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ERIK ESTRADA

Regulation of Smad signaling by the LKB1 interacting protein – LIP1

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Author

Erik Estrada

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Abstract

Recent research has found the rather unknown protein LIP1 to interact with both LKB1 and Smad4, proteins thought to be the cause of the cancer related, inheritable syndromes Peutz-Jeghers syndrome (PJS) respectively juvenile polyposis syndrome (JPS). Could LIP1 be a novel unexplored link between the two syndromes? This study examines the interaction between LIP1 and the Smads and how LIP1 affects Smad-regulated genes. Interaction between LIP1 and Smad4 but not Smad2 or Smad3 could be shown. Also, no correlation could be found between LIP1 levels and TGF-β transcriptional response. It was found however that LIP1 can affect the levels of Smad2 and Smad4 and a hint of a possible domain for this function was discovered.

Keywords

Smad, TGF-β, LIP1, LKB1, PJS, JPS, cancer

Supervisors

Anita Morén

Ludvig Institute for Cancer Research in Uppsala

Scientific reviewer

Lars-Gunnar Larsson

Institutionen för växtbiologi och skogsgenetik, SLU

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala

Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Regulation of Smad signaling by the LKB1 interacting protein – LIP1

Erik Estrada

Sammanfattning

Det finns många cancerrelaterade gener i vårt genom. En av dem kodar för kinasproteinet LKB1 och tros vara orsaken till den ärftliga sjukdomen Peutz-Jeghers syndrom - PJS. Syndromet tros bero på en olycklig mutation som förstör funktionen hos LKB1 och som återfinns hos nästan alla patienter. Förutom de visuella symptomen ökar denna sjukdom risken att få cancer mångfaldigt.

Det LKB1 interagerande proteinet LIP1 trodde man inte hade någon betydande roll förrän man fann att det även interagerade med proteinet Smad4. Smad4 är känt inte bara som en viktig del i den cancermotverkande TGF-β signalvägen utan är också i kopplat till sjukdomen ”juvenile polyposis syndrome” – JPS – där en stor del av patienterna bär en muterad Samd4 gen. JPS är väldigt lik PJS både till symptom och den ökade cancer risken. Detta gör LIP1 till ett mycket intressant protein då det kan vara en koppling mellan de två syndromen.

Smad4 tillhör en grupp om 7 stycken signalmediatorer i TGF-β signalvägen och i det här projektet undersöktes interaktionen mellan LIP1 och andra Smad-proteiner för att undersöka om LIP1 kan interagerar med dem också. Det undersöktes också om LIP1 påverkar uttrycket av de gener som regleras av Smad-proteinerna.

För att kunna bestämma interaktionen mellan proteinerna användes antikroppar för att specifikt kunna fiska upp ett av dem, t.ex. LIP1. Om andra proteiner, t.ex. Smad4, följer med LIP1 vid fiskningen så tyder det på att detta protein kan binda till LIP1. Med hjälp av denna metod kunde man dock inte se något mer än den redan funna interaktionen mellan LIP1 och Smad4. Däremot påvisades det att LIP1 kan påverka nivåerna av flera Smad-proteiner i cellen och genom att klippa upp LIP1 proteinet i mindre bitar hittades ett område på proteinet som möjligen kan vara orsaken till denna påverkan.

För att se om LIP1 påverkar genuttrycket kopplades koden till ett detekterbart protein till en Smad-reglerad gen. Det gick dock inte att se någon koppling mellan mängden LIP1 och uttrycket av denna gen. Projektet kommer fortsätta inom gruppen tills kopplingen mellan LIP1 och Smad-proteinerna är helt klarlagd.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet April 2006

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ABBREVATIONS USED

aa	aminoacid
ALK	activin receptor-like kinase
BMP	bone morphogenic protein
β–gal	β-galactosidase
CDK	cyclin dependent kinase
Co-IP	co-immunoprecipitation
DMEM	dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
ECL	enhanced chemiluminescense
EMT	epithelial to mesenchymal transition
EtOH	ethanol
IB	Immunoblott, i.e. western blott
JPS	juvenile polyposis syndrome
GDF	growth differentiation factor
LIP1	LKB1 interacting protein 1
LRR	leucine rich repeats
MAD	mothers against decapentaplegic
MAPK	mitogen activated protein kinase
MetOH	methanol
MIS	Muelerian inhibiting factor
MH1	MAD homology 1
MH2	MAD homology 2
NIS	Nischarin
PCR	polymerase chain reaction
PJS	Peutz-Jeghers syndrome
PDB	Protein Data Bank
PMSF	phenyl methyl sulphonyl fluoride
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TβR	TGF-β receptor
TGF-β	transforming growth factor-β
Tris	tris-(hydroxymethyl)-aminomethane

1. INTRODUCTION

1.1 Cancer, a global burden

Everyone has been in contact with cancer. Mostly we come in touch with cancer through older relatives, though sadly not always since it can strike any age. It's one of the great fears in the modern world, substituting famine and war in the more wealthy countries, and is the cause of 7 million or 12.5% of worldwide deaths every year (WHO).

Some groups of people are more susceptible than others, but in the end, one third of us will contract cancer before we die. Only in Sweden, over one hundred patients are diagnosed with cancer every day (Cancerfonden 2005). Worldwide more than 11 million cancer patients are recorded every year. In reality the figure is much bigger since cancer can escape early diagnosis and some of the 200 varying types of cancers keep themselves stealthy and harmless throughout life. Also far from all of the inhabitants of our world can afford or even have access to a physician to diagnose the disease.

Because of the heavy impact that cancer has on the whole world it has become a top priority subject for research. The why and how we get cancer can save many lives by preventive methods. But the question is both as simple and complex as life itself. The causes of cancer are innumerable and in about 10% of cases the answer is hidden in our inherited genes (WHO).

Through our knowledge today we are roughly preventing 30% of all cancers mostly by informing the public about the risks of exposure to carcinogens such as tobacco and UV-radiation (WHO). Early detection is also a life saver as surgery and therapy can cure cancer before it becomes fatal. Even preventive surgery can occur sometime when the patients know they have the genes that predispose them to a special kind of cancer. We still need to put a lot more effort in this fight unless the trend, showing that over a stunningly 10 million will die from cancer by the year 2020, is to come true. But what is this disease that can threaten us so in our modern world full of bullet proof fortresses such as medical centres, healthcare planning and vaccinations?

1.1.1 Is cancer evolution?

Cancer is inevitably an end state for any complex multicellular organism. Being multicellular necessitates cooperation between many different kinds of cells. The more complex an organism is the more constrains are put upon the single cell in terms of function, when to divide and even when to die. The cell sacrifices its individual freedom for the good of the whole organism. In an organism as complex as the human every cell is highly specialized and cannot survive without the others. The location, number and function of every cell type need to be in balance for the body to work, and if the body stops working the cells die.

When it comes to single-cell organisms, what we call cancer would simply be called evolution. One cell suddenly acquires a mutation that gives it the upper hand against its neighbours. This mutation may somehow give the cell a decreased death rate, an increased birth rate or even both of these. The mutant cells will soon dominate over the others and prosper until the food runs out or a daughter cell gets another mutation generating an even better adaptation.

The scenario with limited food is the one most similar to what we know as cancer. Mutations that give short-sighted growth benefits to a cell but in the long run bring starvation and death. This is exactly what will happen also in a multi cellular organism. The cells in our body acquire mutations all the time. Some are repaired, some lead to cell death and some are harmless; but some, just some, may cut the constraints that are put upon the cell. With this advantage the cell will start to divide, grow and spread through the body as cancer. Short-

sighted faster growth is an evolutionary success for the cell in order to spread and dominate but in the end the cell cannot survive without the body it tries to outgrow.

1.1.2 Oncogenes and Tumour Suppressors

Because of the inevitability and fatality of cancer the body has developed many safeguard systems that will make it less likely to succumb to cancer. These security measures are meant to provide the organism with enough time to develop, breed and nurture its offspring. The safeguards mainly work by stopping a cell from dividing or even kill cells that act suspiciously. These safeguards and other genes that prevent or slow down malignant tumour development are called tumour suppressors and are roughly divided into to two groups, caretakers and gatekeepers.

The caretakers are as the name implies genes meant to do maintenance and repair of the DNA. If they fail their function the cell will have an increased rate of mutations that may lead to cancer. Since tumourigenesis requires more than one misfortunate mutations, it is considered that a loss-of-function among caretakers usually is the first step toward a rapidly growing tumour. Exposure to mutagens has a similar effect which is why mutagens also are carcinogens. The gatekeeper genes exist to stop cells from becoming a tumour. They function by constraining cell growth or trigger apoptosis – a pre-programmed process to kill cells that malfunction, are too many or threaten the organism in other ways.

Table 1 shows a possible progress of tumourgenesis started with a loss of function in a caretaker gene making harmful mutations more frequent. The damaged cell will soon commit suicide through apoptosis, thus killing the tumour before it is born, unless this function is lost before it can be triggered. The inability to commit suicide must be obtained early as tumourigenesis triggers apoptosis. Most cells also need a loss of function in the cell cycle control in order to bypass it and be able to replicate. The ability to bypass the cell cycle control and evade apoptosis is what more or less defines a tumour cell. The tumour now consists of an immortal and growing lump of cells. The rest of the mutations give the tumour the ability to grow faster and bigger and finally form metastasis.

