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Development of new tools for live imaging of BMP signalling in the *Drosophila melanogaster* wing

Bachelor's thesis
12 EAP

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BMP signalling pathway has a central part in animal development. It is responsible for many different functions during animal development including cell proliferation, adult tissue homeostasis, apoptosis, early axial patterning. To study the dynamics of BMP signalling new and more precise tools are needed. During this bachelor's thesis, a LlamaTag based tool was created. Which can be used to observe BMP signalling pathway protein Mothers against Dpp (Mad) movement *in vivo* in fruit fly *Drosophila melanogaster* using live imaging method.

Key words: *Drosophila melanogaster*, BMP, Mothers against Dpp (Mad), LlamaTag

CERCS: B350 Development biology, growth (animal), ontogeny, embryology

Uudse tööriista arendamine reaalajas BMP signaaliraja jälgimiseks *Drosophila* tiiva arengus

BMP signaalirajal on keskne roll looma arengus. BMP signaalirada vastutab erinevate funktsioonide eest kogu looma arengu jooksul. Näiteks BMP kontrollib rakkude proliferatsiooni, apoptoosi, hoiab homoöstaasi ja määrab ära varajase kehatelgede mustri. Selleks, et uurida BMP signaaliraja dünaamikat on vaja luua uusi ja täpsemaid meetodeid. Selle bakalaureusetöö käigus loodi LlamaTag'il põhinev meetod, mis võimaldab vaadelda BMP signaaliraja valgu Mothers against Dpp (Mad) liikumist *in vivo* äädikakärbses *Drosophila melanogaster*, kasutades *live imaging* meetodit.

Märksõnad: *Drosophila melanogaster*, BMP, Mothers against Dpp (Mad), LlamaTag

CERCS: B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

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Abbreviations

AP – After pupariation

BMP – Bone morphogenetic protein

Dpp – Decapentaplegic, fly equivalent of BMP

eGFP – enhanced green fluorescent protein

Mad – Mothers against Dpp

pMad- Phosphorylated Mad

TGF-β – Transforming growth factor type β

Tkv – Thickveins, type 1 membrane bound receptor

Introduction

Animal development is widely studied area in biology, from embryonic development to organogenesis. A part of animal development, tissue morphogenesis is often directed by evolutionarily conserved signalling proteins including bone morphogenetic proteins (BMPs). It has been shown that BMPs are highly conserved in multiple species. For example, *Drosophila* equivalent BMP, decapentaplegic (Dpp) is a functional ortholog to human BMP2 and BMP4 (Padgett *et al*., 1993). BMP signalling is responsible for directing cell proliferation, tissue size control and cell differentiation among many other functions.

Animal developmental process is dynamic. During development the cells are proliferating, differentiating and reorganizing in order to form fully functional organs. Tissue morphogenesis is tightly connected to growth factor signalling. Gui *et al*., 2019 used *Drosophila* pupal wing as a model to study tissue morphogenesis and BMP signalling coordination. However due to lack of tools to detect BMP signal *in vivo*, precise dynamics of how tissue growth and BMP signalling affect each other remains unclear. BMP signalling starts when ligand-bound receptor phosphorylates Mothers against Dpp (pMad), which then creates a complex with co-Smad Medea and is translocated into nucleus where it regulates the expression of many target genes. This new tool is based on the ability to detect Mad in the nucleus, which shows activated BMP signal. In this thesis we plan to design a new tool for live imaging to detect BMP signalling *in vivo*.

Newly developed tagging technique LlamaTag was used as a base for designing our new tool for live imaging. LlamaTag is a single domain antibody (nanobody) from llamas (Bothma *et al*., 2018; Hamers-Casterman *et al*., 1993). LlamaTag was fused to Mad, which was raised against enhanced green fluorescent protein (eGFP). Mad-LlamaTag fusion protein and eGFP is then transfected into *Drosophila* S2 cells, where they will be translated into fully functioning proteins. Mature eGFP is then able to bind to Mad-LlamaTag fusion protein in seconds, resulting in an accumulation of GFP signal in nucleus when BMP signalling pathway is active. This new tool should allow us to study tissue dynamics and BMP signalling using live imaging. The goal of this thesis is to develop LlamaTag based tool which allows us to study BMP signalling dynamics *in vivo* in *Drosophila melanogaster* using live imaging. Thesis was made in the Institute of Molecular and Cell Biology Chair of Developmental Biology

1 Literature overview

1.1 Overview on *Drosophila* wing development

Developing *Drosophila* wing is great model for studying, for example, how tissue size and patterning is controlled by BMP signalling, due to wing's apparent simplicity. Like many other insects, *Drosophila melanogaster* goes through two stages of development.

First, during larval stage wing's development takes place inside the larva in a region called wing imaginal disc. At this point the wing consists of 2-dimensional single layer of epithelial cells. Normal wing development includes the correct patterning of wing veins. There are total 4 main longitudinal veins (L2-L5) and 2 incomplete longitudinal veins, which do not reach the wing margin. In addition, there are 2 cross-veins, anterior cross-vein and posterior cross-vein (ACV and PCV respectively) (Celis, 2003). Already in wing imaginal discs, there are some cells identified as pro-veins (Figure 1 a). The main driving force in wing imaginal disc development is the formation of long-range Dpp morphogen gradient between longitudinal veins L3 and L4 together with signalling protein Hedgehog, Notch and epidermal growth factor. BMP signalling pathway type 1 receptor thick veins (Tkv) helps regulate the range of long-range Dpp gradient. The levels of Tkv is lower near the center of the Dpp morphogen gradient and higher in lateral part of the wing imaginal disc(T. Lecuit and Cohen, 1998).

