In vitro expression of *Drosophila melanogaster* formins

A thesis submitted towards partial fulfillment of

BS-MS dual degree programme

by

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled "*In vitro* expression of *Drosophila melanogaster* formins" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Pasupureddy Rahul under the supervision of Dr. Aurnab Ghose, Asst. Professor, IISER Pune, during the academic year 2011-2012.

Supervisor: Dr. Aurnab Ghose
Assistant Professor,
Biology Division, IISER Pune

Date:

Place:

Declaration

I hereby declare that the matter embodied in the thesis entitled '*In vitro* expression of *Drosophila melanogaster* formins' are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Aurnab Ghose, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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1. Abstract

Actin cytoskeletal regulation, a crucial feature of cellular remodeling, is a dynamic process and has been extensively investigated. Formins, a class of actin nucleators, have been demonstrated to actively regulate actin nucleation and are implicated in many essential functions such as cell division, migration, adhesion, etc. Given the variety of functions that are performed by formins and the fact that multiple homologues are present in eukaryotes, it becomes important to understand the isoform specific mechanisms of regulating actin nucleation and the redundant roles they play. In silico analyses of *Drosophila melanogaster* genome have identified six FH2 domain containing putative formin proteins. Three formins: Diaphanous, Cappuccino and Disheveled associated activator of morphogenesis (DAAM) have been characterized *in vitro* and are shown to have the ability to nucleate actin while the other three: Fhos, Formin3 and CG32138 remain uncharacterized. To investigate their actin nucleation capability *in vitro*, we have attempted to clone and over-express the FH1-FH2 fragments of these novel formins, and will use these constructs in future to see how they regulate actin polymerization through *in vitro* pyrene actin assays.

2. Keywords: actin polymerization, *Drosophila melanogaster* formins, *in vitro* actin assay

3. Introduction

Actin regulation is an important feature of cytoskeletal dynamics. Complex cellular functions such as cell morphogenesis, motility, exocytosis and endocytosis, cytokinesis, etc. occur with the help of actin cytoskeletal reorganization. Such restructuring of actin cytoskeleton requires a coordinated activity of several actin binding proteins. One of the first identified actin nucleator is the Arp2/3 complex. It consists of seven subunits including two actin related proteins (Arp2 and Arp3) which closely resemble actin monomers. From the dendritic nucleation model (Pollard, 2007), actin filaments are nucleated by Arp2/3 complex at an angle of 70º to pre-existing filaments. It is natively present in an inactive form and gets activated upon association with the SCAR/WAVE (suppressor of cAMP receptor/WASP-family verprolin homologous protein) complex and members of the WASP (Wiskott - Aldrich Syndrome Protein) family (Suetsugu et al., 2002). Spire is also shown to be involved in actin nucleation. Spire, a protein containing four WH2 (WASP Homology 2) domains, binds up to four actin monomers thus forming a nucleation center for further actin polymerization (Quinlan, Heuser, Kerkhoff, & Mullins, 2005). More recently Cordon-Blue (Cobl) a protein involved in brain morphogenesis has been shown to be involved in actin regulation (Ahuja et al., 2007). Cobl contains three WH2 domains and they are shown to bind to actin monomers in a 1:1 ratio thus forming a trimer which acts as a nucleation center. While Arp2/3 complex appears to be a universal actin nucleator, others such as Spire and Cobl act specifically (Renault et al., 2008). Apart from these main branches of nucleators small GTPases, capping proteins, etc. also play a role in regulating actin dynamics.

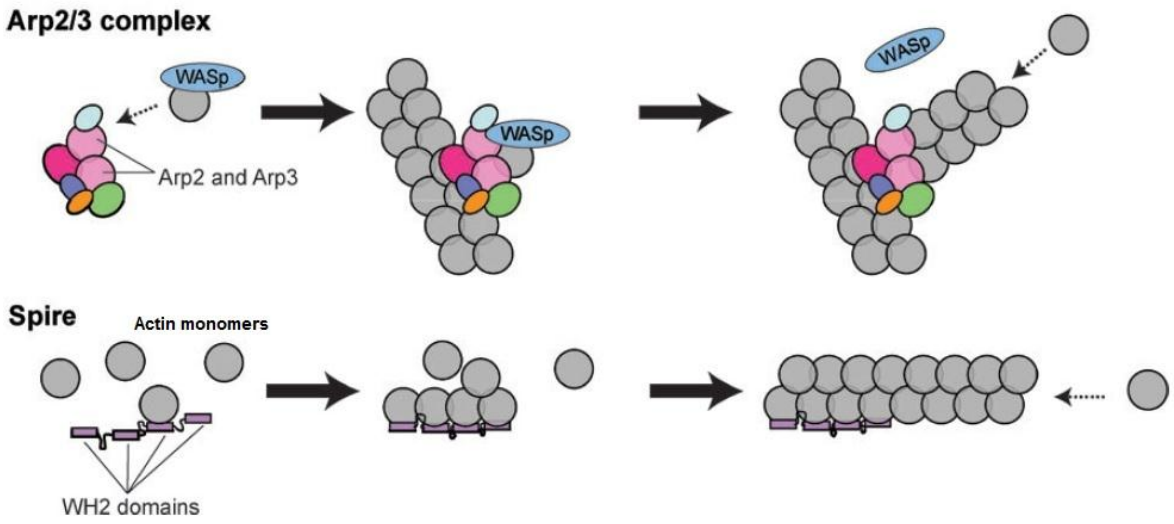


Figure 1: Mechanism of actin nucleation by Arp2/3 complex and Spire (Modified from Goode & Eck, 2007)

Formins, another class of actin nucleating proteins nucleate straight chain bundles. The name formins is derived because of their apparent role found initially in the formation of the limbs and kidneys (Woychik et al., 1990). Unlike other classes of nucleators such as Arp2/3 complex or Spire, formins form dimers and remain associated with the growing end of actin fibers. Formins are characterized by presence of the highly conserved FH2 domain. Majority of the studies show that FH2 domain is the minimal region required for actin nucleation capability (Pring et al., 2003; Takeya and Sumimoto, 2003, Mattila and Lappalainen, 2008). FH2 domain initiates filament assembly and remains associated with the fast-growing barbed end, facilitating rapid addition of actin subunits while protecting the growing end from capping proteins. FH1 domain, a proline rich region, binds to profilin units thus effectively sequestering G-actin monomers thereby, increasing the actin nucleation rate of the formins. Other domains such as DID (Diaphanous Inhibitory Domain), DAD (Diaphanous Autoregulatory Domain) are also present though not very well conserved. DID-DAD interactions are auto-inhibitory in nature and are responsible for the native inactive state of diaphanous

related formins (DRFs) (Copeland et al., 2007). Rho GTPases bind to the GBD (GTPase Binding Domain) domain, which gets activated and severs the DID-DAD interactions, thereby rendering the formin active (reviewed in Higgs, 2005).

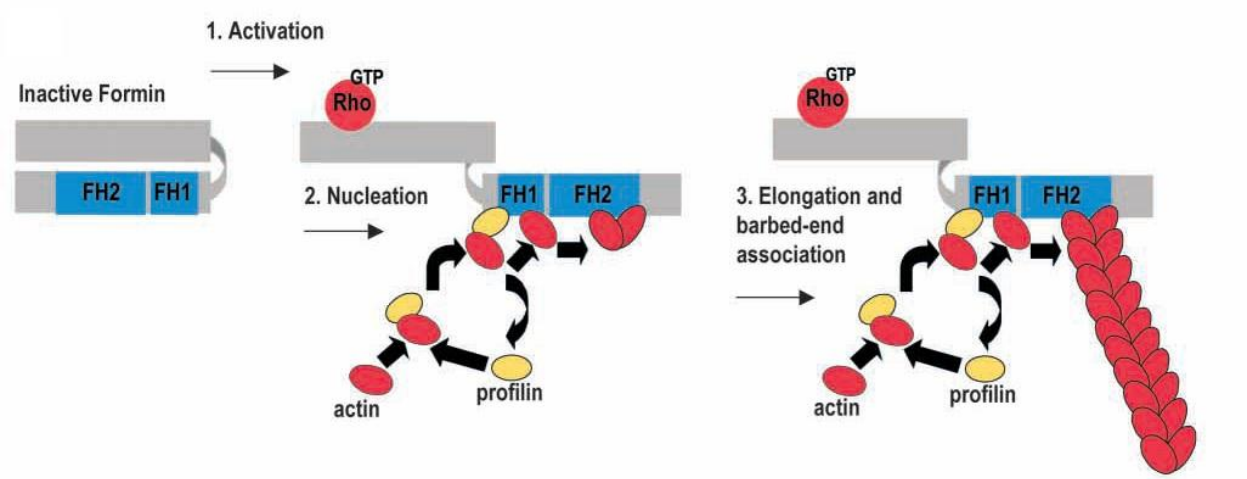


Figure 2: Mechanism of formin activation and actin nucleation (Reproduced from Evangelista, Zigmond, & Boone, 2003)

There are seven subfamilies of formins present which are classified based on phylogenetic analysis and the mechanism of actin nucleation:

1. Diaphanous related formins (DRFs)
2. Formin like proteins in leukocytes (FRLs)
3. DAAM family
4. Delphilins
5. Inverted formins (INFs)
6. Formins (FMNs)
7. Formin homology domain-containing protein (FHOD)

Bioinformatic studies have revealed six formins in *Drosophila melanogaster* genome: diaphanous (Dia - CG1768), cappuccino (Capu - CG15420) DAAM

(CG14622), formin homologue over-expressed in Spleen (Fhos - CG32030), Formin3 (CG33556) and CG32138. Dia corresponds to the DRF subfamily, while DAAM corresponds to the DAAM subfamily and Form3 corresponds to FMN subfamily.