Opposite to tumour suppressors are genes that can work to the benefit of tumourigenesis. These are called tumour promoters or oncogenes. Most of them are genes altered through mutations that render them active when they should not, so called "gain of function" mutations. Some, though, work as normal genes but start to promote cancer when other cell functions start to fail. Normal genes that can become oncogenes during tumourigenesis are called proto-oncogenes.

A lot of cancer research put the emphasis in finding these cancer affecting elements and to in detail understand how they work. Those facts would give us the answers as to why certain tumours are more common than others, how to protect ourselves better against it and also how to treat the condition better. If you know you have inherited oncogenes that make you more susceptible for certain cancers, you can have regular check ups to discover the cancer early and treat it before any harm is done.

Type of mutation	Type of Gene	Gene function	Consequence
Loss of function	**Caretaker**	**DNA repair**	**Accumulation of mutations**
Loss of function	**Gatekeeper**	**Programmed cell death**	**Evasion of apoptosis**
Loss of function	**Gatekeeper**	**Cell cycle control**	**Limitless replicative potential**
Loss of function	**Gatekeeper**	**Growth signalling**	**Insensitivity to anti-growth signalling**
Gain of function	**Proto-Oncogene**	**Growth signalling**	**Self-sustained growth signalling**
Gain of function	**Proto-Oncogene**	**Growth signalling**	**Self-sustained angiogenesis**
Gain of function	**Proto-Oncogene**	**Differentiation**	**Tissue invasion and metastasis**

Table 1. Possible progress of tumourgenesis

1.2 Transforming Growth Factor–β and its dual role in cancer

The transforming growth factor–β (TGF–β) pathway is one of special interest because of its ability to function both as a suppressor and promoter of tumours (Wakefield and Roberts 2002). The TGF–β cytokine is an important messenger protein that tells the cells when they should grow, divide, differentiate or die. It can regulate the growth of a new blood vessel and the closing of a wound. TGF–β instructs the cells to differentiate, grow and divide in order to close a wound and to stop when the wound is closed. (Moustakas, Pardali et al. 2002)

Due to its function TGF–β seems wrongly named as it is actually a potent inhibitor of growth and proliferation of many cell types. Almost all cells secrete TGF–β but respond to it quite differently depending on the cell type and its state of differentiation. A common response though is cell growth inhibition for higher concentrations of the protein – high levels of TGF–β relates to a high density of cells secreting it. This represents the tumour suppressor activity of the TGF–β pathway. In order to quickly grow big and dense, tumours must be able to ignore the signal or stop producing TGF–β (Massague 2000).

As expected, a dysfunction in the TGF–β pathway is very commonly found in pancreatic and colon cancers and at lower frequency in many other tumours in the body.(Massague, Blain et al. 2000) The TGF–β pathway is important for tumourigenesis not only because of the loss of the tumour suppressor activity but also because of a gain in tumour promoter activity. A single mutation can push the cell several steps in the progress of becoming a malignant tumour (see Table 1) (Wakefield and Roberts 2002).

If a cell suddenly becomes insensitive to TGF–β it begins to produce more TGF–β in order to compensate for the loss of its effect. This together with the tumour's high cell density will cause a surplus of the growth factor that will benefit the tumour in two ways. The high concentration of TGF–β will trigger growth of new blood vessels, angiogenesis, which is necessary to provide a growing tumour with nutrients. Large amounts of TGF–β will also inhibit the proliferation and even inactivate T-lymphocytes that otherwise could attack and destroy the tumour (Muraoka-Cook, Dumont et al. 2005).

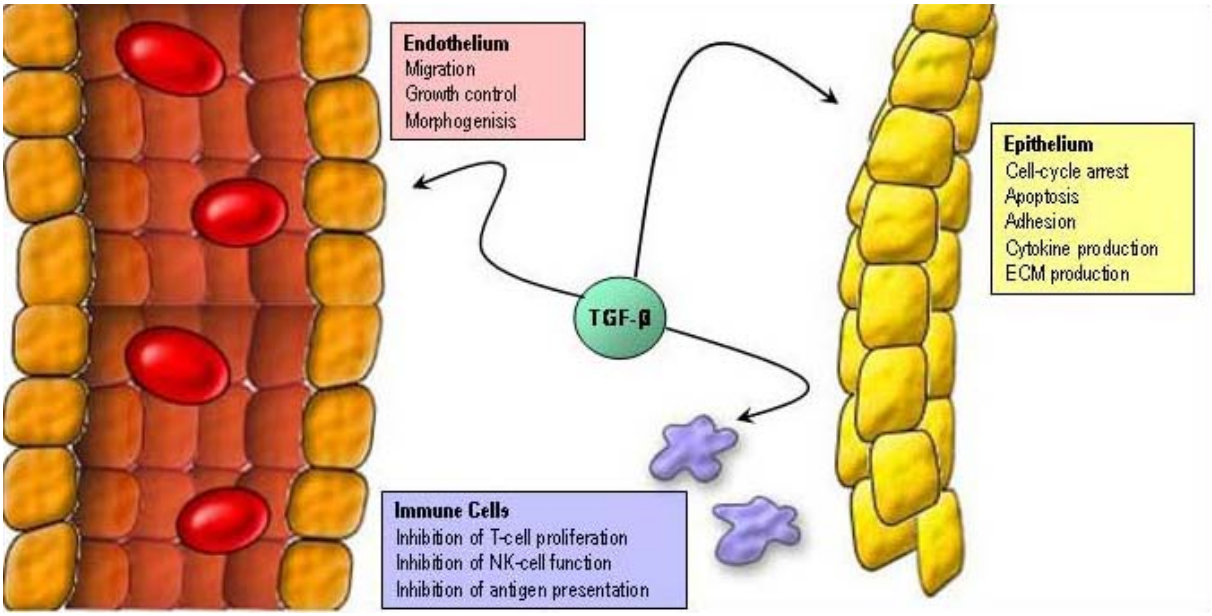

Figure 1. The different roles of TGF-β in cancer

Another effect of TGF−β on solid tumours of epithelial origin is a well known phenomenon called epithelial mesenchymal transition, EMT, where the epithelial cells start to differentiate and migrate as if closing a wound. EMT will cause otherwise harmless benign epithelial cancer, adenoma, to become malign and invasive, carcinoma. The invasive or metastasis phase of a tumour is the hardest to cure or treat which leads to a very high death rate(Muraoka-Cook, Dumont et al. 2005).

1.3 TGF−β signalling

The TGF−β superfamily of cytokines induces a multitasking signalling pathway that controls many cellular processes such as apoptosis, differentiation and proliferation among others. The TGF−β pathway plays important roles both in embryonic development and tissue homeostasis in the adult body.

The diversity of this pathway is obtained through a large set of ligands and receptors in addition to the complex signal transduction system. The signal carrying proteins, Smads, are prone to regulation from many other cellular mechanisms, all not yet fully understood. Smads also interact with different transcription factors in the nucleus thus the growth and differentiation state of the cell affect the response. This complexity is needed to provide specificity to this pathway that can induce so many crucial responses.

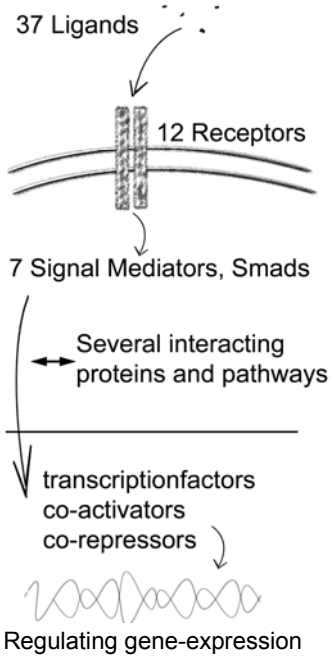

Figure 2. The TGF-β pathway
A very generic and simple cartoon of the TGF-β pathway.

1.3.1 The ligand

The TGF−β family of ligands includes 34 members such as TGF−β, activin, inhibin, nodal, bone morphogenic protein (BMP), Muelerian inhibiting factor (MIS) and growth diffetentiation factor (GDF) (Feng and Derynck 2005). They all share some common sequence and structural features. One similarity found in all of these cytokines are six well-conserved cysteine residues creating three sulphur bridges resulting in a compact structure of each monomer known as the "cystine knot" (Sun and Davies 1995). When active these cytokines form a dimer that can bind and bring together two type I and type II serine/threonine kinase receptors on the cell surface. Each ligand can bind specific combinations of receptors, and each unique complex can lead to different effects (Feng and Derynck 2005).