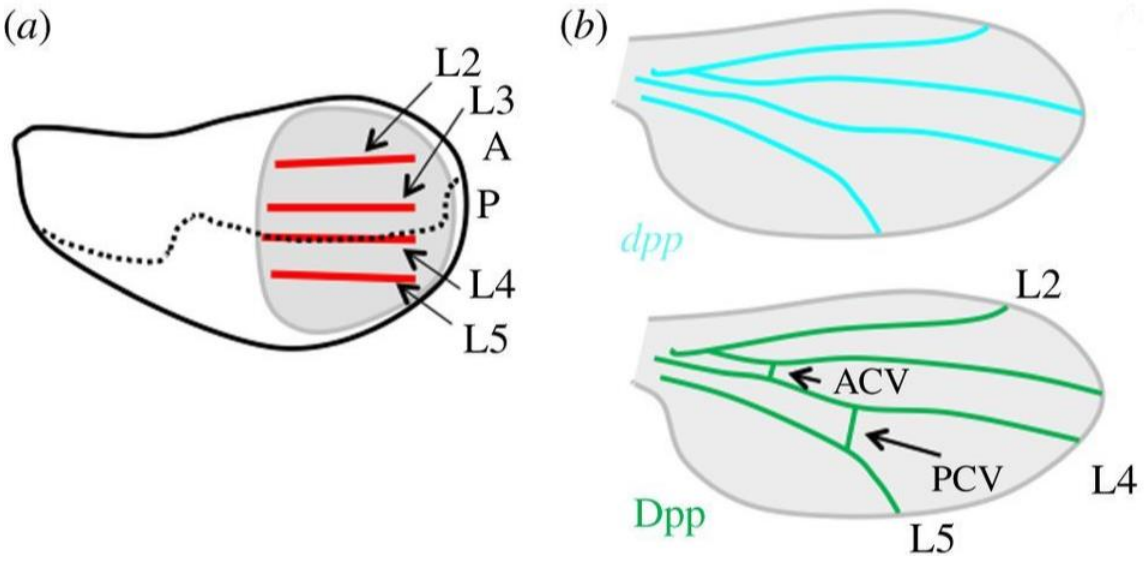

Figure 1. ***Drosophila* wing development**. (a) Development of wing veins of third-instar larva wing imaginal disc. Dpp long-range morphogen gradient separates anterior (A) and posterior (P) sides of the wing imaginal disc (dotted line). Red lines represent longitudinal veins L2-L5. The grey circle represents the wing pouch. (b, TOP) Dpp expression in longitudinal veins (blue) during early pupal stage. (b, BOTTOM) Dpp signal in all veins, including longitudinal veins (L2-L5) and anterior and posterior cross veins (ACV and PCV respectively). Modified by Shimmi *et al*., 2014.

Second, the next step in wing development takes place in pupal stage. In early pupal stage the single layered wing imaginal disc forms into two-layered 3D wing through apposition (0-10 hours after pupariation (AP)), inflation (10-20 hours AP) and second apposition (~20 hours AP). During pupal stage Dpp signalling is shown to be necessary in tissue proliferation and differentiation of vein cells, including cross veins (De, 1997; Gui *et al.*, 2019; Wartlick *et al.*, 2011). Both long-range signalling and short-range signalling of Dpp is required for normal wing vein patterning.

1.2 BMP signalling pathway

Bone morphogenetic protein (BMP) belongs to the transforming growth factor type β (TGF-β) superfamily. These extracellular proteins regulate animal development including *Drosophila* wing patterning, controlling cell proliferation and tissue growth, adult tissue homeostasis, apoptosis, in both vertebrates and invertebrates. There are many similarities between vertebrate and invertebrate BMPs. For example, human's BMP2 and BMP4 can substitute decapentaplegic (Dpp) in *Drosophila* as shown in Padgett *et al.*, 1993. This thesis focuses mainly on *Drosophila's* BMP decapentaplegic (Dpp) pathway. Dpp pathway consists mainly of ligand (Dpp), type 1 and type 2 receptors (Tkv and punt respectively), receptor-regulated Smad (R-Smad), Mothers against Dpp (Mad), and common mediator Smad (Co-Smad), Medea (Figure 2).

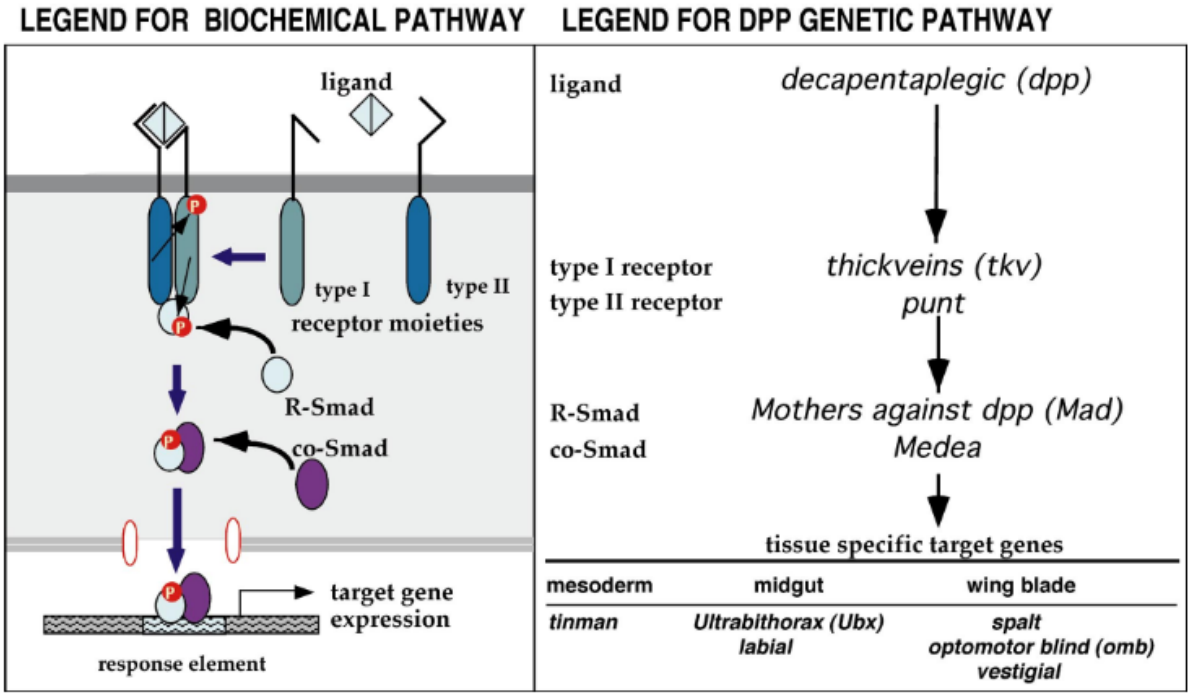

Figure 2. **Simplified BMP signalling pathway.** Modified by Raftery and Sutherland, 1999.

Signalling is initiated when the Dpp binds to type 1 receptor Tkv. Type 2 receptor then phosphorylates ligand-bound type 1 receptor to activate it. Type 1 receptor is then able to

phosphorylate R-Smad (Mad). After this phosphorylated Mad (pMad) associates with co-Smad (Medea). This pMad/Medea complex is then translocated to nucleus where it binds to target genes like spalt (sal) and optomotor blind (omb) based on distinct pMad concentrations. (Thomas Lecuit *et al.*, 1996; Raftery and Sutherland, 1999)

1.3 LlamaTag systems: new tools for reading out BMP signal *in vivo*

In recent years new tools have been developed to live image protein localization in *Drosophila* (Bothma *et al*., 2018; Harmansa *et al.*, 2017). The half-life of fluorescent proteins *in vivo* is >40 minutes in *Drosophila* (Hazelrigg *et al*., 1998). For example, compared to the half-life of about 8 minutes of fly transcription factor Fushi Tarazu (Edgar *et al.*, 1987). This means that we need new tools that allows us to get fluorescent signal much faster. Bothma *et al*., 2018 developed new tagging method, which is based on nanobodies, which they named LlamaTag. Nanobodies are smallest naturally functioning single domain antibodies. These antibodies contain only one variable domain, though surprisingly these nanobodies exhibit broad antigen binding possibilities. (Muyldermans, 2001) Using Bothma *et al*., 2018 nanobody tagging method, LlamaTag and transcription factor Mad were fused and raised against enhanced green fluorescent protein (eGFP). (Figure 3) This brings an advantage to avoid the long maturation time of fluorescent protein fusions and allow precise observation of transcription factor dynamics *in vivo*. Mad protein localized in nucleus can be used as an intracellular marker to observe Dpp activity (Tanimoto *et al.*, 2000).

