 5'Flag-Dof-486F,515F.pUAST.

5'Flag-Dof-486F.pAlter was generated by ssDNA mutagenesis of 5'Flag-Dof.pAlter with the oligo 5'ACGGCGGAGTTTATGGAGATGTCCAG. Similarly, mutagenesis with the oligo 5' ATCTCAACTTCATAAGTGTGGAAACCGAGGAC produced 5'Flag-Dof-515F.pAlter, and the use of both oligos one mutagenesis reaction generated 5'Flag-Dof-486F,515F.pAlter. The corresponding pUAST constructs were created by inserting a 3252 bp EcoRI-SpeI fragment from each mutant into an EcoRI-XbaI pUAST vector.

5'Flag-Dof[1-802]-95F,486F,515F.pUAST

The mutations in 5'Flag-Dof-95F.pAlter and 5'Flag-Dof-486F,515F.pAlter were combined by inserting a 1595bp Bsu36I-XhoI fragment from 5'Flag-Dof-486F,515F.pAlter into the corresponding sites of 5'Flag-Dof-95F.pAlter. A XhoI digest of the resulting construct was

treated with Klenow, ligated to XbaI linkers (NEB # 1082], and digested with XbaI and EcoRI to generate a 2455 bp fragment, which was inserted into an EcoRI-XbaI pUAST vector.

5'Flag-Dof.pAT-Hygro

A 3252bp EcoRI-SpeI fragment of 5'Flag-Dof.pAlter was treated with Klenow and ligated to a Asp718 digestion of pAT-Hygro (Allard et al., 1996), that had been treated with Klenow to produce blunt ends. A clone containing Dof under the control of the actin5C promoter was identified by restriction enzyme analysis.

lamda-Btl.phsT-Neo

pAT-Hygro (Allard et al., 1996) was digested with BamHI and a 3597bp fragment containing the tubulin polyA sequence was recircularized. The resulting plasmid was linearized with NotI, treated Klenow and religated to destroy the NotI site. This derivative of pAT-Hygro was used to generate a vector with blunt ends by digestion with XbaI and treatment with Klenow. The vector was ligated to an EcoRI-AscI fragment encoding lambda-Btl , also treated with Klenow, that was derived from pUAST-λbtl.3'Asc (see construction of 5'Torso^{4021}-Dof.pUAST). A clone containing lambda-Btl upstream of the tubulin polyA sequence was digested with NotI and ligated to the NotI cassette of pV9 (Allard et al., 1996), which contains the heat shock promoter and neomycin resistance gene. The orientation of the promoter in the resulting plasmid, lamda-Btl.phsT-Neo, was determined by restriction analysis.

Constructs for transient transfection of Schneider cells.

The constructs used for the transient transfection were derived from the above constructs. Details can be provided on request. See also Battersby et al., 2003.