1.3.2 The receptor

In humans there are seven type I receptors, also known as activin receptor-like kinases (ALK), numbered from 1 to 7 and five type II receptors (Massague and Weis-Garcia 1996). The pathway can be split into two parallel groups. One group is activated by TGF−β, activin, and nodal and here the type II receptor binds the ligand before forming the complex with the ALK-4,-5 or -7 type I receptor (Moustakas, 2001 #8). The other group is activated by BMPs, GDF and MIS, and here the type I receptors ALK-1,-2,-3 and -6 bind the ligand first before forming complex with the type II receptor (Shi and Massague 2003). Binding of the ligand brings the receptors to close proximity and creates a functional configuration of the intracellular kinase domain of the receptors, thus facilitating phosphorylation of the type I receptor by the type II receptor. This activates the kinase of the type I receptor. The active type I receptor can now in turn phosphorylate the signal mediators, the Smads.

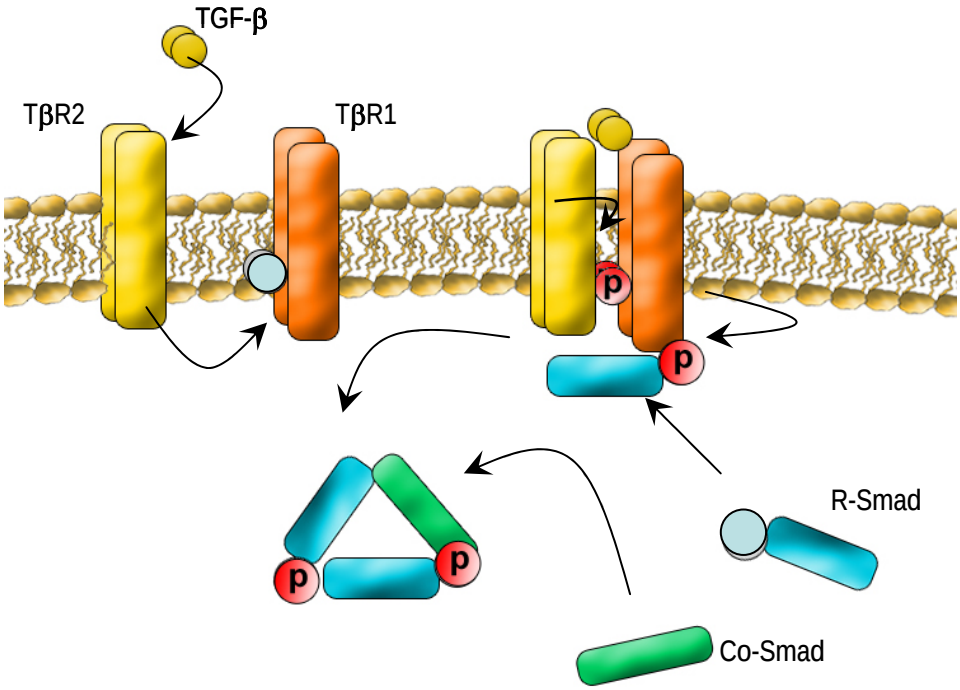

Figure 3. Activation of the Smads by the TGF-β ligand. *The ligand brings together the two receptors, enabling the type II receptor to phosphorylate the type I receptor. The active type I receptor can now phosphorylate and activate the Smads, which carry the signal to the nucleus and change the expression of certain genes. (see Figure 2)*

1.3.3 The signal mediators, Smads

The Smads got their name from their orthologs MAD in *D. melanogaster* and Sma in *C. elegans* that were discovered first (Derynck, Gelbart et al. 1996). They act as signal mediators in the TGF−β pathway and are divided into groups by their functions. R-Smads, Smad 1,2,3,5 and 8, are activated by the type I receptor after which they form a complex with themselves and the Co-Smad, Smad4 (Kretzschmar and Massague 1998). This complex can now travel into the nucleus and together with various transcription factors affect the expression of hundreds of genes (Massague and Wotton 2000). As the opposite to R-Smads there exist the inhibitory I-Smads, Smad 6 and 7 (Shi and Massague 2003; Feng and Derynck 2005). They lack the phosphorylation site and inhibit the TGF−β signal from passing it on to an R-Smad, by blocking the receptors (Huse, Muir et al. 2001). This function serves to end signalling and the I-Smads can also trigger an ubiquitin-mediated degradation of the activated receptor complex.

The Smads can travel in and out of the nucleus, so in their inactive form R-Smads are tethered to the cytoplasm by a Smad anchor for receptor activation protein, SARA (Xu, Chen et al. 2000). SARA functions by binding the Smads in the cytoplasm, blocking the nuclear import signal in the MH1 domain and helping to recruit Smads to the receptor. Upon activation the R-Smads not only gain increased affinity for Smad4 but also loose their affinity for SARA which stays behind in the cytoplasm. The Smads are a group of about 500 amino acids long proteins with two highly conserved regions (Kretzschmar and Massague 1998; Feng and Derynck 2005). The Mad homology 1, MH1, domain can be found at the N-terminal and has the ability to recognize DNA sequences – mostly CAGAC. In Smad2 and Smad7 this sequence specific DNA-binding activity is less respectively gone.

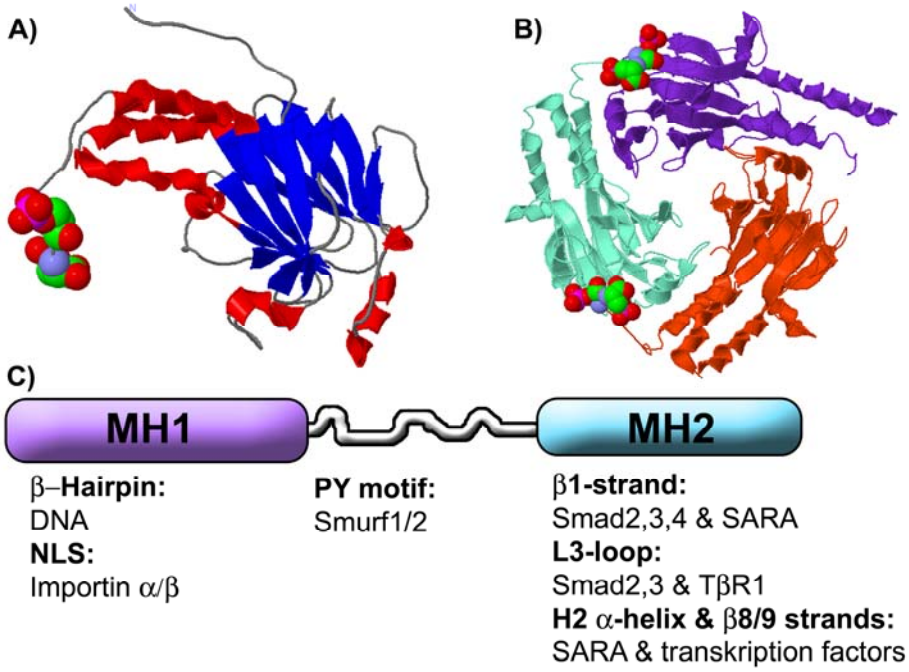

Figure 4. Smad structure and motifs.
A) Smad2 structure viewed as cartoon in Jmol with data from PDB. SxS motif is viewed as balls. α-helices are marked in red and β-strands in blue. B) Smad2-Smad complex structure viewed as cartoon in Jmol with data from PDB. Red and green proteins are the two phosphorylated Smad2 molecules while the purple one is Smad4. C) A cartoon image of Smad2 with its two main domains MH1 and MH2. Interacting motifs and their ligands are listed beneath the domain in which they are located.

A linker domain chains the MH1 domain together with the C-terminal MH2 domain that is responsible for activation, nuclear import/export, oligomerization and the interaction with the receptor and other proteins. The MH2 domain is the most conserved domain in all Smads although it also differs a bit from Smad to Smad. The MH2 domain of R-Smads contains a L3 loop that is specifically recognized by the type I receptor and a Ser-X-Ser motif at the very C-terminus that becomes phosphorylated (Feng 2005; Kretzschmar 1998; Moustakas 2001). The I-Smads lack the Ser-X-Ser motif and therefore block the receptor competitively.

Both MH1 and MH2 show interaction with many proteins in the nucleus and among them are plenty of transcription factors or co-transcription factors. And although the linker is variable between the Smads it contains a conserved PY motif that is recognized by the E3 ubiquitin ligases, Smurfs (Moustakas 2001). As Smurfs ubiquitinate the Smads, they promote Smad degradation by the proteosome. The linker also contains multiple phosphorylation sites that are thought to function as points for "crosstalk" with other signalling pathways, such as the mitogen activated protein kinase (MAPK) and cyclin dependent kinase (CDK), although these mechanisms are not fully understood (Feng 2005; Moustakas 2001).

Experiments show that the interaction between Smads and the CAGAC sequence is too weak to allow the Smad complex alone bind to DNA, unless several CAGAC repeats exists at the right distances. Instead it seems that the Smads regulate gene expression by binding transcription factors and co-transcription factors and thus stabilizing the large DNA binding complex (Chai, Wu et al. 2003).