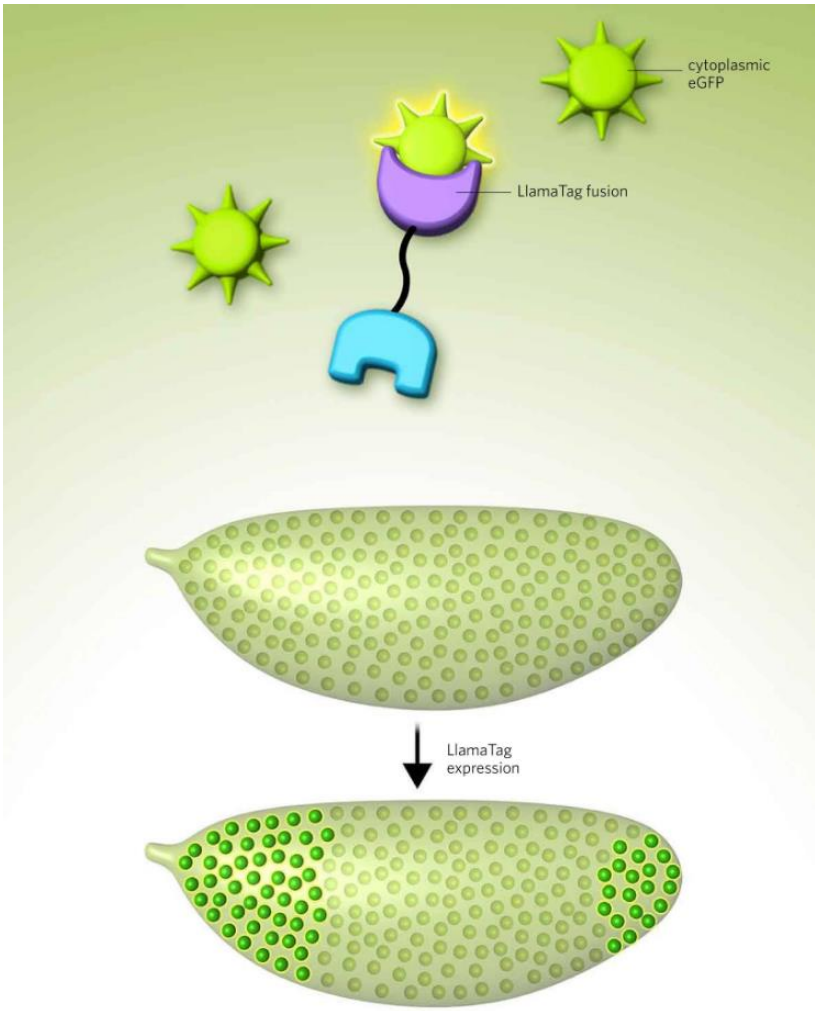

Figure 3 **LlamaTag system.** TOP: LlamaTag (purple) grab cytoplasmic GFP (green), localizing it to the fused Mad (blue) and increasing GFP fluorescence intensity.
BOTTOM: In this example, the LlamaTaged protein is a transcription factor involved in patterning of the early fruit fly embryo. Recruitment of the readily available GFP to the tagged transcription factor therefore causes the nuclei containing the factor to glow brightly. (Bothma et al., 2018)

2 Experimental part

2.1 Aim of the thesis

The main goal of this thesis is to develop a new tool that allows us to study BMP signalling dynamics *in vivo* in *Drosophila melanogaster*. To achieve this, following sub-goals were made:

1. Generating recombinant plasmid containing LlamaTag-Mad fusion protein.
2. Testing the ability of LlamaTag as a reporter of BMP signal in S2 cells.

2.2 Materials and methods

This thesis was built on the idea that we can make our own tool to study Dpp activity through pMad concentration *in vivo*. As explained by Bothma *et al.*, 2018 the reason we need new tools for live imaging is the slow maturation step of fluorescent protein fusions to transcription factors *in vivo*. Our tool aims to completely by-pass this problem, by creating Llama-tagged Mad fusion protein that is raised against eGFP. Mature eGFP is located in cytoplasm and whenever Llama-tagged Mad is translated, eGFP is then able to bind to LlamaTag within seconds. (Figure 3)

2.2.1 Materials

LlamaTag fly stocks (kindly provided by Dr. Hernan G. Garcia from UC Berkeley, USA)

• LlamaTag 1: yw; Sp/Cyo; SnaNB

• LlamaTag 2: yw; Sp/Cyo; HbNbCRISPR(6)/TMb,C

DNA purification kit (Thermo Fisher Scientific)

Taq Polymerase DNA Polymerase (Thermo Fisher Scientific)

Gel extraction kit (GeneJET Gel Extraction Kit)

DreamTaq Green PCR Master Mix (Thermo Fisher Scientific)

Nuclease free H2O

5 U/µl T4 DNA ligase (Thermo Fisher Scientific)

10×T4 DNA ligase buffer (Thermo Fisher Scientific)

pIB/V5-His vector (Thermo Fisher Scientific; plasmid V802001)

XhoI restriction enzyme (Thermo Fisher Scientific)

HindIII restriction enzyme (Thermo Fisher Scientific)

E. coli DH5α

LB agar plates containing 100 µg/ml ampicillin

GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific)

Primer sequence:

- Mad fragment: 1. Forward (Mad-linker)
  5’GGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCTGGTATTTTATTTTGACTG
  ATAG 3’
  2. Reverse (XhoI-Mad) 5’CTCGAGATGTTGCCCAGGTTAGCTATTCGGAGCC3’
- LlamaTag fragment: 1. Forward (HindIII-LlamaTag)
  5’AAGCTTGATGGCCCAGGTTCAGCTGGT3’
  2. Reverse (Linker-LlamaTag)
  5’ACCAGAACCACCACCAGAACCACCACCAGAACCACCCGACGAGACAGT
  GACCTGAG3’

Thermal cycler

0.2-ml PCR tubes

2.2.2 Methods

2.2.2.1 Designing primer

To fuse LlamaTag and Mad we designed primers that include restriction enzyme sites and overlapping sequence. In the forward primer for nanobody fragment we added HindIII restriction enzyme site sequence 5’AAGCTT3’. In the reverse primer, we added overlapping sequence 5’GGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCTGGT3’. Forward primer of the Mad fragment included the overlapping sequence, which is 5’GGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCTGGT3’ and to the reverse primer we added XhoI restriction enzyme site 5’CTCGAG3’. The overlapping sequence (marked in red) is used to fuse together LlamaTag fragment and Mad fragment (Figure 4).