Dia, the most studied *D.melanogaster* formin, is found to be actively involved in cytokinesis. Dia localizes at the sites of membrane invagination in early embryos and is involved in furrow formation and pole cell formation (Afshar, Stuart, & Wasserman, 2000; Castrillon & Wasserman, 1994). Capu, another heavily studied formin is required in regulating the timing of ooplasmic streaming (Emmons, Phan, & Calley, 1995; Manseau & Schupbach, 1989). Capu is also shown to associate with Spire to organize microtubule and microfilament dynamics in developing embryos. Also it has been shown that the KIND domain of Spire strongly inhibits the FH2 domain of Capu *in vitro* (Quinlan, Hilgert, Bedrossian, Mullins, & Kerkhoff, 2007; Rosales-nieves et al., 2006). DAAM has been shown to be involved in correct patterning of tracheal tubes (Matusek et al., 2006) and is also found to be an important regulator of neuronal growth and filopodial formations (Matusek et al., 2008; Prokop et al., 2011). DAAM has also been extensively characterized about its *in vitro* actin nucleating capabilities (Barkó et al., 2010). Of the other three less characterized formins, Form3 has been shown to be involved in cellular rearrangement during tracheal fusion(Tanaka et al., 2004). Recently it has been shown that Fhos takes part in regulating programmed cell death in larvae(Lucas et al., 2012). The following table below summarizes the putative roles of the six *drosophila* formins:

	CG Annotation	**Putative functions**	***In vivo* studies**	***In vitro* studies**	**References**
Diaphanous (dia)	CG1768	Formation of contractile ring during cytokinesis; Regulation of membrane invagination	Yes	Yes	(Afshar et al., 2000; Castrillon & Wasserman, 1994)
Disheveled associated activator of morphogenesis (DAAM)	CG14622	Regulating tracheal tube patterning and development, Axonal morphogenesis	Yes	Yes	(Barkó et al., 2010; Matusek et al., 2006, 2008; Prokop et al., 2011)

Cappuccino (capu)	CG15420	Regulates the timing of ooplasmic streaming, Coordinates microtubule and microfilament dynamics	Yes	Yes	(Emmons et al., 1995; Manseau and Schupbach, 1989,Quinlan et al., 2007; Rosales-nieves et al., 2006)
Formin Homologue Over-expressed in Spleen (Fhos)	CG32030, CG5797, CG5775	programmed cell death in larvae	No	No	(Lucas et al., 2012)
CG32138	CG32138, CG6807	-	No	No	
Fomin3 (form3)	CG33556	actin reorganization during tracheal fusion	Yes	No	(Tanaka et al., 2004)

Table 1: Putative roles of *D.melanogaster* formins

In order to understand actin nucleation activity *in vitro*, various techniques have been developed. Pyrene actin assays have been the standard to understand actin nucleation. In this technique, actin monomers are labeled with the fluorophore pyrene (N-1-pyrene iodoacetamide). The protein of interest is then introduced along with energy supplements. Pyrene-labeled actin incorporated into a filament fluoresces significantly greater than a labeled monomer in solution, thus the amount of fluorescence accurately represents the polymerization state of actin. This simple technique accurately depicts the kinetics of actin polymerization; however, it cannot help represent the organization/structure of the actin networks being formed.

Bead binding assays provide an alternative to this problem. In this assay, the protein of interest is bound to a bead with the help of a tag and is thus concentrated onto the beads. Once placed in fluorescently labeled actin (rhodamine labeled) and supplemented by energy sources, one can visualize the actin nucleation as it occurs.

Ex vivo assays represent a better picture of the regulatory aspects of actin nucleation in a system mimicking *in vivo* conditions. In these assays, cytoplasmic extracts containing co-factors and extra energy currencies are added into the assay,

which gives a much precise view of the actin polymerization. Further one can deplete such putative cofactors and see how actin nucleation is affected.

While three of the six *D.melanogaster* formins have been extensively studied about their actin nucleation capabilities *in vitro*, relatively less light has been shed on the actin regulation capabilities of the other *D.melanogaster* formins. Formins of different subfamilies employ different mechanisms of regulating actin polymerization. The *C.elegans* formin CYK-1, which contains the FH2 domain, has been shown to be a poor actin nucleator (Neidt, Skau, & Kovar, 2008). Similarly it has been shown that only the core FH2 domain of DRF formins inhibit actin polymerization (Shimada et al., 2004). These studies suggest that mere presence of FH2 domain doesn't necessarily imply a role in actin nucleation and that FH1 domain is also indispensable in regulation. Thus it is essential to understand the mechanism of actin nucleation and regulation by the FH1+FH2 domains of these uncharacterized *D.melanogaster* formins.

The experimental strategy of the project is as follows:

- PCR amplify FH+FH2 fragments from fly preps/DGRC clones.
- Clone these inserts into a suitable bacterial expression vector
- Optimize protein over-expression

Once the large scale over-expression and the purification of these proteins is achieved, the recombinant proteins will be used to study actin nucleation using *in vitro/ex vivo* assays.

4. Materials and Methods

4.1 Primer Design:

Primers for PCR were designed to amplify the FH1+FH2 fragments of the six *D.melanogaster* formins. The primers were positioned in such a way that overhangs are present upstream of FH1 domain and downstream of FH2 domain, which facilitate in correct folding of the insert. For confirmation of clones with correct orientation of insert through colony PCRs, a forward primer in GST region and a reverse primer inside each of the six inserts were designed. For amplification of Dia, Capu and Fhos FH1+FH2 fragments, primers were designed with BamHI restriction site, while for Form3 and Capu, EcoRI restriction site was chosen, all to be cloned into pGEX 2TKcs vector. For amplification of CG31238 FH1+FH2 fragments, primers were designed with NotI restriction site, to be cloned into pGEX 4T3 vector. The sequences of the primers used are listed in Appendix.

4.2 Extraction of DNA from flies:

As there are no intronic regions present in the six formin genes of *D.melanogaster,* FH1+FH2 fragments can directly be extracted through PCR from single fly preps of wild type (Canton S strain) *Drosophila melanogaster* flies. Flies were squished in a buffer (10mM Tris, pH 8.2; 25mM NaCl; 1mM EDTA; 200µg/ml Proteinase K), incubated at 37ºC for 1hr and heat inactivated at 95ºC for 10 min. The supernatant was then used as a template for PCR reactions. Conditions for PCR were standardized separately for each formin fragment, changing parameters such as temperature, $MgCl_2$ concentration and DNA template concentration.

All DNA samples were analyzed on EtBr stained 0.8% agarose gels. Agarose and SDS-PAGE gels were analysed using Syngene Automated Gel Documentation system (Syngene). DNA purity and quantification were done using Nanodrop ND-2000/2000c spectrophotometer (Thermo Scientific) at optical density A_{260}. DNA preps were done using standard alkaline lysis buffers as well as Qiagen Plasmid Mini/Midi Kits (Qiagen). The protocol for DNA prep with alkaline lysis buffers is as follows:

2ml of overnight inoculated culture was pelleted down at 10,000 rpm for 10min in a centrifuge at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 100 µl of ice-cold Solution I by vigorous vortexing. 200 µl of freshly prepared Solution II was added and the contents mixed by inverting the tube rapidly. Care was taken to not vortex the samples. After storing the tubes on ice for 5min, 150 µl of ice-cold Solution III was added and gently vortexed in an inverted position for 10 sec. After storing the tubes on ice for 3-5 min, the samples were centrifuged at 12,000g for 5 min and the supernatant transferred to a fresh tube. 450 µl of phenol: chloroform was added and mixed by vortexing. The aqueous phase was separated from the organic phase into a fresh tube after centrifugation at 12,000g for 2min. 450 µl of chloroform was added and mixed by vortexing. After centrifugation at 12,000g for 2min, the aqueous phase was transferred to a fresh tube. The double-stranded DNA was precipitated with 2 volumes of ethanol at room temperature. After mixing by vortexing, the tubes were stored on ice for 5min and later centrifuged at maximum speed for 5min at 4ºC. The supernatant was then removed carefully and the pellet of double-stranded DNA was rinsed with 1ml of 70% ethanol. The supernatant was then removed and pellet was air dried for 10min. The nucleic acids were then finally resuspended in TE (pH 8.0) containing RNAse A (20 µg/ml). After vortexing briefly, the prep was stored at 4ºC

Solutions used:
Solution I: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)
Solution II: 0.2 N NaOH, 1% SDS (to be freshly prepared prior to use)
Solution III: 5M Potassium Acetate - 60ml, Glacial Acetic Acid - 11.5ml, H_2O - 28.5ml

PCRs were performed using *Pfu* polymerase and *Pfx* polymerases. Standard PCR cycle conditions were used unless otherwise specified as follows: 1. Initial Denaturation - 94^0C for 2min; 2. Denaturation - 94^0C for 15sec; 3. Annealing - Primer T_m for 30sec; 4. Extension - 72^0C @ 1kB/min; 5. Goto step 2 - repeat 30 cycles; 6. Final extension - 72^0C for 5min; 7. Hold 4^0C

4.3 DGRC Clones:

Clones available for the formin genes and other cofactors of formins were ordered from DGRC (Drosophila Genomics Resource Center); the below table summarizes the details of the ordered clones. Once revived, these clones were directly used to amplify the FH1+FH2 fragments and for experiments.