1.4 LKB1 and PJS

Another multifunctional protein known to act as a tumour suppressor is the PAR4 homologue LKB1 (Marignani 2005). This serine/threonine kinase works, as PAR4 in *C.elegans*, together with other proteins to establish cell polarity (Baas, Smit et al. 2004). Little more was known of the behaviour of this protein in mammals before it was linked to the Peutz-Jeghers syndrome (PJS). Patients with this autosomal dominant condition suffer, apart from more harmless symptoms, a hundredfold higher risk of developing cancer in the gastrointestinal tract. It was shown that a vast majority of the PJS patients had inherited a loss-of-function mutation of LKB1's catalytic domain (Jishage, Nezu et al. 2002; Marignani 2005). This mutation has also been found in tumours from non PJS patients suggesting that the protein functions as a tumour suppressor. Exactly how this works is still unclear except that the suppressing activity lies in the catalytic part of the protein.

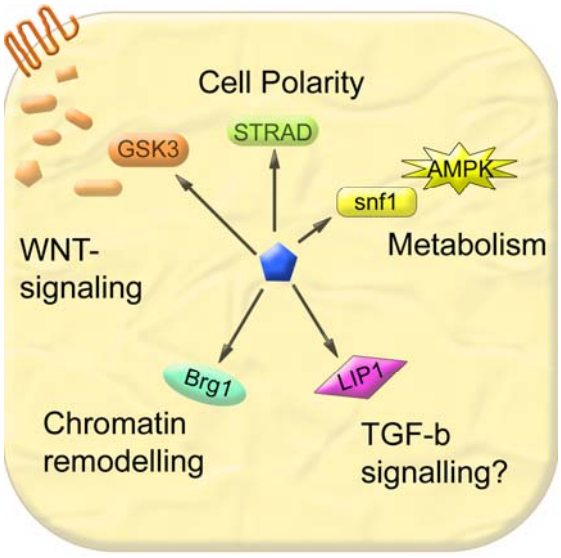

Figure 5. LKB1, a multitasking protein
LKB1, the blue pentagon, affects many processes in the cell.

Recent research have uncovered LKB1 to be another "jack-o'all-trades" protein that plays roles in many cell processes, thus making the puzzle even more complicated. Up to this date LKB1 is thought to partake in regulation of cell polarity, chromatin remodelling, Wnt signalling, cell cycle arrest and energy metabolism among others (Shaw, Kosmatka et al. 2004; Marignani 2005). Moreover, a protein named LIP1 - LKB1 interacting protein 1 - was recently found to form a complex with both LKB1 and Smad4 (Smith, Rayter et al. 2001). This suggests that LKB1 is somehow linked to the TGF−β pathway through Smad4, with LIP1 as the bridge. It also hints about a correlation between PJS and the similar juvenile polyposis syndrome, JPS, that is caused by Smad4 deficiency. Maybe these new findings could clear the fog around LKB1's tumour suppressor activity.

1.5 LIP1 – a bridge?

Very little is known about LIP1. It seems to be of some importance to cell core functions as it is widely expressed in cells from almost any tissue. Early on it was proposed to regulate the cellular localization of LKB1 as this otherwise nuclear protein shows in high levels in the cytoplasm when co-expressed with LIP1 (Smith, Rayter et al. 2001). With this new information about the LKB1-LIP1-Smad4 complex it seems, though, that there is more to this protein than previously discovered. The 1099 long aminoacid sequence of LIP1 gives very little information about the function of the protein as few and nondescript motifs can be recognized. Six tandem leucine rich repeats, LRRs, close to the N-terminus could be involved in protein interaction though the LIP1 LRRs shows low similarity to other known LRRs with that function. In the middle of the protein there is a glutamic acid rich region of 70 aminoacids and further down the C-terminal there is a possible but not very probable leucine zipper. The most similar known proteins are IRAS-1 and its mouse orthologue Nischarin, suggested to be involved in cell migration. The two proteins share a somewhat weak homology with LIP1 and also have the LRRs and E-rich domains in the N-terminal part of the protein (Smith, Rayter et al. 2001).

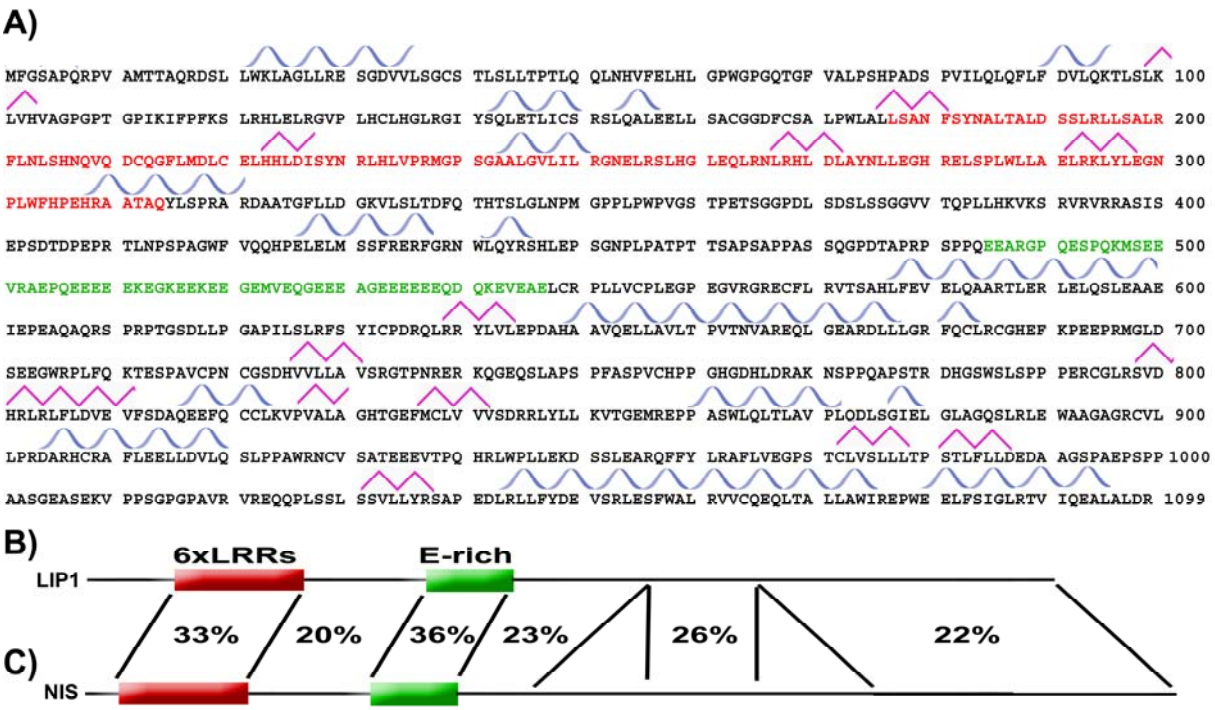

Figure 6. The LKB1 Interacting Protein 1 – LIP1
A) Predicted secondary structure above LIP1s 1099 aminoacid sequence. Blue spirals symbolizes α-helices and purple zig-zag β-strands. The six times leucine rich repeats are marked in red and the E-rich domain in green. B) A schematic cartoon of LIP1 and its likeness with Niscarin in percent. C) Schematics of Niscarin (Adapted from Smith, Rayter et al. 2001)

1.6 Aim of this study

The aim of this study is to examine the connection between LIP1 and the Smads, to find how LIP1 affects Smad signalling and also map the interaction domain on both proteins. This degree project aims at creating deletion mutants of Smad2, map a crude interaction domain with co-immunoprecipitation, find an effect on signalling pathway with reporter-assays and screen the phenotype of stable knock-down clones in which LIP1 is depleted using short interfering RNA (siRNA).

2.MATERIALS AND METHODS

2.1 Cells and plasmids

2.1.1 Cell lines

293T is the cell line primarily used for the protein assays in this degree project. This is a human embryonic kidney cell line 293 transformed with an adenovirus which carries a temperature sensitive SV40 large T antigen co-selected with neomycin. These cells have the ability of being highly transfectable (more than 50% tranfection efficiency) with calcium phosphate or lipid based transfection protocols.

The HepG2 cell line was used in the luciferase assays. HepG2 is a well established and commonly used human hepatocarcinoma cell line with epithelial morphology. Furthermore, the human epidermal keratinocyte cell line HaCaT was used to create stable clones.

2.1.2 Cell culturing

The 293T, HepG2 and HaCaT cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) from Sigma with 10% foetal bovine serum and 1% penicillin-streptomycin.The incubator atmosphere contained 5% CO_2 and kept a constant 37 ºC temperature.

2.1.3 Plasmids

Protein assays were primarily done using the vector constructs pcDNA3 and pDEF.
The constructs contained either a FLAG- or a 6xMYC-tag that was incorporated to the N-terminal of the insert protein. The retro super plasmid, pRS, was used as vector for the siRNA sequence used to knock down LIP1 in the stable clones. In the luciferase/β–gal assays two reporter-gene constructs were used. One TGF–β inducible luciferase reporter vector called pGL3-$CAGA_{12}$ and one control vector, pSV–β-Galactosidase.

2.2 Deletion mutants

2.2.1 Smad2 mutagenesis

A pcDNA3-FLAG-Smad2 construct was used as template for the mutagenesis. Primers with the introduced mismatches (Figure 7 and Table 3) were ordered from Sigma. The reaction mixture of 50 µl contained 50 ng template plasmid, 0.2 mM dNTP, 2 µl DMSO, 0.3 µM of both sense and antisense primers and 1 µl Pfu Turbo DNA polymerase together with 5 µl of its 10xbuffer, both from NE Biolabs. The mutagenesis reaction took place in Biorads' MJ Mini thermocycler with the program shown in Table 2. The mixture was then treated with the endonuclease Dpn1 which digested all parental plasmids leaving only mutant plasmids. The plasmid mixture was then transformed into *E.coli* which was grown over night on agar plates and then screened for the mutant cleaving site.