2.2.2.2 Generating the recombinant vector construct

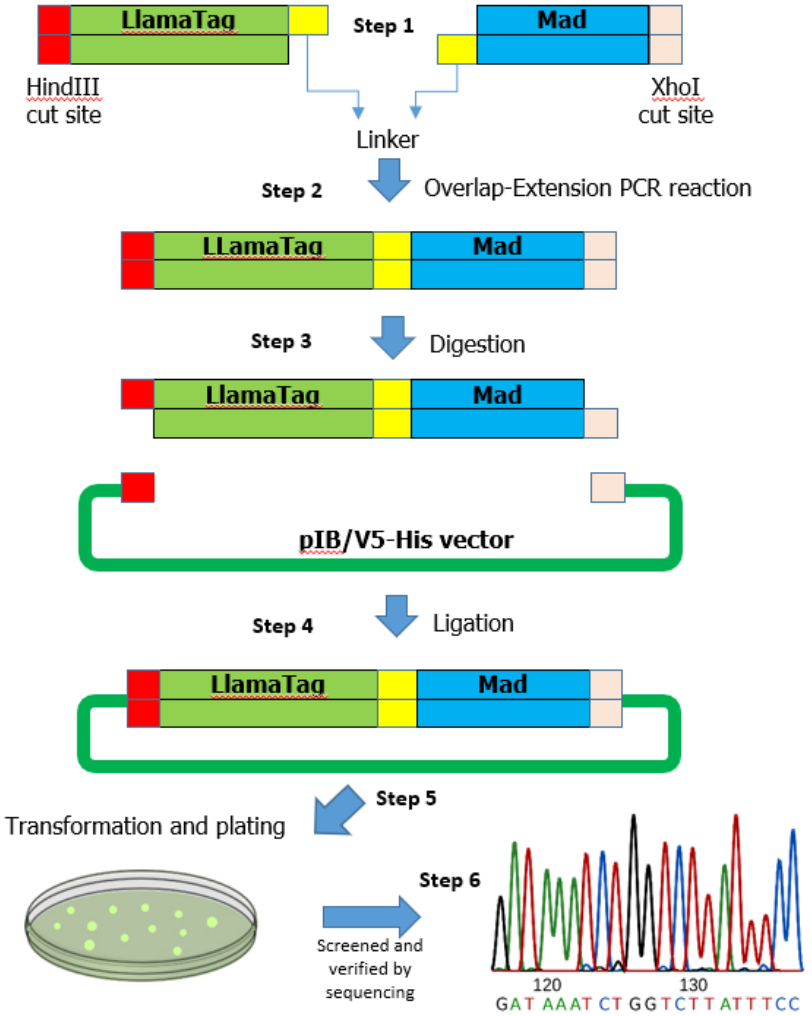

Figure 4. **Workflow for generating the recombinant plasmid construct. Step 1 U**se PCR to add restriction sites (XhoI and HindIII) and overlapping sequence (Linker, yellow) to LlamaTag and Mad fragments. **Step 2** Fuse together LlamaTag and Mad fragments using overlap extension PCR. **Step 3** is the digestion of pIB/V5-His vector and LlamaTag-Mad fragment by restriction enzymes XhoI and HindIII. **Step 4** Ligate LlamaTag-Mad fragment into pIB/V5-His vector. **Step 5** Transform the bacteria. **Step 6** Confirm the recombinant vector using restriction enzyme analysis and DNA sequencing.

Step 1 in generating the recombinant vector construct is to amplify the nanobody fragment and Mad fragment. This PCR will add restriction enzyme sites and overlapping sequence to the nanobody fragment and Mad fragment (Figure 4 Step 1).

The PCR mix (50 µl) for amplifying nanobody fragment/ Mad fragment if following:

- 25 µl of DreamTaq G2 Green Master Mix
- 1 µl of 10 µM Forward primer HindIII-Nanobody/ Forward primer Mad-linker (0.2 µM)
- 1 µl of 10 µM Reverse primer Nanobody-Linker/ Reverse primer Mad-XhoI (0.2 µM)
- 1 µl of DNA (Nanobody, genomic) (50 ng/µl)
- 22 µl of nuclease free water

PCR was carried out using the following program:

Initial denaturation		94°C	5 minutes
30 cycles	denaturation	94°C	30 seconds
	Annealing	68°C	30 seconds
	Elongation	72°C	60 seconds
Final extension		72°C	7 minutes
Hold		4°C	∞

Step 2, fuse amplified Nanobody fragment to Mad fragment using overlap extension PCR (Figure 4 Step 2).

The following PCR mix (50 µl) is used:

- 25 µl of DreamTaq G2 Green Master Mix
- 1 µl of 10 µM 10 µM Forward Primer HindIII-Nanobody (0.2 µM)
- 1 µl of 10 µM Reverse primer Reverse Primer Mad-XhoI (0.2 µM)
- 1 µl of DNA (Amplified nanobody)
- 1 µl of DNA (Amplified Mad fragment)
- 21 µl of nuclease free water

Overlap extension PCR was carried out using the following program.

Initial denaturation		94°C	5 minutes
30 cycles	denaturation	94°C	30 seconds
	Annealing	68°C	90 seconds
	Elongation	72°C	60 seconds
Final extension		72°C	7 minutes
Hold		4°C	∞

Step 3, purify the fusion fragment using gel agarose electrophoresis and gel extraction kit. DNA concentration is measured by using Nanodrop.

Step 4, digest the fusion fragment and plasmid pIB/V5-His with both restriction enzymes (XhoI and HindIII) (Figure 4 Step 3). 1 µg of fusion fragment and 1 µg of plasmid pIB/V5-His are digested using 10 U of restriction enzyme. The mixture is incubated at 37°C for 2 hours. Digested fusion fragment and plasmid is purified using gel agarose electrophoresis and gel extraction kit. NanoDrop is used to measure DNA concentration.

Step 5, ligate digested fusion fragment into pIB/V5-His vector (Figure 4 Step 4). Combine 50 ng of pIB/V5-His, 3:1 molar ratio of digested fusion fragment, 1 µl T4 DNA ligase, 1 µl of 10x T4 DNA ligase buffer (1x), up to 20 µl of nuclease free water. The mixture is incubated at 4°C overnight.