Clone ID	Flybase Accession no.	Details
LD14246	FBcl0154646	Dia full length cDNA clone
RE67944	FBcl0227457	DAAM full length cDNA clone
SD08909	FBcl0290082	$Fhos^{192-4182}$ cDNA clone
AT04875	FBcl0018107	CG32138 full length cDNA clone
GH12052	FBcl0111697	$Form3^{1649-5153}$ cDNA clone
IP05727	FBcl0349500	$Form3^{20-716}$ EST
SD10157	FBcl0281408	$Spire^{1-1753}$ cDNA clone
FI03875	FBcl0474791	$Spire^{1474-3063}$ cDNA clone
LD06785	FBcl0157991	*D.melanogaster* cofilin twinstar cDNA clone full length

Table 2: Details of the DGRC clones

4.4 Cloning Strategies:

PCR fragments were cloned into expression vectors pGEX-2TKcs and pGEX 4T3 (Vector maps provided in Appendix). For PCR products which contained non-specific bands, the band corresponding to the expected size was gel extracted using QIAquick Gel Extraction Kit (Qiagen). Insert specific PCR conditions are mentioned in the results section. Fhos PCR insert and pGEX 2TKcs vector were digested with BamHI for 2hrs, with the vector additionally subjected to Calf Intestinal Phosphatase (CIP) treatment at 37ºC for 45 min. Form3 insert and pGEX 4T3 vector were digested with EcoRI for 3hrs and the vector was subjected to CIP treatment at 37ºC for 45 min. Ligations were performed with Takara Mighty Mix Ligation Kit (Takara Bio Inc.) at 16ºC for 30min. Heat shock transformation protocol was used to transform the ligated products into *E.Coli* DH5α competent cells.

The cloning strategy involved digestions with a single restriction site and colony PCRs were done to screen and select colonies with the insert ligated in the correct orientation. The primers for colony PCR were positioned such that the forward primer is present in the GST domain of the vector (named GST f) in the direction 5'->3', while reverse primer is present in the insert (named direction confirmation reverse primer or dcr) in the direction 3'->5'. The protocol for colony PCR is as follows: Mix together the following on ice: 38 µl sterile distilled water, 5µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0% Triton X 100), 3µl of 25 mM MgCl2, 1µl of 10 mM dNTPs, 1µl of 20 µM forward primer, 1µl of 20 µl reverse primer, 0.2-1µl of Taq polymerase. Add a small amount of colony to each tube and pipet up and down to mix. Set up a standard PCR reaction and check for the expected DNA fragments.

An alternate strategy was also used, named Seamless Cloning (Lu, 2005). As described in this technique, primers were designed such that the insert is PCR-amplified with vector overhangs on both sides and the vector is PCR-amplified with insert overhangs on both sides. This leads to great specificity in cloning and also removes the requirement of traditional ligase enzymes. Background colonies formed because of self-ligation were eliminated by digesting the vector and insert mixture with Dpn1 enzyme (which cleaves methylated GATC sites i.e. all the contaminations excluding the PCR amplified DNA). After digesting for 2hr this reaction mixture was directly used for further steps such as transformation.

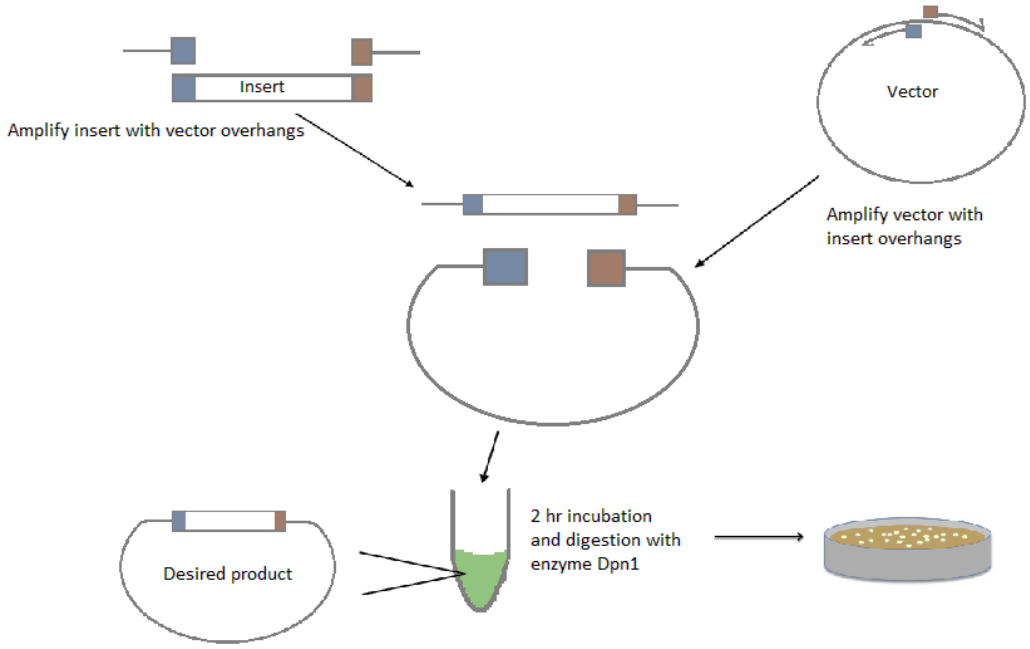


Figure 3: Seamless Cloning. Schematic showing the principle of seamless cloning

4.5 Protein over-expression and purification:

For over-expression of Fhos and Form3 proteins, the clones were transformed using heat shock transformation protocol into *E.Coli* BL21-CodonPlus® (DE3) RP strain of competent cells. Cells were grown in LB (Luria Broth) medium at 37ºC for 4hrs and induced at OD_{600} of about 0.6-0.8 by IPTG (Isopropyl-β-D-thio-galactoside) induction. Protein samples were prepared as follows: 2ml of culture from each of the induced samples were pelleted down. 6X SDS loading dye was added to the pellets and incubated at 95ºC for 20min. The samples were then spun at 13,400 rpm for 5min and supernatants were used for loading on a SDS-PAGE gel.

As the size and conformation of each of the six proteins are different, standardization of conditions such as IPTG concentration, temperature and time of incubation need to be optimized individually for each protein. Once the conditions for over-expression are standardized at small scale, these conditions are then employed to induce large scale cultures.

5. Results

5.1 Identifying the FH1+FH2 domains

Bioinformatics studies give the details of the formin homology domains of the six *D.melanogaster* formins. Uniprot databases give precise information about the positions and boundaries of individual domains of the six formin proteins. The table below reviews the domains present in the six *D.melanogaster* formins.

	Total protein length	GBD/ FH3 domain	FH1 domain	FH2 domain	DAD domain	Region chosen for cloning
Dia transcript variant B, mRNA	1091aa	372aa (59-431)	84aa (512-596)	400aa (601-1001)	32aa (1022-1054)	566aa (477-1043)
DAAM transcript variant C, mRNA	1455aa	-	-	468aa (942-1410)	-	572aa (883-1455)
Capu transcript variant A, mRNA	1060aa	-	80aa (480-560)	436aa (585-1021)	-	617aa (436-1053)
Fhos transcript variant A, mRNA	1393aa	-	-	467aa (883-1350)	-	538aa (839-1377)
CG32138 transcript variant A, mRNA	1183aa	484aa (76-559)	-	402aa (687-1088)	34aa (1136-1169)	468aa (640-1108)
Form3 transcript variant B, mRNA	1644aa	-	-	508aa (342-850)	-	587aa (311-898)

Table 3: Domains present in the six *D.melanogaster* formins
(Numbers in brackets denote the amino acid positions)

The protein sequence alignment of the FH2 domains of six *D.melanogaster* formins reveals a good amount of sequence similarity across them (Fig 4).