Temp.	Time	Repeats
95ºC	3min	
95ºC	30s	<|
58ºC	1min	|X18
68ºC	15min	>|
68ºC	15min	
4ºC	Inf.	

Table 2. Thermocycler program used for mutagenesis

Primer	Sequence
1.MH1-stop-Xho1 ►	GAGACACCAGTTTAGCCTCGAGTATTAGTGCCCCG
2.MH1-stop-Xho1 ◄	CGGGGCACTAATACTCGAGGCTAAACTGGTGTCTC
3.EcoR1-MH2 ►	CTTACTCAGAACCTGAATTCTGGTGTTCGATAGC
4.EcoR1-MH2 ◄	GCTATCGAACACCAGAATTCAGGTTCTGAGTAAG
5.Linker PCR ►	ACCAGAATTCCCTCCAGATATTAGTGCCCCGACACACCG
6.Linker PCR ◄	TATGCTCGAGAACACTAAAATGCAGGTTCTGAGTAAGTAACTGGC

Figure 7. Cartoon map of Smad2 with primers ordered from Sigma.
Table 3. Primers ordered from Sigma. *Mismatched nucleotides are marked with yellow.*

2.2.2 PCR of linker region

Again primers with mismatch were ordered from Sigma. The reaction was mixed as the mutagenesis reaction using Pfx DNA polymerase instead of Pfu TURBO and incubated in thermocycler with the program shown in Table 4.

Temp.	Time	Repeats
95ºC	3min	
95ºC	30s	< |
48ºC	30s	| X5
68ºC	45s	> |
95ºC	30s	< |
52ºC	30s	| X35
68ºC	45s	> |
68ºC	5min	
4ºC	Inf.	

Table 4. Thermocycler program used for PCR

2.2.3 Bacterial transformation

Electro-competent *E.coli* of the strain DH5α was prepared according to a standard protocol. The prepared DH5α were quickly frozen in dry ice with ethanol, stored at -80 ºC and thawed on ice before transformation.

For each transformation the frozen aliquots of 200 µl electro competent *E.coli* was carefully thawed on ice and mixed with approximately 300-500 ng plasmid DNA – no more than 3 µl plasmid reaction solution – and placed in electroporation cuvets from Biorad with a 1 mm path. For electroporation, the electroporator aggregate from Invitrogen was used set at 50 µF and 150 Ω. Immediately after shocking, 500 µl of LB was added and the bacteria were incubated 37 ºC on a shaker in for 1h. When the cells had recovered they were plated on LB-agar plates (10 g bactotryptone, 5 g yeast extract, 5 g NaCl, 15 g bacto-agar in 1 L H_2O and autoclaved) containing 200 µg/ml ampicillin for selection and incubated in 37 ºC over night. The next day 6 colonies were picked from the plate to be screened for the right DNA insert and were inoculated in 3 ml LB with ampicillin (200 ug/ml) for at least 15h at 37 ºC on a shaker.

2.2.4 Mini plasmid extraction with Cetyl Trimethyl Ammonium Bromide (CTAB)

1.5 ml of *E.coli* culture was centrifuged for 5min at 5000 rpm. After removing the supernatant the pellet was resuspended in 200 µl STET buffer (8% glucose, 5% Triton X100, 50 mM EDTA, 50 mM Tris pH 8.0). Then 4 µl of Lysozyme was added and the sample was incubated for 5 min at RT before heating it to 95 ºC for 50s. The lysate was spun down at 13000 rpm for 10 min and the pellet was removed using a toothpick. To precipitate the DNA, 8 µl of 5% CTAB was addded followed by 5 min centrifugation at 13000 rpm. The pellet was suspended in 1.2 M NaCl solution and forceful vortexing. The suspension was then precipitated with 0.75 ml ethanol and centrifuged for 10 min at 13000 rpm. Finally the plasmids were suspended in 50 µl H_2O.

2.2.5 DNA agarose gel eletrophoresis

The extracted plasmid DNA was screened for the correct insert size using 0.8% agarose-gel electrophoresis. 0.4 g agarose mixed with 50 ml 0.5x TBE (0.89 M Tris and 0.89 M Boric acid) was boiled and left to cool in a gel tray after addition of SYBR Safe DNA gel Stain. The restriction enzyme reaction that was incubated at 37 ºC for 2h consisted of 8 µl plasmid DNA, 1 µl 10x NEbuffer 2 from New England Biolabs and 1 µl restriction enzyme mix (1.5 µl XhoI or 1.5 µl XhoI and 1.5 µl EcoRI, depending on the sites to look for, in 10 µl H_2O – all enzymes from New England Biolabs). One clone with the correct digestion pattern was picked for sequencing.

2.2.6 Sequencing

Big Dye v1.1 protocol was followed to prepare the plasmids for sequencing. The actual sequencing was then performed by staff proficient with the sequencer.

2.2.7 Construction of final plasmids

Mini plasmid DNA containing the mutation was chosen and digested, now using almost all plasmid material. 44 µl of mini plasmid DNA solution and 5 µl 10x NEbuffer2 was digested with 1 µl EcoR1 and 1 µl Xho1 restriction enzymes. Again the restriction reaction was incubated at 37 ºC for 2-3h and then separated on a 0.8% agarose-gel with 0.5x TBE buffer. The DNA fragment was cut out from the gel and put in a dialysis-tube (3.5 kDa cutoff) together with 0.5 ml 0.5x TBE. To elute the DNA from the agarose gel, the tube was put into the gel-tray with running current. The same was done to get a digested vector plasmid with the right restriction-endings. The DNA was precipitated with NaAcetate and ice cold EtOH, incubated at -80 ºC and centrifuged at 13000 rpm for 20 min and then diluted in water. These crudely purified DNA fragments were then ligated by mixing 1 µl (40 ng) vector, all of the digested insert suspended in 7.5 µl, 0.5 µl T4 DNA ligase and 1 µl 10x T4 ligase buffer. This reaction was incubated at 16 ºC over night. Next day the ligation solution was transformed into *E.coli* and later successful colonies were screened as above. One colony containing the right plasmid was inoculated in 50 ml LB with 200 ug/ml ampicillin at 37 ºC over night. 1.5 ml of these bacteria were frozen with 70% glycerol at -80 ºC and the rest were used in the Midi-V100™ Ultrapure Plasmid Extraction System from Viogene to prepare pure DNA for mammalian transfection.

2.3 Protein Assays

2.3.1 Cell transfection

293T cells were transiently transfected with plasmids for the protein interaction assays using calcium phosphate protocol. One day before transfection cells were seeded onto Sarstedt 6 well plates - approximately $2x10^5$ cells and 2 ml medium in each well - and incubated overnight. One well per condition was used. For every condition 90 µl of plasmid solution was mixed with 100 µl freshly shaken 2xBBS (50 mM BES pH 6.95 (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 mM NaCl and 1.5 mM Na_2HPO_4) and 10 µl 6,25 mM $CaCl_2$. Empty vector plasmids were used to make every condition in an experiment contain equal amounts of DNA. The solution was given 20 min to start forming crystals while the cell media was changed to a new one with 10% newborn calf serum instead of foetal calf serum. After 20 min the plasmid solution was added to the cells. The cells were incubated for 3h at 37 ºC before switching back to the normal media. After 40h the cells were confluent and ready to harvest.

2.3.2 Protein extraction

The cells were lysed with 200 µl solubilizing buffer (0.5% Triton X-100, 0.5% Deoxycholate, 20 mM NaCl and 10 mM EDTA) per well. After 20 min on ice to completely lyse the cells the solution was centrifuged at 13 000 rpm for 10 min in a cold centrifuge. The insoluble pellet was thrown away and the protein concentration in the soluble supernantant was measured using Bradford protocol. To prepare samples for SDS-PAGE about 25 µl of the lysate, depending on the protein concentration, was mixed with equal volume of 2x loading buffer so that each sample was of equal total protein content, thus minimizing errors from varying cell density.

2.3.3 Co-immunoprecipitation

For immunoprecipitation assays, 1% trasylol and 1% 2 mM PMSF were added to the solubilizing buffer before harvesting the cells and 400 µl buffer was used per well. After centrifugation 25 µl total cell lysate was saved and balanced as above. The rest of the cell lysate was mixed with 1.5 µl of monoclonal mouse antibody against either FLAG or MYC, or rabbit polyclonal antibody against LIP1 – DRAKE – depending on which protein to pull down. After 2h in an end-over-end machine at 4 ºC 25 µl of 50% proteinA-sepharose dissolved in solubilizing buffer was added and the lysate was put 1h more in the turning machine. Then the samples were centrifuged at 13 000 rpm, the supernantant carefully removed and 1ml solubilizing buffer was added. After doing this washing step two more times the solution was pipetted into new tubes and a last wash now with a high salt buffer (550 mM NaCl, 20 mM Tris pH 7,5, 1% Triton X-100). All buffer was completely removed using a syringe needle with a smaller diameter than the sepharose beads. The beads were then suspended in 30 µl of 2x loading buffer and western blotted.