Step 6, transform the ligation reaction into *E. coli* and select the colonies on LB agar plate containing 100 µg/ml ampicillin (Figure 4 Step 5). For transformation I use 5 µl of ligation mixture into 100 µl of competent cells. I plate 100 µl of the transformed bacteria to the ampicillin (100 µl/ml) containing agar plate.

Step 7, isolate the plasmids using GeneJET Plasmid Miniprep Kit.

Step 8, screen the colonies for recombinant vector using restriction enzyme analysis. DNA sequencing was used to confirm the correct vector (Figure 4 Step 6).

2.3 Results and discussion

2.3.1 Using PCR to add restriction sites and overlapping sequence to the ends of Mad and nanobody fragments

The goal of the PCR experiment was to amplify the Mad and Nanobody fragments, while also adding restriction enzyme sites and overlapping sequence to the end of the Mad and LlamaTag fragments.

The size of amplified Mad fragment was 746 bp and the expected band was between 1000 bp and 500 bp. (Figure 5) The size of amplified LlamaTag was 369 bp and the expected band was below 500 bp. There was single correct sized band visible. (Figure 6)

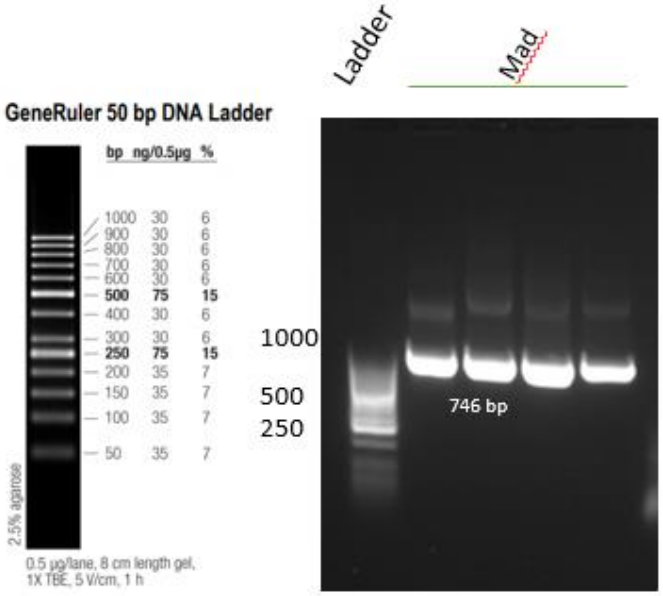

Figure 5. **PCR results of Mad fragment.** PCR was used to add XhoI restriction site and overlap sequence to Mad fragment. Bands are in expected size (746 bp). All 4 Mad fragments are the same and used in future experiments.

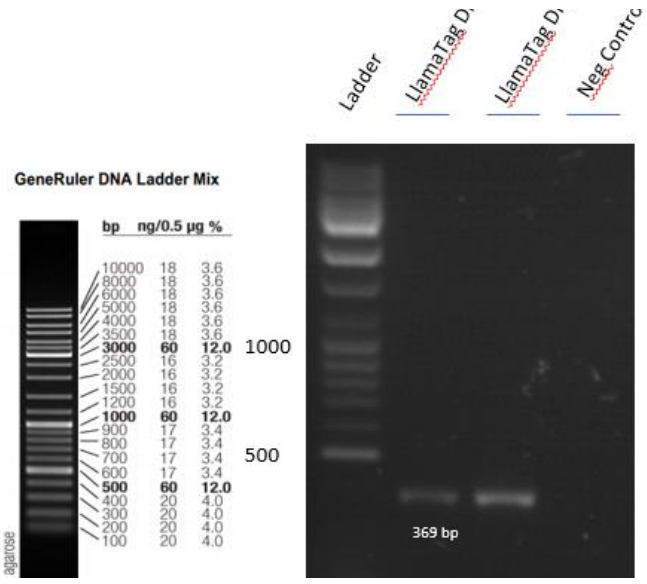

Figure 6. **PCR results of LlamaTag fragment.** PCR was used to add HindIII restriction site and overlapping sequence to LlamaTag fragment. Bands are in expected size (369). There is no notable difference between LlamaTag 1 and LlamaTAg 2.

2.3.2 Using overlap extension PCR to fuse together amplified Mad and nanobody fragments via overlapping sequence

The overlap extension PCR is a valuable technique used to fuse together two or more DNA fragments via overlapping sequences. In our case the overlapping sequence acted as a bridge for fusing amplified Mad and nanobody fragments. The combined size of the fusion fragment was 1123 bp. The brightest band was slightly above the 1000 bp band, which was expected. There were also some higher non-specific bands visible and some smearing (Figure 7). To avoid the possible problems caused by these non-specific bands, the fusion fragment was extracted from the gel. The DNA concentration after purification for Mad-LlamaTag 1 was 25.6 ng/µl and 11.7 ng/µl for Mad-LlamaTag 2.

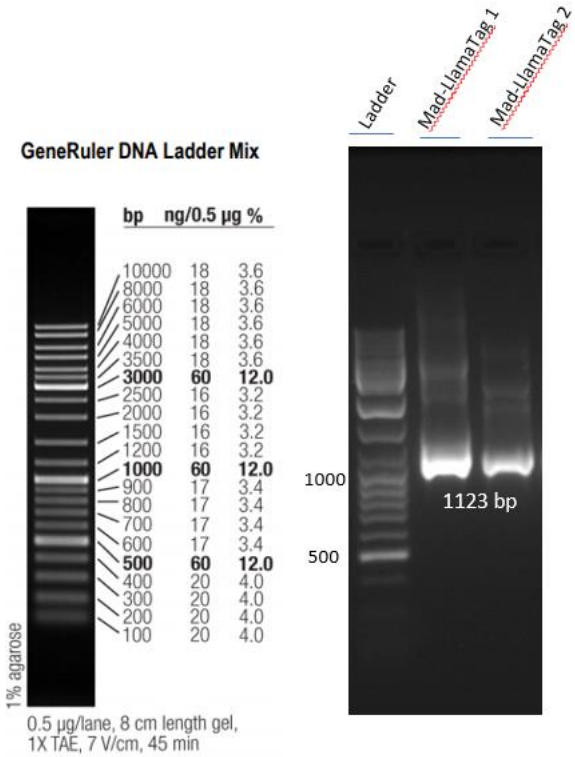

Figure 7. **Overlap extension PCR results of Mad-LlamaTag fusion fragment.** Mad (746 bp) and LlamaTag (369 bp) fragments were fused together via overlapping sequence, thus forming Mad-LlamaTag fusion fragment (1123 bp). Bands are in expected size, slightly above 1000 bp band. Due to smearing and two non-specific bands, the correct sized bands were extracted from the gel.