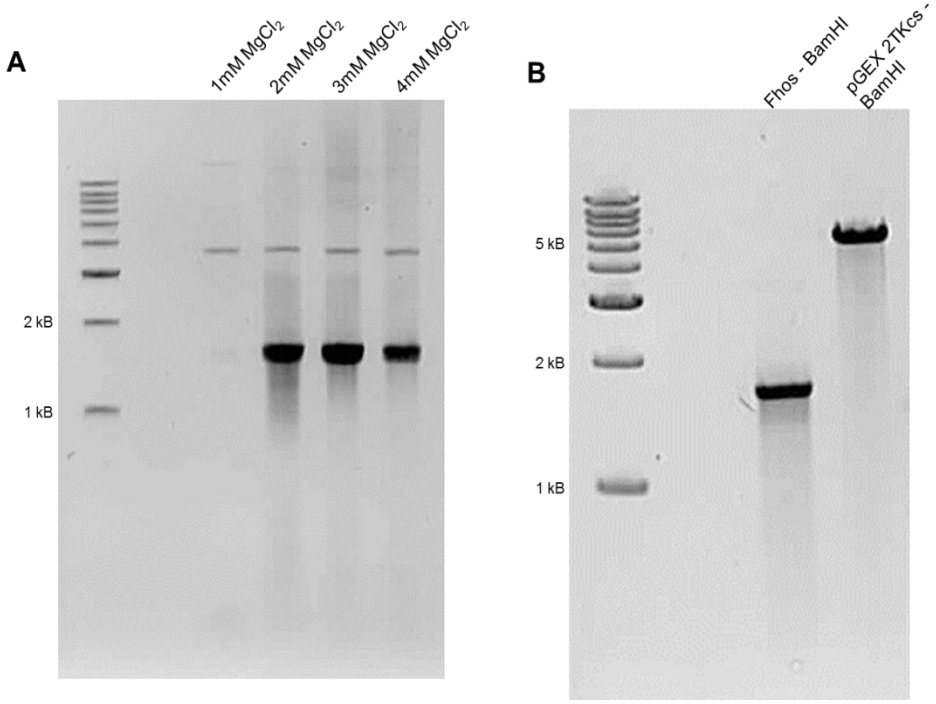
Figure 4: Alignment of the six *Drosophila melanogaster* formin protein sequences using ClustalW software

NCBI databases provided the required DNA sequences of the six formin transcript variants. After aligning with the protein sequences, appropriate primers were designed to amplify the regions as specified in the table above.

5.2 Cloning of Fhos into pGEX 2TKcs:

Fhos FH2 domain was extracted from DGRC clone SD08909. The optimal PCR yield was obtained at 2mM of $MgCl_2$ concentration, with an annealing temperature of 58ºC (Fig 5A). The insert was sequenced and confirmed as Fhos FH2 fragment. The insert (1.6kB) was digested along with the vector (5kB) with the restriction digestion enzyme BamHI (Fig 5B). The ligations were performed with Takara Mighty Mix Ligation Kit (Takara Bio Inc).

Colony PCRs were done according to the protocol described above. Random colonies were picked up and PCR amplified. Three colonies that have shown the correct band size (1kB) have been selected and DNA midi prep was done using Qiagen Midiprep Kit (Qiagen). The clones were again reconfirmed by restriction digestion with BamHI and shown the insert and vector bands (1.6kB and 5kB respectively) (Fig 5C).



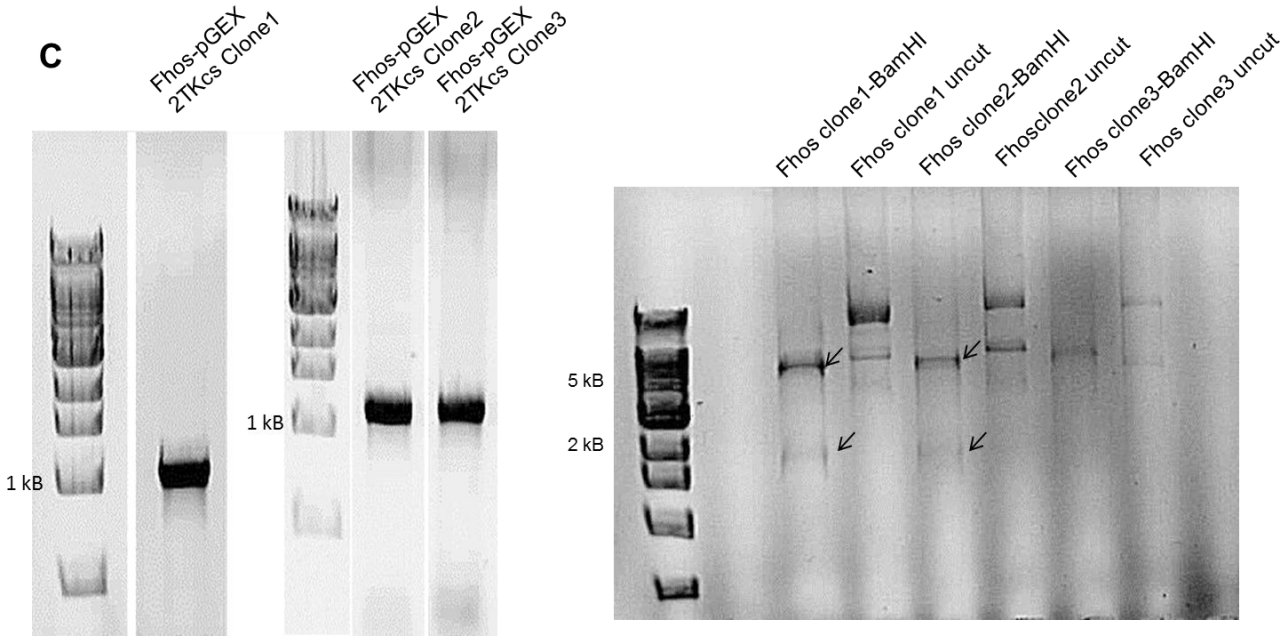


Figure 5: Cloning of Fhos into pGEX 2TKcs

5A. PCR of Fhos FH2 fragment from DGRC clone SD08909. 5B Digestion of the Fhos insert and pGEX 2TKcs vector with BamHI restriction enzyme. 5C Colony PCRs showing 1kB fragment corresponding to the correct orientation of the insert. Confirmation of the three screened clones was done by digestion with BamHI restriction enzyme. Bands are visible at 5kB and 1.6kB

5.3 Cloning of Fhos into pGEX 4T3:

The seamless cloning technique was employed to clone Fhos insert into pGEX 4T3 vector. The insert and vector were amplified through PCR with Platinum *Pfx* polymerase (Invitrogen). The following conditions gave the optimal yield: Fhos insert- 2mM $MgSO_4$ at T_m - 59ºC; pGEX 4T3- Two-step PCR (Invitrogen) at 4mM $MgSO_4$ (Fig 6A and 6B).The products were then digested with Dpn1 restriction enzyme for 2hrs and used for subsequent experiments.

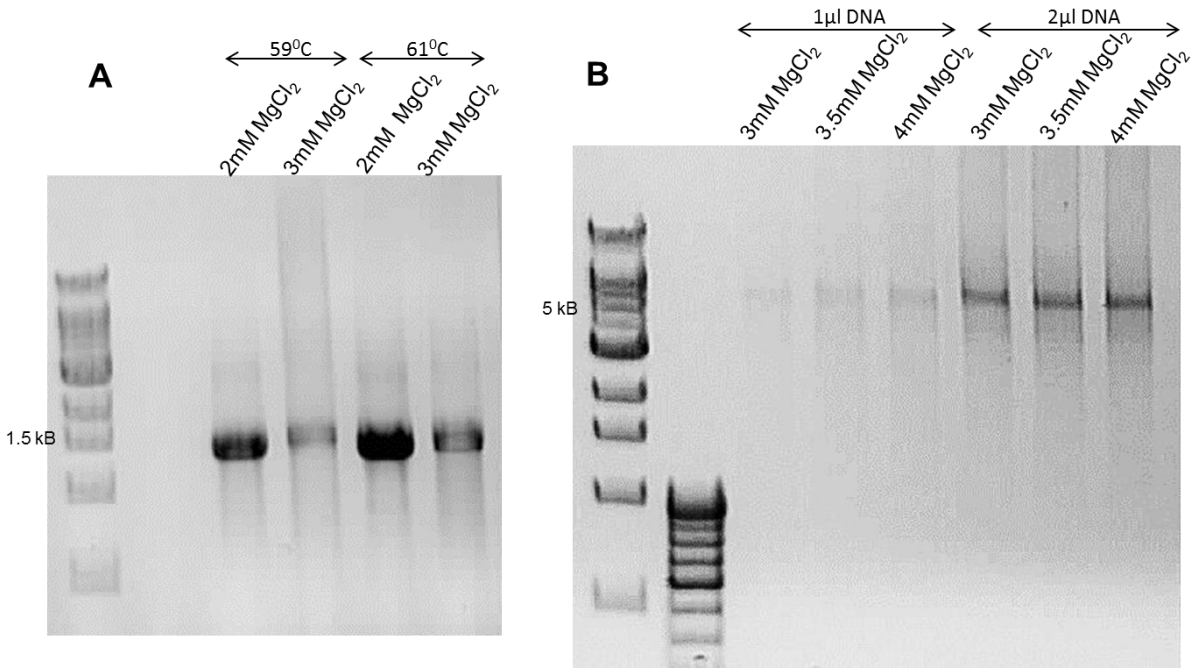


Figure 6: Seamless cloning of Fhos clones into pGEX 4T3

6A. PCR of Fhos insert gave a 1.4kB band. 6B. PCR of pGEX 4T3 gave a 5kB band

5.4 Fhos protein over-expression:

IPTG induction was employed to induce the Fhos protein. Small scale cultures were grown to OD_{600} of 0.6-0.8. IPTG concentrations ranging from 0.6mM to 2mM were tested. Fast induction was employed wherein cultures were incubated for ~4hrs at 37ºC with vigorous shaking. Protein samples, prepared as mentioned above, were loaded onto a 10% SDS-PAGE gel. The results show (Fig 7) mild amount of overexpression at the expected size of 89.7kDa.