2.3.4 SDS-PAGE and western Blot

The protein extracts were electrophoresed with SDS-PAGE using an 8% polyacrylamide gel in Tris-Glycine running buffer (0.025 M Tris, 0.192 M Glycine and 0.1% SDS) and then electrotransfered onto a Whatman Protran nitrocellulose transfer membrane (0.2 µm) using an aggregate from Biorad. Semidry buffer contained (39 mM Glycine, 48 mM Tris, 0.0375% SDS and 20% methanol). The membrane was then blocked for 0.5h using TBS-T (0.5 M Tris-HCL pH 8.0, 1.38 M NaCl, 0.027 M KCl and 0.05% Tween-20) with 5% BSA. After blocking, the membrane was briefly washed with TBS-T and probed over night in TBS-T with monoclonal mouse antibody against FLAG or MYC. After being washed in TBS-T for 30 min the membrane was probed a second time with a monoclonal anti-mouse-IgG with horse-radish peroxidase attached to it from Amersham Biosciences. Another 45min of washing before the membrane was put in a fresh mix of equal amounts of ECL - enhanced chemiluminescense - solutions (A: 435 ml ddH_2O, 50 ml 1M Tris-HCl pH 8.5, 10 ml Luminol solution (1 g luminol/10 ml DMSO), 5 ml π-coumaric acid (0.2 g/5 ml DMSO), B: 100 mM Tris pH 8.5) and exposed on X-ray film (Fujifilm).

2.3.5 Reporter assay

HepG2 cells were seeded onto a 12-well plate one day before transfection. The transfection followed as described above with changes in the volumes. Every condition was done in double triplicates, one with TGF–β stimulation and one without. A master tube with sufficient transfection solution for 7 wells was prepared for each condition. Each tube contained 1.75 µg luciferase reporter plasmid, 1.4 µg β-galactosidase plasmid, LIP1 plasmid and empty vector plasmid, all dissolved in 315 µl H_2O before adding 350 µl BBS and 35 µl $CaCl_2$. 100 µl transfection solution was then added to the HepG2 cells after the media switch. 5 to 6h later the medium was switched back. At the end of next day, 16h before harvesting, the cell

medium was changed to a nutrient poor medium (0.5% FCS in DMEM and 1% penicillin-streptomycin) with or without 2.5 ng/ml TGF−β. When harvesting cells, 250 µl lysis buffer from BDH Pharmingen was used. For the luciferase reading, 25 µl lysate was mixed with 25 µl luciferase assay substrate buffer from BDH Pharmingen and light emission was immediately measured. Both mixing and measuring was done automatically by an EGRG Wallac multilabel counter. The β-galactosidase reading was done by adding 25 µl 2xβ-gal buffer (200 mM Na-Phosphate pH7.3, 2 mM $MgCl_2$, 100 mM β -mercaptoethanol and 1.33 mg/ml o-nitro-phenyl-galactoside) and incubated until it took a clear yellow color, approximately 10-40 min. Then, the absorption at 420 nm was measured using the ERGRG Wallac multilabel counter. This reading was then used as a baseline for how good the transfection or cell growth had been. The luciferase/β−gal ratio was calculated for each well and used for comparison between the conditions.

3. RESULTS

3.1 The construction of Smad2 deletion mutants

Deletion mutants of Smad2 were needed in order to investigate which part of Smad2 LIP1 affects or interacts with. These plasmid constructs that would express a selected part of the protein were going be created by ligating a part of the Smad2 sequence with a pcDNA3 plasmids with incorporated FLAG-tag sequence. From what is known about the Smads it was naturally to choose the three domains of Smad - MH1, linker and MH2 – for a rough mapping. The MH1 and MH2 mutants were generated by mutagenesis introducing restriction sites and stop codons inside the Smad2 sequence of a template plasmid while the middle linker piece was simply amplified with PCR (see Figure 8). The MH1 and MH2 domains could then easily be cut out from the template plasmid and ligated into a new plasmid with the start codon and tag sequence already in place.

Sequencing showed that the mutagenesis worked flawlessly but when the restricted plasmid was run on an agarose-gel too few bands showed up. More investigative sequencing revealed that the EcoR1 restriction site was missing on the template plasmid. Because of this there was no easy way to remove the FLAG-tag from the MH1 domain. This problem didn't matter much in this study since the plan was to use FLAG-tagged deletion mutants anyway. The linker region, whose sequence was amplified with PCR, and MH2 domain was generated according to the plan though. Unfortunately, due to some problem with the ligase enzyme occurring during the time of this project, the constructs were never completed. The mutated plasmids were frozen for future use when the ligation problem is solved.

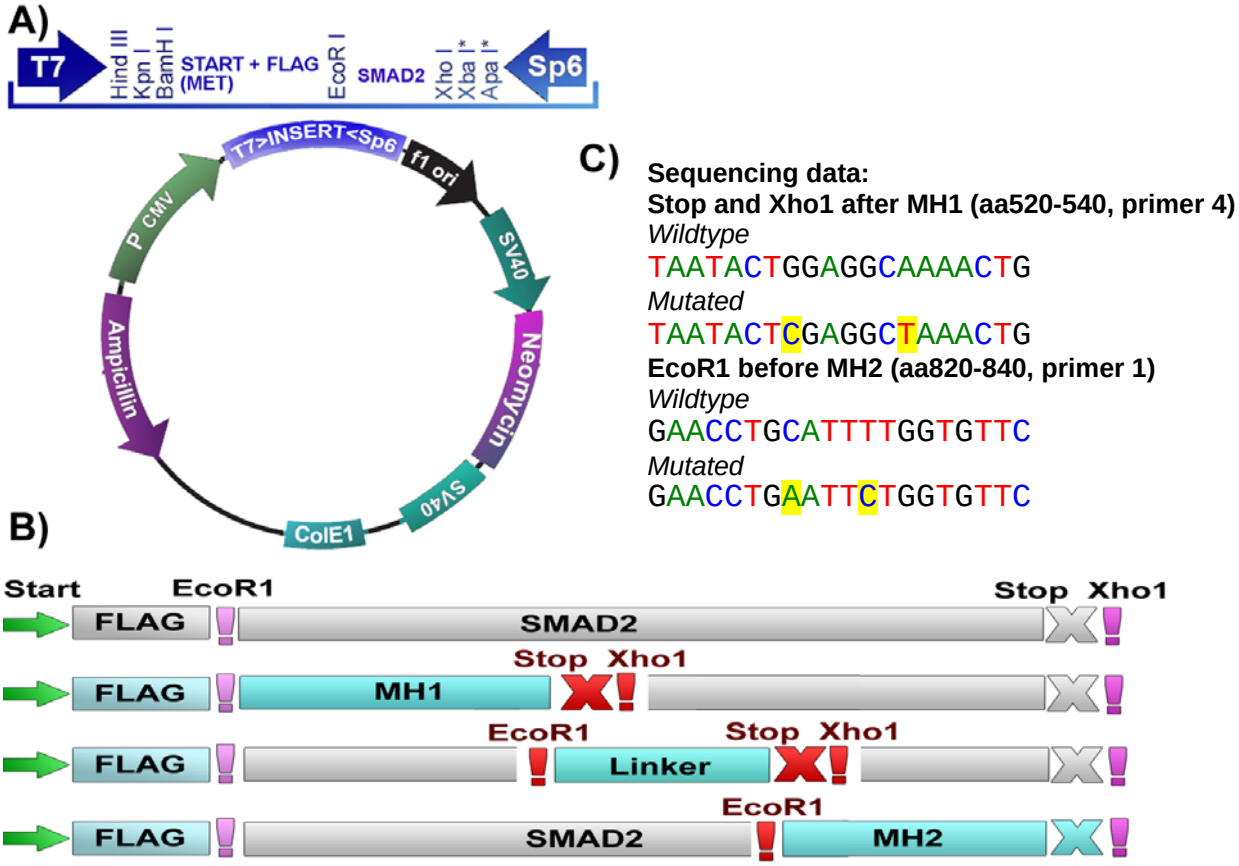

Figure 8.Mutagenesis of Smad2

A) The pcDNA3 vector that contained the Smad2 template and which was going to be used for the deletion mutants. The EcoR1-site between the flag-tag and the insert has mutated. B) Cartoon of deletion mutants. Deletion mutants are in cyan and introduced mutations in red. C) Sequencing data using primers from table 2 show that mutagenesis was successful. Mutations are marked in yellow.

3.2 LIP1 affects Smad levels

293-T cells were transiently transfected and the protein extracts were analysed with SDS-PAGE and western blotting. The amounts of Smads are compared with and without co-expression with LIP1. Later co-expression with active ALK5 was also added to see how an active signal affects the interaction with LIP1 and the Smads (Lane 3, 6 and 9 in Figure 14). LIP1's negative effect on Smad2 and Smad4 stability was quite clear while the effect on Smad3 seemed to be vauge and ambigous with active ALK5 (Lane 4-6 in Figure 14 B and E). When co-expressed with LIP1, Smad2 was visibly reduced through all experiments though not as much as Smad4 which vanished completely when using pcDNA3 vectors in 239-T cells. The effect became drastically less or gone when the pcDNA3 vector was switched for a pDEF vector as shown in Figure 10 and 14. pDEF is a vector that contains an elongation factor promoter unlike the cytomegalovirus promoter in pcDNA3.