2.3.3 Using restriction enzyme digestion on pIB/V5-His vector and fusion fragments.

1 µg of pIB/V5-His vector and fusion fragments were digested by 10 U of restriction enzyme (XhoI and HindIII). Digestion of pIB/V5-His vector results in two linear DNA fragments. The big fragment is about 3.5 kb and small fragment is about 50 bp. As a result, on Figure 8 we can see one clear bright band above 3000 bp and one barely visible band below 3000 bp.

After digestion of fusion fragment, we can see two clear bands above 1000 bp.

The correct sized bands (inside blue box) were excised and purified using gel extraction kit. DNA concentration was measured using NanoDrop and was following: 29.8 ng/µl for plasmid, 55 ng/µl for Mad-LlamaTag 1 fragment and 54 ng/µl for Mad-LlamaTag 2 fragments.

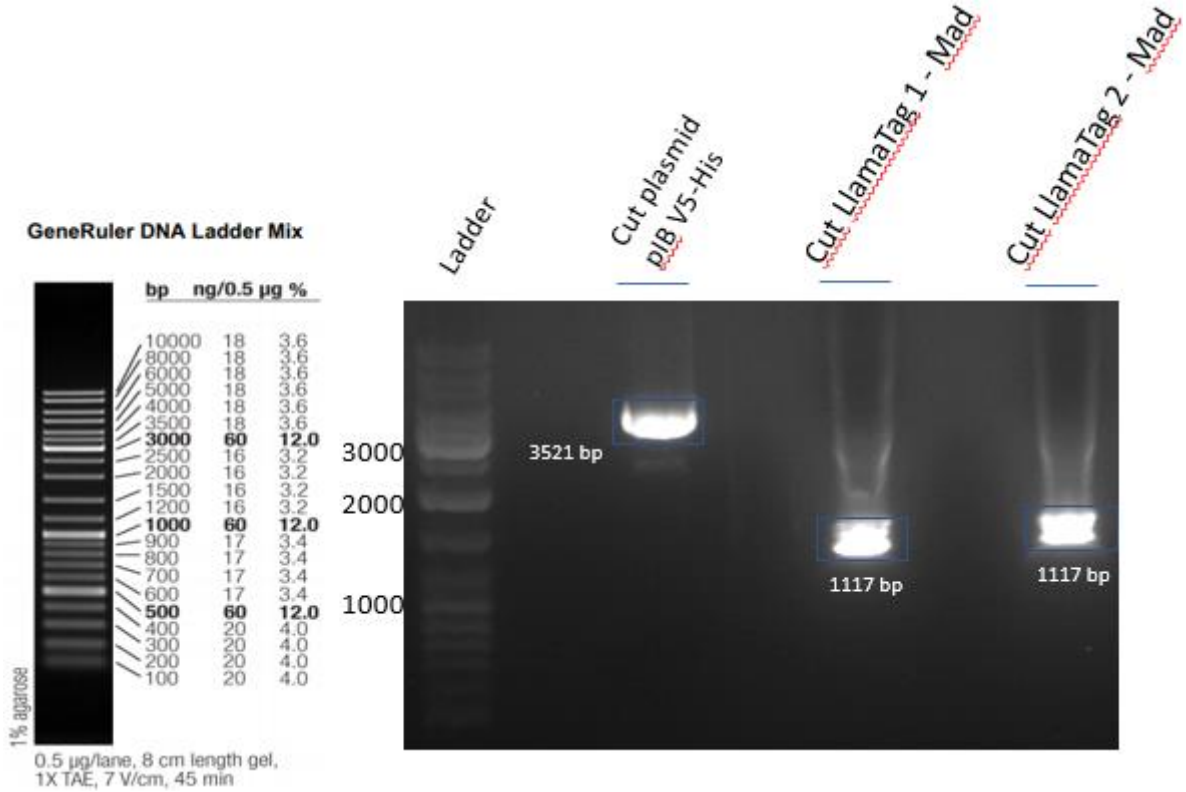

Figure 8. **Results of digestion of pIB/V5-His vector and fusion fragments.** Both the plasmid and Mad-LlamaTag fusion fragment, were digested by restriction enzymes XhoI and HindIII. Area inside blue box was excised and purified using gel extraction kit.

2.3.4 Ligation of insert into vector and transformation.

Ligation is used to fuse together the vector and insert, circularizing the vector, thus making the vector acceptable for transformation. We ideally used vector to insert ratio 1:3. We set up the following control reactions in Table 1.

Table 1. **Control reactions used in transformation**

Vector	Ligase	insert	Result	Note
+	+	+	Ligation reaction	Main reaction
+	+	-	Quantifies undigested + re-ligated vector	Negative control for the background
+ (not digested)	-	-	Verifies transformation procedure, antibiotic, cell competency	Negative control

In the transformation step we set up two different negative controls. One negative control was used to check the antibiotic resistance of the bacteria. The second negative control was used to check for the quality of transformation.

In our experiment, we observed that the bacterial colonies from the transformation resulted in the positive control and the sample plates, without in the negative control plate.

2.3.5 Using restriction enzymes to verify if the target gene of interest was successfully cloned.

Restriction enzyme cutting is often used to verify the existence of target gene in recombinant vectors after cloning. On Figure 9 two clear bands for each colony can be seen. The band above 3000 bp is digested plasmid and the band above 1000 bp and below 1500 bp is inserted fusion fragment. There were no smearing or other abnormal bands.