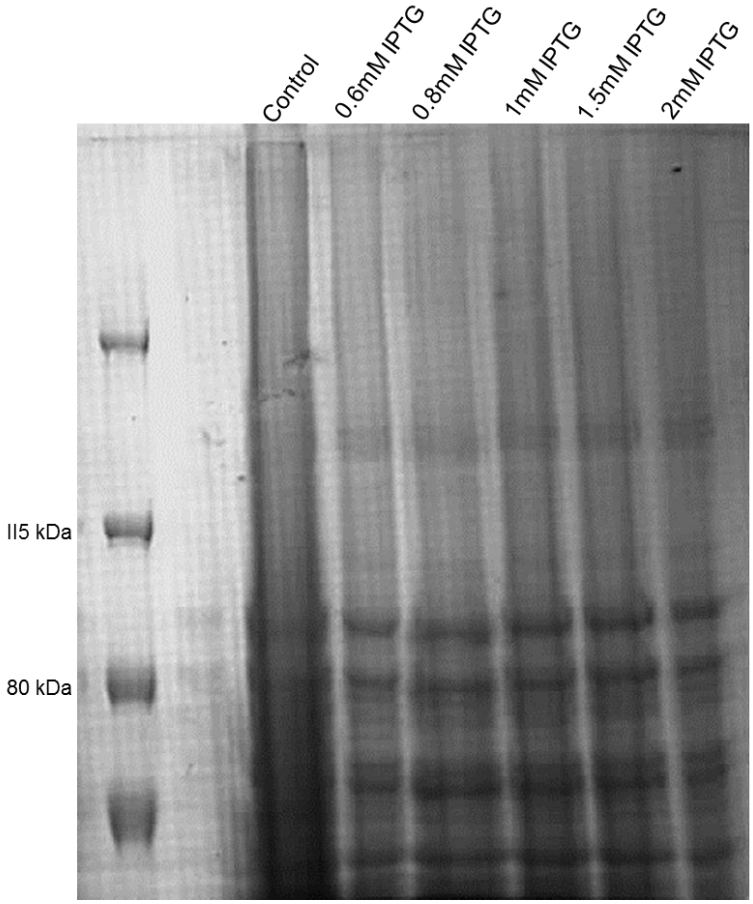


Figure 7: Protein overexpression profile of Fhos

5.6 Cloning of Form3 into pGEX 2TKcs:

Single fly preps were used to extract the Form3 fragment from *D.melanogaster* genome (Fig 8A). PCR conditions are as follows: 3mM $MgCl_2$ at T_m-58ºC. This fragment was sequenced to confirm Form3 FH2 fragment. This fragment, along with the vector pGEX 2TKcs was digested with EcoRI restriction enzyme for 2hrs (Fig 8B) and ligated using Takara Mighty Mix Ligation Kit (Takara Bio Inc). The colonies were randomly selected and DNA prep was done using Qiagen mini prep (Qiagen). Digestion of the midi prep samples with EcoRI gave one clone with correct insert and vector band sizes (2kB and 5kB respectively) (Fig 8C). We are repeating the minipreps and confirming the insert's orientation through diagnostic digestion.