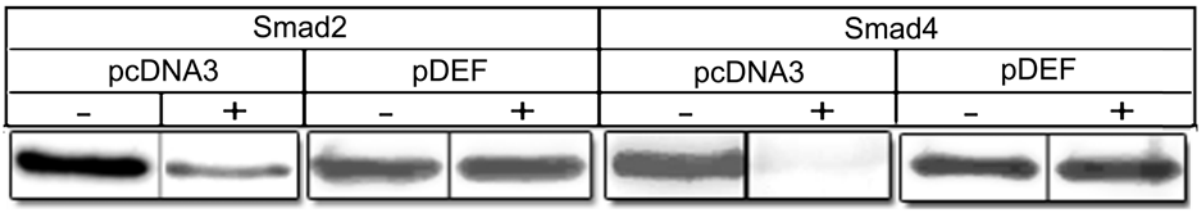

Figure 10. Smad2 and Smad4 are destabilized by LIP1. *Total cell lysates from 293-T cells transfected using pcDNA3 vector versus pDEF. Transfection with or without LIP1 is marked with + or -. The effect of LIP1 on Smad2 and Smad4 is greatly reduced when using pDEF as vector.*

3.3 Domain mapping of LIP1

No mapping for interaction domains could be done on Smad2 without the deletion mutants, but attempts to find the domain on LIP1 that affect Smad2 were made with the already existent deletion mutants of LIP1. Lacking knowledge of LIP1's functional domains, the deletion mutants were instead used to examine the recognizable motifs. Therefore the three mutants consisted of one with the N-terminus and the LRR's (1-318), one with everything but the LRR's (313-1099) and one only with the C-terminus and leucine zipper (598-1099). None of these (lane 3 and 4, Figure 11) seemed to have the same destabilizing effect on Smad2 as the wildtype. (Lane 2, Figure 11) The C-terminus mutant (not shown) was almost of the same size as Smad2 which made it difficult to separate them on the gel, but there was little reason to believe it would affect Smad2 since the Δ-N-terminus mutant did not. This hints that a functional domain may lay close by or across the cuts at residue 313-318. New deletion mutants are needed in order to investigate this.

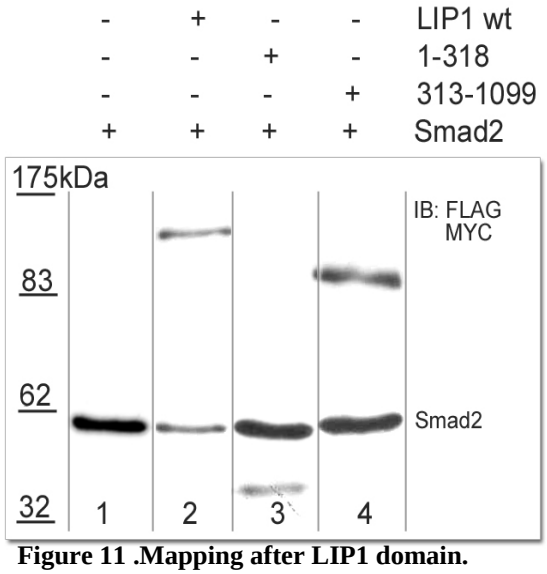

Figure 11 .Mapping after LIP1 domain.
Total cell lysates from 293-T cells transfected with Smad2 and LIP1 deletion mutants (1-318 and 313-1099, referring to amino acid residues). None of the deletion mutants in lane 3 and 4 affects Smad2 as the wildtype in lane 2. Lane 1 contains Smad2 alone as control.

3.3 Effect of LIP1 on Smad signaling

A luciferase reporter assay was to measure the effect LIP1 has on TGF−β signaling dependent transkription. HepG2 cells were used for these experiments. The cells were transiently transfected, cultured and subjected to TGF-β 16h before harvesting. Two values were measured, one of luciferase activity and one of β-galactosidase activity. Unlike the luciferase plasmid, the constitutively expressed β-galactosidase vector is independent of TGF−β signaling and was used to normalize the reporter signal in order to reduce the effects of transfection efficiency. The first assay (Figure 12A) showed a trend of decreased CAGAC-transcription with increasing amounts of LIP1 which is consistent with the results that LIP1 critically destabilizes Smad4. This trend, however, couldn't be reproduced. Instead further experiments (Figure 12B,C) with steeper concentration-ladder of LIP1, that was thought to visualize the effect in the first assay better, even showed the opposite result. From these results it would seem that TGF−β dependent CAGAC-promoters are independent of LIP1.

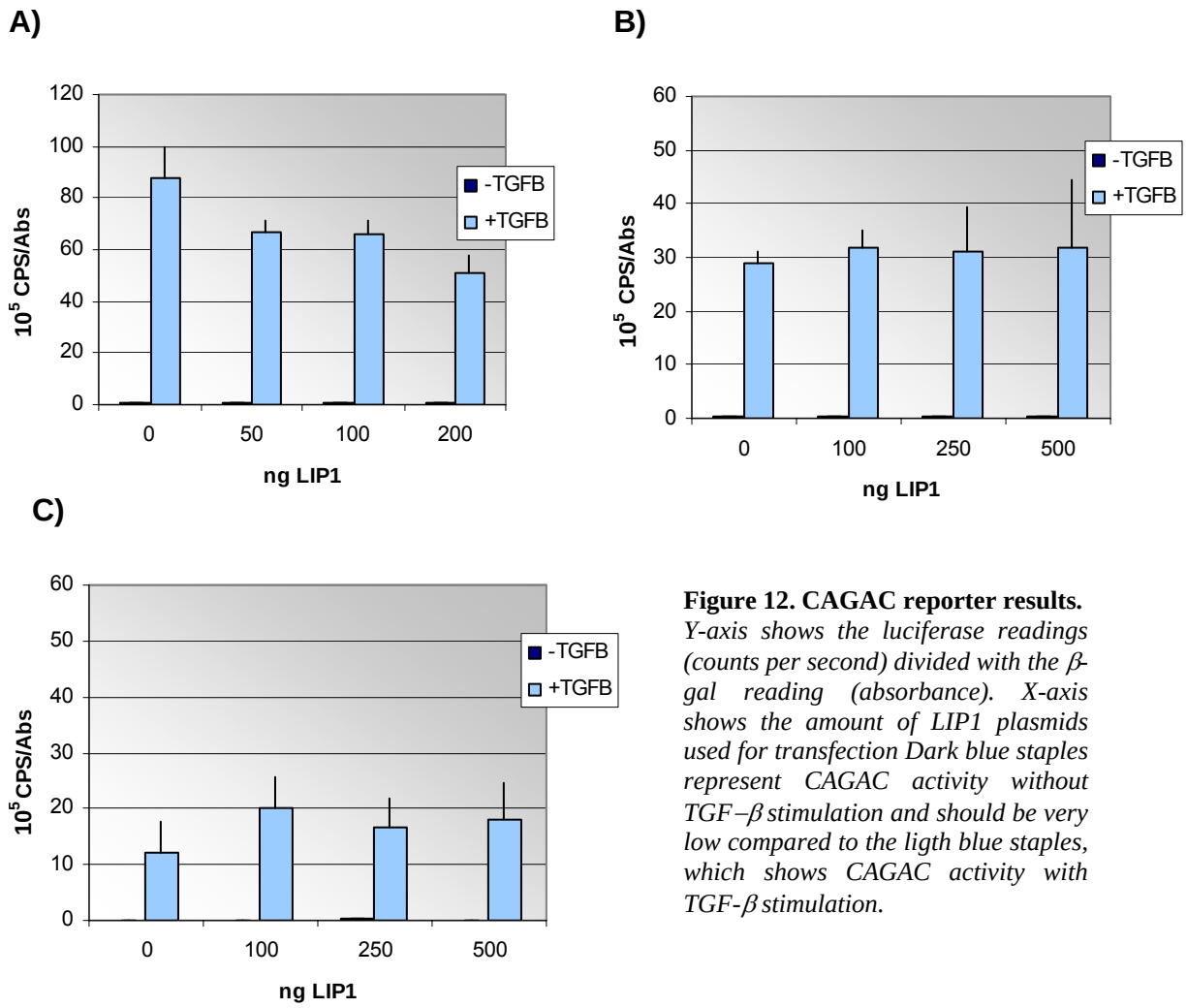

Figure 12. CAGAC reporter results. *Y-axis shows the luciferase readings (counts per second) divided with the β-gal reading (absorbance). X-axis shows the amount of LIP1 plasmids used for transfection Dark blue staples represent CAGAC activity without TGF−β stimulation and should be very low compared to the ligth blue staples, which shows CAGAC activity with TGF-β stimulation.*

3.4 Interaction between LIP1 and Smads

Co-immunoprecipitation, co-IP, assays were used to see whether LIP1 interacts directly with Smad2. In these assays 293-T cells were used and protocol for co-IP was followed, except a few changes in the washing conditions in order to get rid of pesky background bands of 6xMYC-LIP1. The first experiments where made using pcDNA3 as vector, but it proved impossible to use Smad4 as a positive control with this setting, since Smad4 was thoroughly degraded when co-expressed with LIP1 (Figure 14B lane 7-9). Later the pcDNA3 vector was switched for pDEF to stabilize the Smads. In every assay conducted, with pcDNA3 or pDEF vector, LIP1 failed to pull down Smad2 and vice versa. (Figure 13 and lane 1-3 in Figure 14) In a combined assay with all the three Smads -2, 3 and 4- only Smad4 was pulled down together with LIP1. Constitutively active ALK5 was added as a condition for co-IP to see if complex forming Smads affect the result, and it did. Smad4 failed to precipitate together with LIP1 when ALK5 was active.