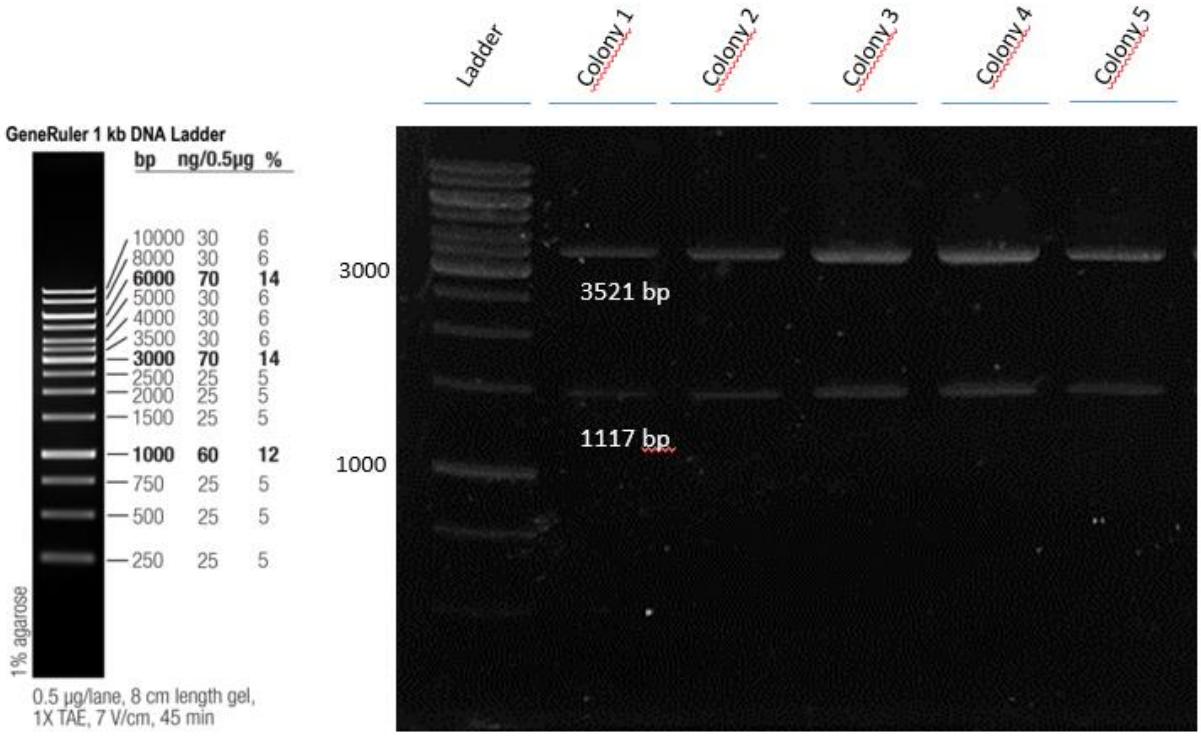

Figure 9. **Restriction enzyme screening for target gene in recombinant vector.** Recombinant vector (4.5 kb) was digested using restriction enzymes XhoI and HindIII, resulting in two smaller fragments. Big fragment is the vector backbone (3.5 kb) and small fragment is our inserted Mad-LlamaTag fusion fragment (1.1 kb). All the colonies show the same result, indicating that the Mad-LlamaTag fusion fragment was successfully cloned into pIB-V5/His vector.

2.3.6 DNA sequencing was used to confirm the results of restriction enzyme analysis.

DNA sequencing is fast method for determining the sequences of different DNA fragments. In our result we compared the DNA sequence of fusion fragment with the original Mad and LlamaTag DNA sequence, using ncbi nucleotide BLAST sequence alignment tool, which showed 98-99% similarity between the fusion fragment and original Mad and LlamaTag fragment.

We conclude that we have successfully created the recombinant vector construct.

3 Conclusion

The aim of this thesis was to develop new tools for live imaging of BMP signalling in *Drosophila* wing. Based on restriction enzyme analysis and DNA sequencing, we have successfully created recombinant vector containing Mad fragment. Recombinant vector functionality was tested on *Drosophila* S2 cell culture by my colleague and is thus included in Supplementary Information. Preliminary tests on S2 cells show that Llama-tagged Mad works for detecting BMP signals is S2 cell culture (see Supplementary Information). Based on these results, we have successfully developed a new tool, which can be used to study BMP signalling pathway *in vivo*.

Next step in developing this tool further is to introduce Mad-LlamaTag fusion fragment into live *Drosophila melanogaster* using CRISPR/Cas9 system. This tool can then be fully used in live imaging to further study BMP signalling pathway in *Drosophila melanogaster*.

Uudse tööriista arendamine reaalajas BMP signaaliraja jälgimiseks *Drosophila* tiiva arengus

Robin Pau

Resümee

Organismi areng on laialdaselt uuritav teema bioloogias, alates embrüo arengust kuni äädikakärbse *Drosophila* tiiva mustrite moodustumise mehhanismide uurimiseni. Äädikakärbse tiib on hea mudel organ erinevate kasvu mõjutavate signaaliradade uurimiseks Esiteks koosneb tiib oma ehituselt kahest epiteelraku kihist. Teiseks signaalirajad on evolutsiooniliselt konserveerunud erinevate liikide vahel. Lõpuks geneetiliste meetodite abil on võimalik lihtsalt uurida ühe geeni mõjusid.

Peamine uuritud signaalirada *Drosophila* tiivas on Dpp/BMP signaalirada. Paljud artiklid on leidnud mitmeid BMP signaaliraja homolooge erinevates loomades. Näiteks *Drosophila* BMP ligand, Dpp on funktsionaalne ortoloog inimese BMP2 ja BMP4-le. BMP signaaliraja peamised funktsioonid on kontrollida rakkude proliferatsiooni koe suurust, rakkude diferentseerumist, apoptoosi ja hoida homöostaasi.

Looma areng on dünaamiline. Arengu käigus toimub pidev rakkude jagunemine ja ümberpaiknemine. Erinevate kudede areng on tugevalt seotud kasvu faktori signaaliradadega. Kasutades mudelina äädikakärbse tiiba on kirjeldatud morfogeneesi ja BMP signaali omavahelist seost. Täpsete töövahendite puudumise tõttu on ebaselgeks jäänud, kuidas on BMP signaal mõjutab rakkude kasvu ja vastupidi. BMP signaalirada algab kui ligand Dpp fosforüülib transkriptsiooni faktori Mad (pMad). pMad seejärel moodustab kompleksi co-Smad Medeaga, millejärel suundub pMad/Medea kompleks rakutuuma, kus ta reguleerib mitmete sihtmärk geeni ekspressiooni.

Antud bakalaureusetöö eesmärk on uudse tööriista välja töötamine, millega on võimalik reaalajas jälgida BMP signaalirada *Drosophila* tiivas. See tööriist võimaldab tuvastada rakutuumast pMadi, mis näitab aktiveeritud BMP signaalirada.

Uue tööriista väljatöötamisel kasutati uudset valgu märgistamise süsteemi LlamaTag. LlamaTag on ühe domeenne laamadest pärit antikeha. Tööriista väljatöötamiseks esiteks moodustati rekombinantne vektor, mis sisaldab Mad'i DNA järjestusele juurde lisatud LlamaTag'i DNA järjestust. Restriktsiooni analüüsi ja DNA sekveneerimis tulemuste põhjal saab öelda, et suutsime tööriista luua. Lisaks katsetati uut tööriista *Drosophila* S2 rakukultuuris. S2 rakkudes küps eGFP seondus kiiresti Mad-LlamaTag'ile, mis põhjustas GFP signaali kogunemise rakutuuma, mis näitab BMP signaaliraja aktiivsust. Nende tulemuste põhjal võib öelda, et õnnestus välja töötada tööriist, millega on võimalik uurida BMP signaalirada *in vivo*.

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SUPPLEMENTARY INFORMATION

Llama-tagged Mad works for detecting BMP signal in tissue culture cells

To investigate whether the Llama-tag Mad is functional in tissue culture cells, we performed co-transfection analysis of eGFP, a constitutive active form of BMP type I receptor, and LlamaTag-Mad constructs into S2 cells. When Llama-tag Mad and eGFP are transfected into S2 cells, we observed that Llama-tag bound eGFP highly accumulates into the nucleus when the BMP signal is positive. In contrast the majority of eGFP locate in the cytosol without signal. Thus, Llama-tag Mad appears to serve as an excellent system for the readout of BMP signaling in vivo.