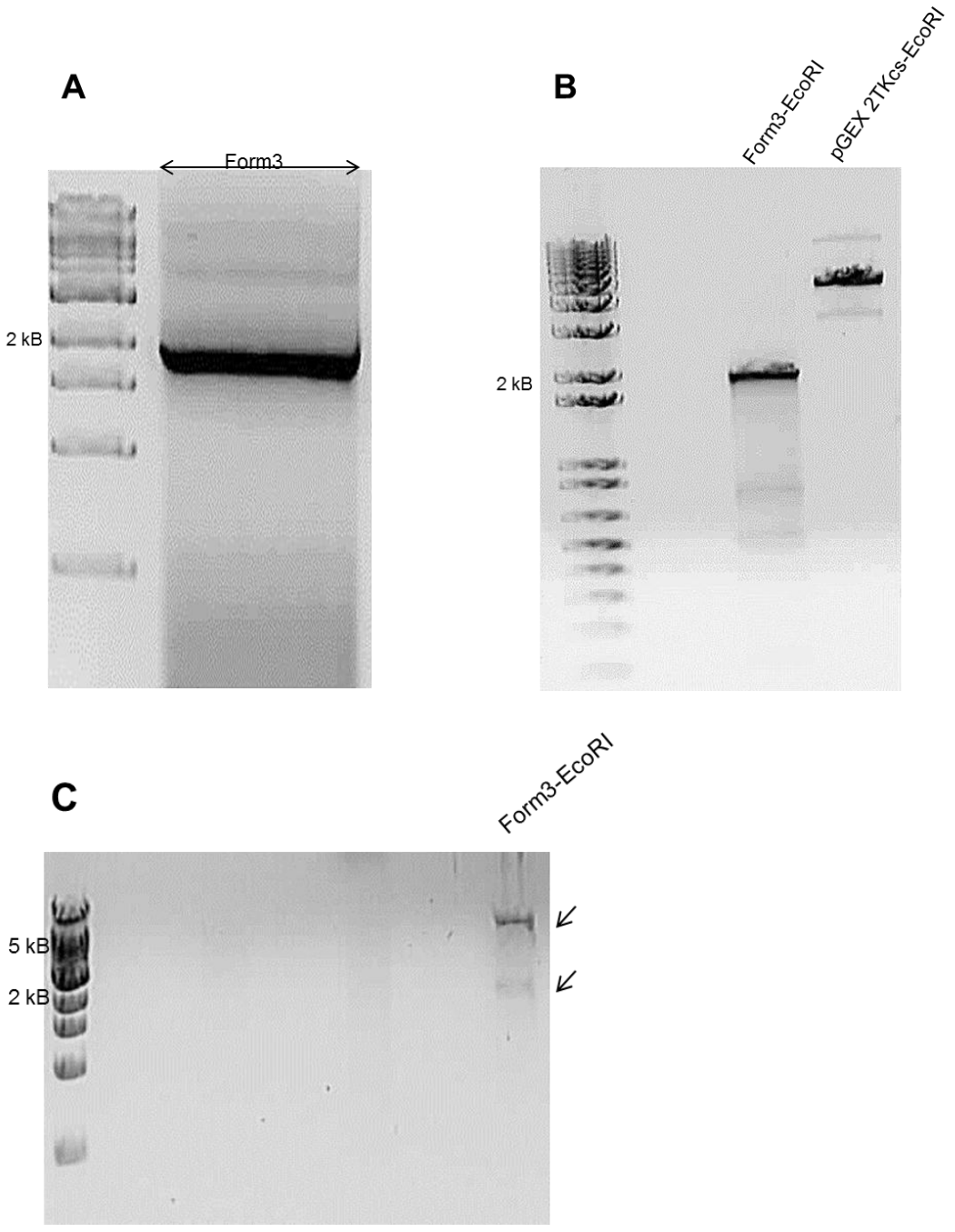


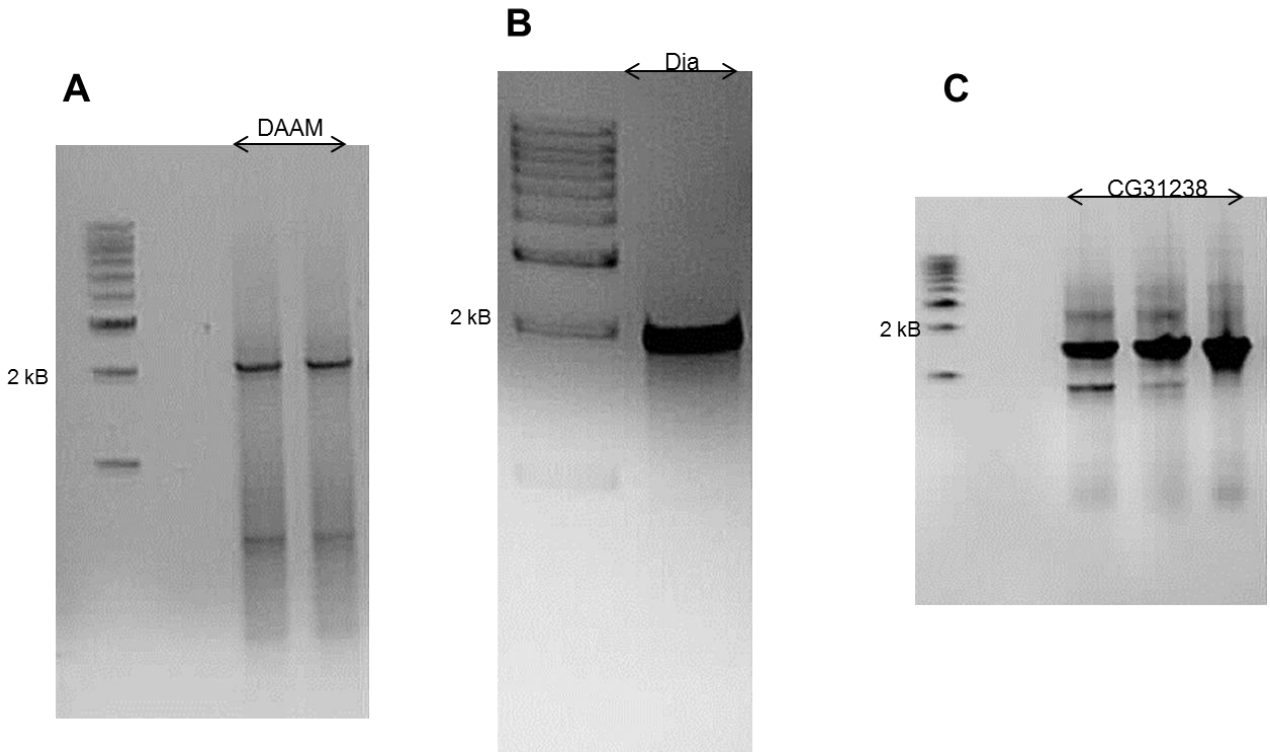
Figure 8: Cloning of Form3 into pGEX 2TKcs

8A.PCR of Form3 FH2 fragment from single fly prep. 8B.Digestion of the Form3 insert and pGEX 2TKcs vector with EcoRI restriction enzyme. 8C.Digestion with EcoRI to check for ligation. Bands are visible at 5kB and 2kB confirming ligation

5.7 Cloning of Dia, DAAM and CG31238:

FH1+FH2 constructs of Dia and DAAM were extracted and PCR amplified from single fly preps (Fig 9A, 9B). PCR conditions are as follows: Dia- 3mM $MgCl_2$ at T_m-58ºC; DAAM- 3mM $MgCl_2$ at T_m-54ºC. AT04875, the CG31238 full length cDNA clone was used to PCR amplify the FH2 fragment (Fig 9C) at a standardized condition of 3mM $MgCl_2$ at T_m-56ºC. Sequencing results have confirmed the Dia and DAAM FH1+FH2 sequences amplified from single fly preps.

The Dia insert and pGEX 2TKcs vector were digested with BamHI restriction enzyme and DAAM insert and pGEX 2TKcs vector with EcoRI restriction enzyme (Fig 9D). The CG32138 fragment and pGEX 4T3 vector were digested with NotI enzyme (Fig 9E). Ligations of these inserts into appropriate vectors have not yet yielded clones with the insert in correct orientation.



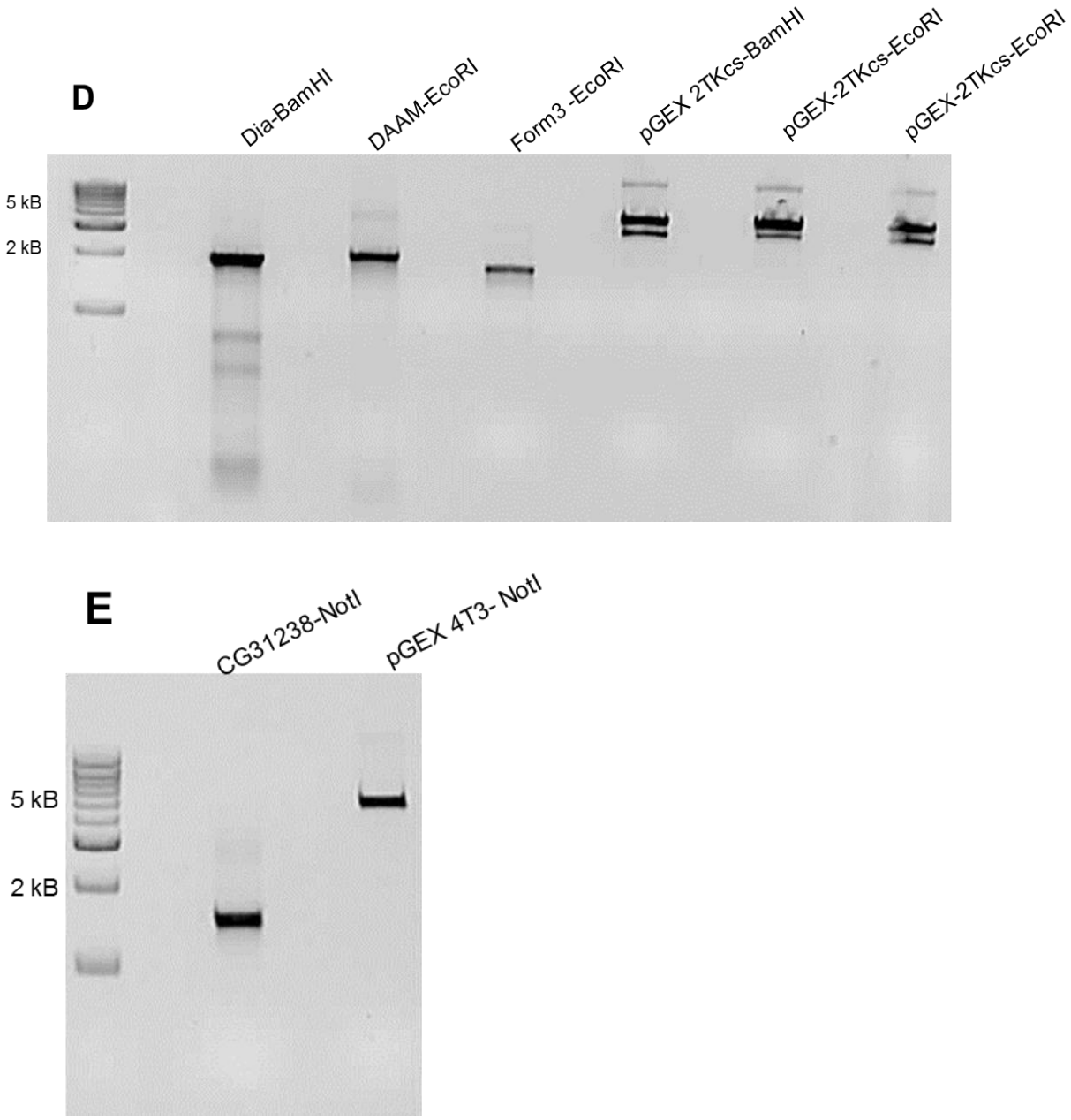


Figure 9: Cloning of Dia, DAAM and CG31238

9A, 9B, 9C. PCR of Dia, DAAM fragment from single fly preps and CG31238 from DGRC clone AT04875. 9D. Digestion of the DAAM insert and pGEX 2TKcs vector with BamHI restriction enzyme and digestion of Dia insert and pGEX 2TKcs vector with BamHI restriction enzyme. Also shown is the Form3 insert and pGEX 2TKcs vector

digested with EcoRI enzyme. 9E. Digestion of CG31238 insert and pGEX 4T3 vector with NotI restriction enzyme.

5.8 Over expression of W2C for use in an *ex vivo* assay to study actin polymerization

The W2C (WAVE2C) protein of the human WASP family of proteins is known for its high affinity to bind and sequester monomeric G-Actin and also activate the Arp2/3 complex. The reason for choosing W2C domain is that, unlike formins which are direct actin nucleators, W2C domain cannot directly nucleate actin and requires Arp2/3 complex for nucleation *in vitro*. Thus this can be used as an ideal positive control to study actin polymerization through *in vitro/ex vivo* assays.

A plasmid pGEX was obtained with the VCA domain (residues 429-498) of W2C cloned into it and also modified by introducing a trans-carboxylase domain and renamed as pGTC (GST Trans Carboxylase) plasmid (Bouslama-oueghlani, Echard, Louvard, & Gautreau, 2007). The protein induced by IPTG induction from the plasmid pGTC has been over-expressed with the following conditions: 37ºC at 4hr with different IPTG concentrations.