Taken together, we conclude that Llama-tagged Mad works for detecting BMP signals in tissue culture cells.

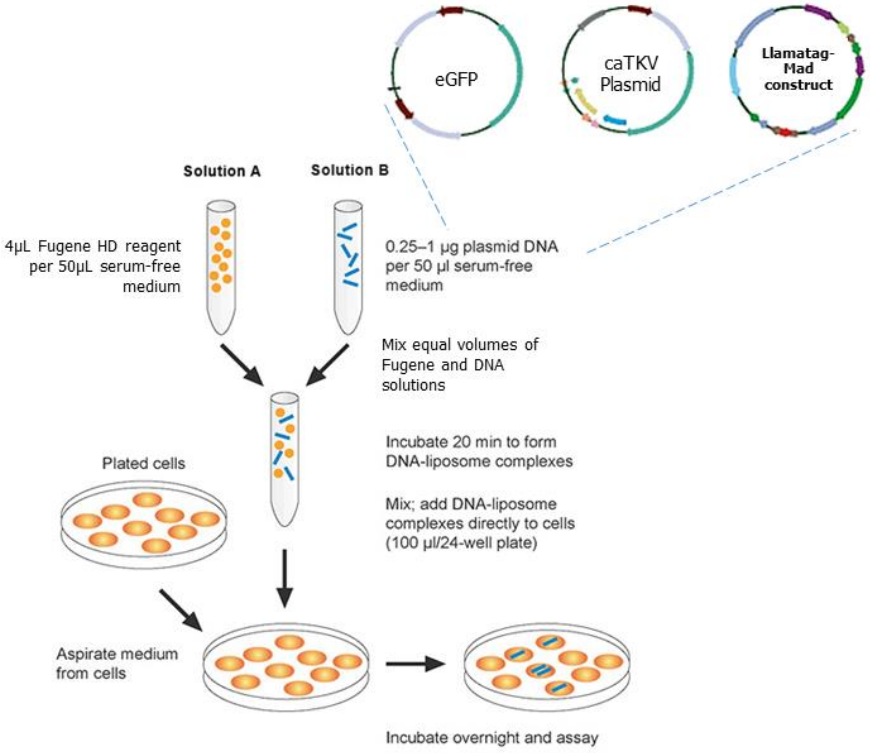

Figure S1. **The procedure to test the ability of LlamaTag as a reported of BMP signal in the S2 cells.**

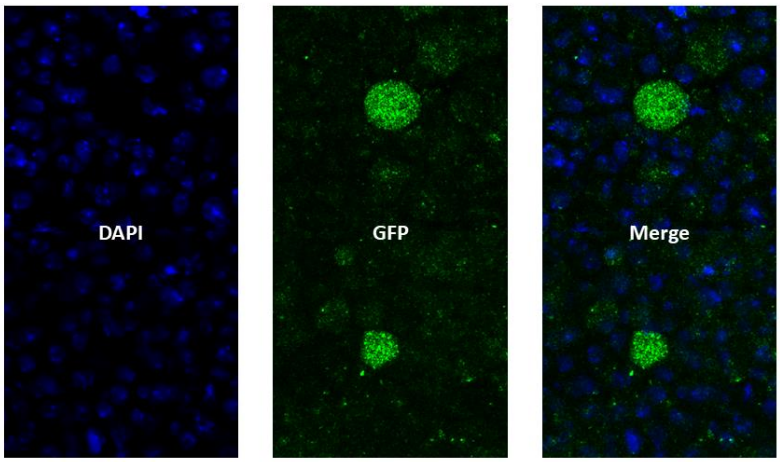

Figure S2. **Transfection of eGFP into S2 cells.** The eGFP was located in the cytosol.

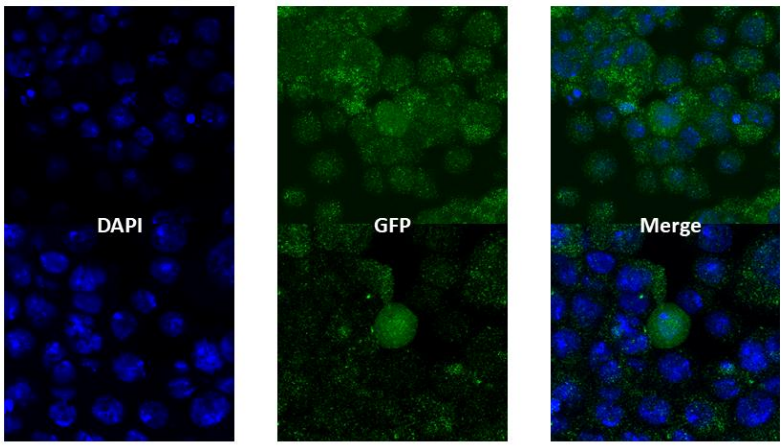

Figure S3. **Co-transfection analysis of eGFP and LlamaTag-Mad constructs into S2 cells without constitutive active form of BMP type I receptor (caTKV).** The Mad-nanobody was not translated. The BMP signal was negative and eGFP was enriched in cytosol.

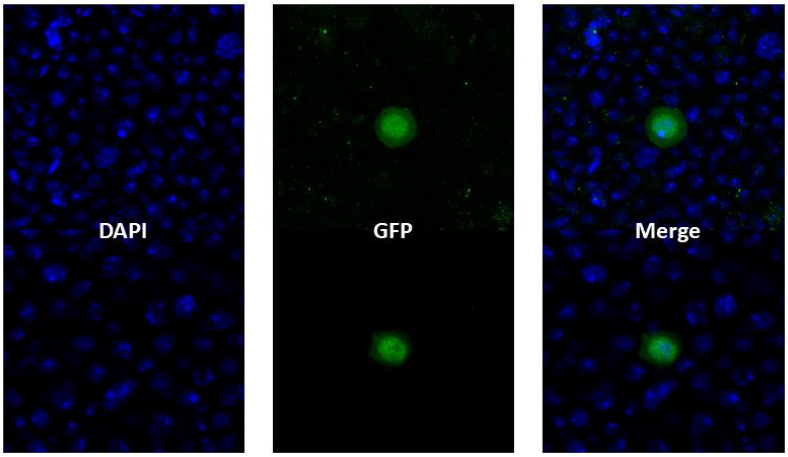

Figure S4. **Co-transfection analysis of eGFP, constitutive active form of BMP type I receptor and LlamaTag-Mad constructs (caTKV) into S2 cells.** The Mad-nanobody was translated, then it bound cytoplasmic eGFP, and Llama-tag Mad-eGFP complex was enriched into nucleus when BMP signal was positive.

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supervised by Osamu Shimmi, Vi Ngan Tran and Tambet Tõnissoo

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10/08/2020