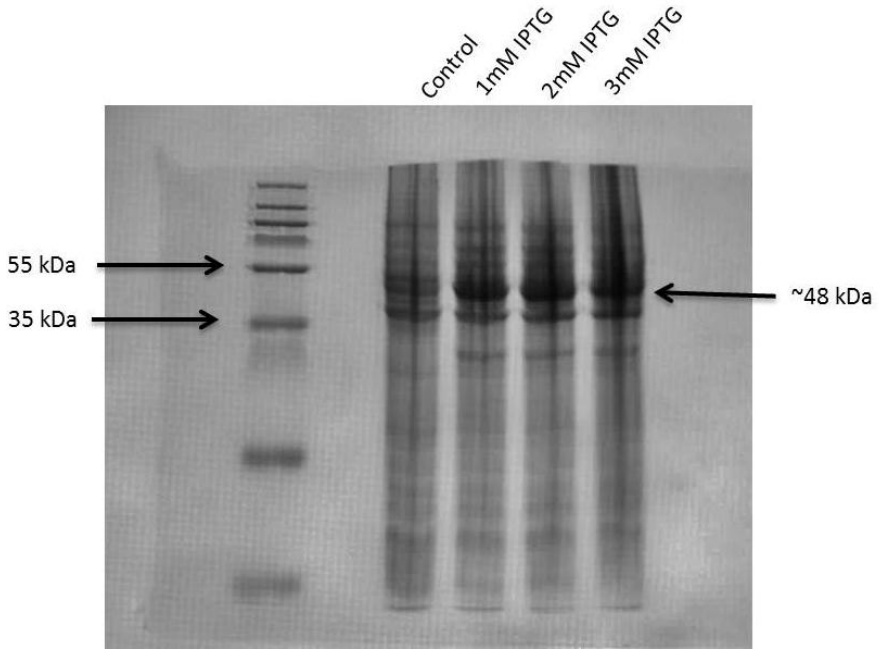


Figure 10: Protein Overexpression of pGTC construct with IPTG induction

1mM IPTG concentration gave the best yield and this condition was used to induce over-expression at large scales (Fig 10). The purified protein has been tested and was further used in an *ex vivo* assay by Subhamita Sarkar (Ph.D. scholar). The *ex vivo* assay involved GTC tagged W2C proteins which were bound to streptavidin coated magnetic Dynabeads (M280). These beads when placed in S2 cell cytosolic extracts supplemented with energy currencies were shown to nucleate actin filaments (unpublished data).

6. Discussions

We were able to extract the FH1+FH2 fragments of five *D.melanogaster* formins. We were also able to clone Fhos and Form3 FH2 fragments into pGEX 2TKcs vector. The protein over-expression of Fhos is being standardized. As is mentioned earlier, the highly conserved FH2 domain acts in different mechanisms to regulate actin nucleation and this study tries to address the role of the three uncharacterized *D.melanogaster* formins in actin regulation.

Limited success with cloning of the other formins could be because of several reasons. Mutations or additional inserts added during PCR amplification from single fly preps could lead to incompatible sticky ends which decreases the chances of successful cloning. A single site cloning strategy was chosen over two site cloning because, the two restriction sites available for cloning into the pGEX 2TKcs and pGEX 4T3 vectors were close to each other which would decrease the chances of a successful digestion and ligation. Seamless cloning proves to be a better alternative to traditional cloning strategies as it is simple and cost-effective because it is less time consuming and doesn't require traditional restriction enzymes or ligase enzymes. Single fly preps are a good way to isolate the FH1+FH2 fragments and several inserts such as Dia and DAAM have been isolated. However, the variability of the amount of contaminants present proves the standardization to be tedious and time taking. Also the location of the genes in the chromosome affects the amount of PCR amplification that can be achieved.

Protein overexpression at small scale gave less amount of overexpression while scaling up the volume gave much lesser expression. Initial trails to over express the protein of interest with BL21-CodonPlus™ cells were ineffective. The problem was attributed to Codon Bias. Codon usage bias means that different strains of cells prefer different set of codons over others for coding the same amino acid. The expression of FH1 domain, a proline rich region would get affected because of such bias as the endogenous reserves of arginine and proline codon's corresponding tRNAs, which are

fewer in *E.Coli* gets depleted soon. This might sometimes lead to slowing or termination of translation or can cause frame-shifts. In order to overcome this problem, another strain *E.Coli* BL21-Codon Plus (DE3)-RP was suggested as this strain contains additional copies of Arg and Pro tRNAs, important for FH1 domain over-expression (Moseley, Maiti, & Goode, 2006). The change to this strain has worked as it helped in over-expression of another formin Bni1p in the lab (Subhamita Sarkar, Ph.D. scholar). Also the conditions being used for optimization need to be varied to cover a wider range of conditions. Different types of induction such as slow induction and fast induction needs to be employed.

The pGTC vector is obtained with the W2C domain inserted and also contains a trans-carboxylase domain. The use of this construct has several advantages. The trans-carboxylase domain, which is endogenously biotinylated, binds very strongly to streptavidin coated magnetic Dynabeads M280 (Invitrogen), which helps in easier purification of the protein while preserving its native state. It has been demonstrated that size of the beads affects the rate of actin-polymerization with smaller beads giving rapid reaction kinetics as compared to larger beads (Bernheim-groswasser, Wiesner, & Golsteyn, 2002). Thus, the M280 Dynabeads which are 2-5 µm in diameter has a major advantage over traditional GST beads which have 90µm in diameter. The *ex vivo* assay described earlier used the cytosolic extracts from S2 cells. S2 cells were chosen because there is less functional redundancy in this cell line and when coupled with RNAi mediated loss-of-function studies, it could prove as a cost-effective alternative to techniques such as immunodepletion.

The assay once developed, can be used to better understand the nucleation capability of these uncharacterized formins. Future work can be done by introducing several cofactors such as profilin, cofilin, etc. into the assay and measure the changes in actin nucleation dynamics. Profilin has been shown to increase the rate of actin nucleation in several in vitro studies (Goode & Eck, 2007; Neidt, Scott, & Kovar, 2009; Yang et al., 2011). Thus it is interesting to see whether these novel formins also increase their rate of actin nucleation in presence of such cofactors. Also the assay once developed can be used to cover a wider range of formins, other than the three novel formins being described here.

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8. References

Afshar, K., Stuart, B., & Wasserman, S. a. (2000). Functional analysis of the Drosophila diaphanous FH protein in early embryonic development. *Development (Cambridge, England)*, *127*(9), 1887-97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10751177

Barkó, S., Bugyi, B., Carlier, M.-F., Gombos, R., Matusek, T., Mihály, J., & Nyitrai, M. (2010). Characterization of the biochemical properties and biological function of the formin homology domains of Drosophila DAAM. *The Journal of biological chemistry*, *285*(17), 13154-69. doi:10.1074/jbc.M109.093914

Bernheim-groswasser, A., Wiesner, S., & Golsteyn, R. M. (2002). The dynamics of actin-based motility depend on surface parameters. *Nature*, *417*(May), 308-311.

Bouslama-oueghlani, L., Echard, A., Louvard, D., & Gautreau, A. (2007). RNAi depleted Drosophila cell extracts to dissect signaling pathways leading to actin polymerization. *Pharmacia*, *70*, 663-669. doi:10.1016/j.jbbm.2007.03.003

Castrillon, D. H., & Wasserman, S. A. (1994). diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. *Development*, *3377*, 3367-3377.

Emmons, S., Phan, H., & Calley, J. (1995). Cappuccino, a Drosophila maternal effect gene required for polarity of the egg and embryo, is related to the vertebrate limb deformity locus. *Genes & Development*. doi:10.1101/gad.9.20.2482

Evangelista, M., Zigmond, S., & Boone, C. (2003). Formins : signaling effectors for assembly and polarization of actin filaments. *Journal of Cell Science*, (ld). doi:10.1242/jcs.00611

Goode, B. L., & Eck, M. J. (2007). Mechanism and function of formins in the control of actin assembly. *Annual review of biochemistry*, *76*, 593-627. doi:10.1146/annurev.biochem.75.103004.142647

Higgs, H. N. (2005). Formin proteins: a domain-based approach. *Trends in biochemical sciences*, *30*(6), 342-53. doi:10.1016/j.tibs.2005.04.014

Lu, Q. (2005). Seamless cloning and gene fusion. *Trends in biotechnology*, *23*(4), 199-207. doi:10.1016/j.tibtech.2005.02.008

Lucas Anhezini, Ana Paula Saita, Mara S.A Costa, Ricardo Guelerman Pinheiro Ramos, C. R. S. (2012). Fhos encodes a Drosophila Formin- like protein participating in autophagic programmed cell death . *Genesis*, 22025. doi:10.1002/dvg.22025.

Manseau, L. J., & Schupbach, T. (1989). cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo. *Genes & Development*, *3*(9), 1437-1452. doi:10.1101/gad.3.9.1437

Matusek, T., Djiane, A., Jankovics, F., Brunner, D., Mlodzik, M., & Mihály, J. (2006). The Drosophila formin DAAM regulates the tracheal cuticle pattern through organizing the actin cytoskeleton. *Development (Cambridge, England)*, *133*(5), 957-66. doi:10.1242/dev.02266

Matusek, T., Gombos, R., Szécsényi, A., Sánchez-Soriano, N., Czibula, A., Pataki, C., Gedai, A., et al. (2008). Formin proteins of the DAAM subfamily play a role during axon growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *28*(49), 13310-9. doi:10.1523/JNEUROSCI.2727-08.2008

Moseley, J. B., Maiti, S., & Goode, B. L. (2006). Formin proteins: purification and measurement of effects on actin assembly. *Methods in enzymology*, *406*(06), 215-34. doi:10.1016/S0076-6879(06)06016-2

Neidt, E. M., Scott, B. J., & Kovar, D. R. (2009). Formin differentially utilizes profilin isoforms to rapidly assemble actin filaments. *The Journal of biological chemistry*, *284*(1), 673-84. doi:10.1074/jbc.M804201200

Neidt, E. M., Skau, C. T., & Kovar, D. R. (2008). The Cytokinesis Formins from the Nematode Worm and Fission Yeast Differentially Mediate Actin Filament Assembly *. *Journal of Biological Chemistry*, *283*(35), 23872-23883. doi:10.1074/jbc.M803734200

Pollard, T. D. (2007). Regulation of Actin Filament Assembly by Arp2 / 3 Complex and Formins, 451-477. doi:10.1146/annurev.biophys.35.040405.101936

Pring, M., Evangelista, M., Boone, C., Yang, C., & Zigmond, S. H. (2003). Mechanism of formin-induced nucleation of actin filaments. *Biochemistry*, *42*(2), 486-96. doi:10.1021/bi026520j

Prokop, A., Sánchez-Soriano, N., Gonçalves-Pimentel, C., Molnár, I., Kalmár, T., & Mihály, J. (2011). DAAM family members leading a novel path into formin research. *Communicative & integrative biology*, *4*(5), 538-42. doi:10.4161/cib.4.5.16511

Quinlan, M. E., Heuser, J. E., Kerkhoff, E., & Mullins, R. D. (2005). Drosophila Spire is an actin nucleation factor. *Nature*, *433*(January).

Quinlan, M. E., Hilgert, S., Bedrossian, A., Mullins, R. D., & Kerkhoff, E. (2007). Regulatory interactions between two actin nucleators, Spire and Cappuccino. *October*, *179*(1), 117-128. doi:10.1083/jcb.200706196

Rosales-nieves, A. E., Johndrow, J. E., Keller, L. C., Magie, C. R., Delia, M., & Parkhurst, S. M. (2006). Coordination of microtubule and microfilament dynamics by Drosophila Rho1 , Spire , and Cappuccino. *Cancer Research*.

Shimada, A., Nyitrai, M., Vetter, I. R., Kühlmann, D., Bugyi, B., Narumiya, S., Geeves, M. a, et al. (2004). The core FH2 domain of diaphanous-related formins is an

elongated actin binding protein that inhibits polymerization. *Molecular cell*, *13*(4), 511-22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14992721

Takeya, R., & Sumimoto, H. (2003). Fhos , a mammalian formin , directly binds to F-actin via a region N-terminal to the FH1 domain and forms a homotypic complex via the FH2 domain to promote actin fiber formation. *Journal of Cell Science*. doi:10.1242/jcs.00769

Tanaka, H., Takasu, E., Aigaki, T., Kato, K., Hayashi, S., & Nose, A. (2004). Formin3 is required for assembly of the F-actin structure that mediates tracheal fusion in Drosophila. *Developmental biology*, *274*(2), 413-25. doi:10.1016/j.ydbio.2004.07.035

Yang, C., Huang, M., Debiasio, J., Pring, M., Joyce, M., Miki, H., Takenawa, T., et al. (2011). Nucleation of Actin Polymerization Profilin Enhances Cdc42-induced. *Cell*, *150*(5), 1001-1012.

9. Appendix

Details of the primers:

Primers for amplifying FH1+FH2 domains

Primer Name	5'	3'
d.Dia f	GGCGCGGATCCCAAGAGGCGGAAGCGAAGGC	
d.Dia r	GGCGCGGATCCGCCAAAGGCTGAGCCCGTT	
d.DAAM f	CGCCGGAATTCGGCAATGGTGCCGTCTCGCCT	
d.DAAM r	CGCCGGAATTCCACGCCATCTGGAAACATATACACC	
d.Capu f	GGATCCGGCCAAACGGAAAGCGAGGATTGC	
d.Capu r	GGATCCGGGTTCCATCGCACAAATACCATG	
d.Fhos f	GGCGCGGATCCGCGCTGGGTGGAGCCATTG	
d.Fhos r	GGCGCGGATCCCCGTTTTTCAGCGTGCGACGT	
d.Form3 f	CGCCGGAATTCCCACCACCGCCCTCTATGC	
d.Form3 r	CGCCGGAATTCGCCCGTTCACGATCCATGC	
d.CG32138 f	GCGGCCGCTTCAGTCCGCTGGGCAGT	
d.CG32128 r	GCGGCCGCCCGTTTGAGGGCAACTGTTG	

Primers used in colony PCR

Primer Name	5'	3'
GST f	CCCAATGTGCCTGGATGCGT	
d.Dia dcr	CGGTAGCGGTTCGCCCTTT	
d.DAAM dcr	ACGGGAACGAGCCACCTC	
d.Capu dcr	CGATTTCGCTGGACGGCAC	
d.Fhos dcr	CGAGTGCTTGTGCACCGTG	
d.Form3 dcr	TCCGCCTGAAGAGCCACGA	
d.CG32138 dcr	GCTGGTCCCCGTTTGTTGCT	

(Note: f –forward primer, r – reverse primer, dcr – direction confirmation reverse primer)

Diagrammatic maps of vectors pGEX 2TKcs and pGEX 4T3:

1. pGEX 2TKcs: The pGEX 2TKcs originated from pGEX 2TK vector with some modifications done in the MCS.

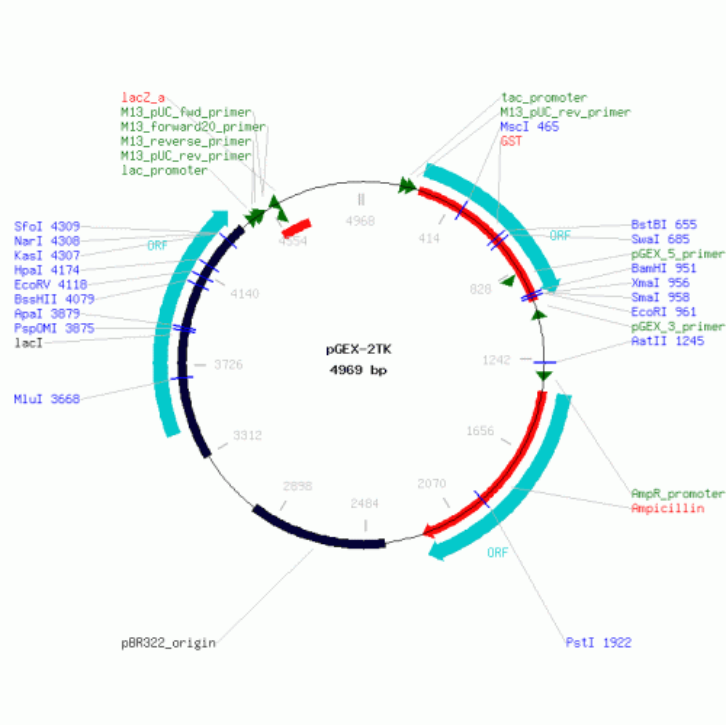

Image reproduced from [LabLife website]

2. pGEX 4T3:

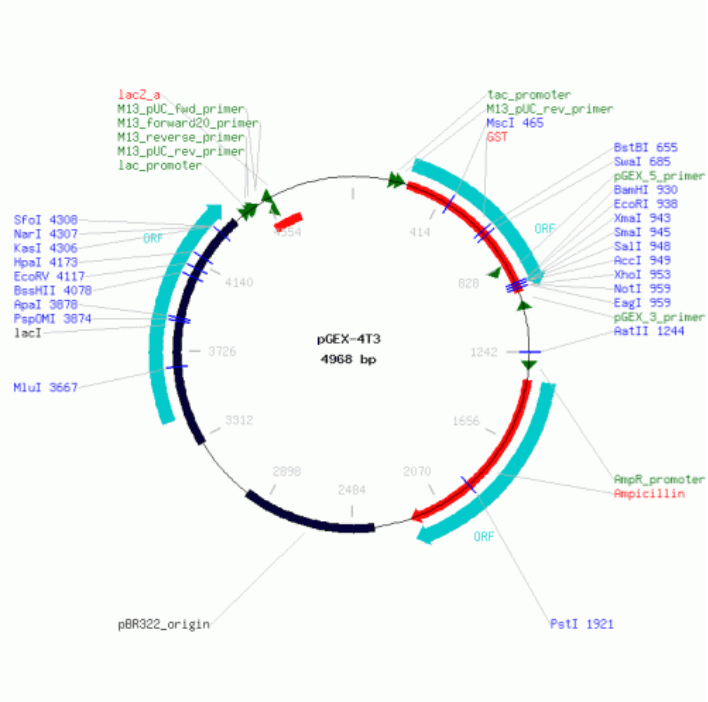

Image taken from [LabLife website]