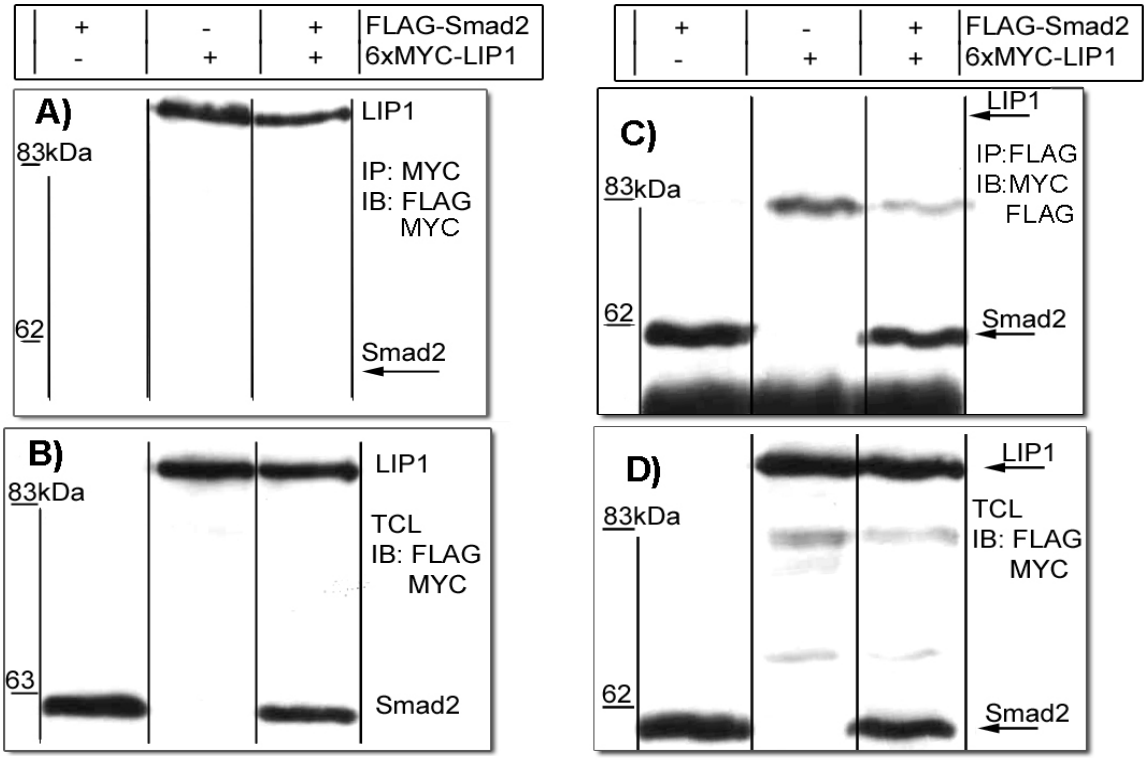

Figure 13. Co-immunoprecipitaion of LIP1 fails to pull down Smad2 and vice versa.
293-T cells transfected with MYC tagged LIP1 and FLAG tagged Smad2 using pcDNA3 vector. A) Co-IP against MYC-LIP1 blotted against MYC and FLAG. No sign of Smad2. B) Total cell lysates from the same experiments as A *show that expression levels are good. C) Co-IP against FLAG-Smad2 blotted against MYC and FLAG. LIP1 cannot be found. D) Again, the total cell lysates show good expression levels for the same experiment as in C.*

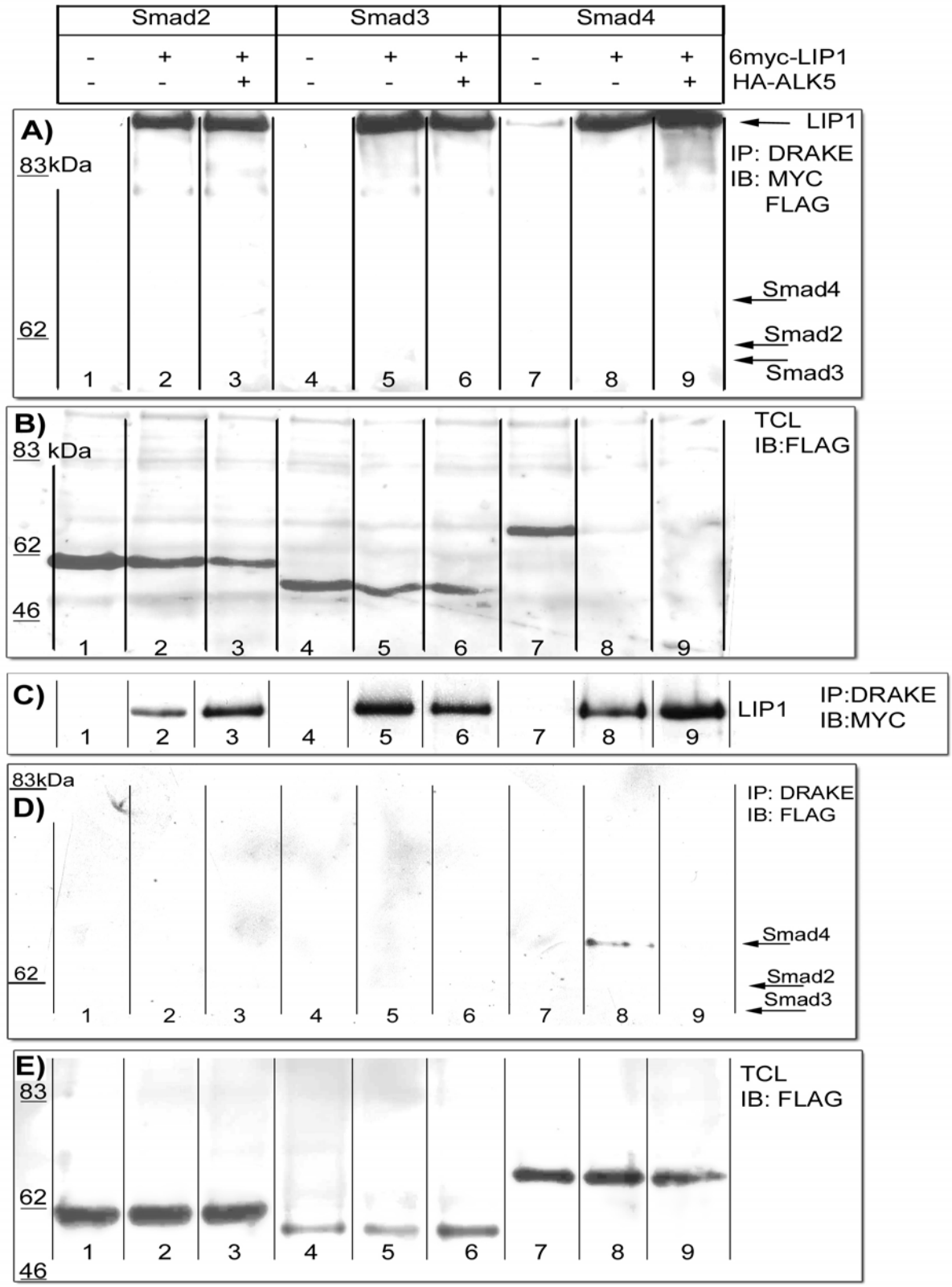

Figure 14. Co-IP against LIP1 with Smad2, Smad3, Smad4 and ALK5.
293-T cells are transfected with FLAG tagged Smads, MYC tagged LIP1 and HA tagged ALK5. Co-IP against LIP1 epitope DRAKE and respective total cell lysates are shown. In Figure A and B, pcDNA3 vector is used. In Figure C, D and E, pDEF is used as vector for all but ALK5 and Smad3 as those constructs were unavailable. A) Co-IP blotted against MYC and FLAG. No Smads. B) TCL for A blotted against FLAG. The posistive control, Smad4 is exhausted by LIP1 why it does not show in A. C) Co-IP blotted against MYC. D) Same Co-IP blotted against FLAG. Only the positive control, Smad4, shows. E) TCL for D blotted against FLAG shows good expression levels.

4. DISCUSSION

Aim and previous work

The aim of this study was to investigate the connection between the LKB1 interacting protein, LIP1, and core elements of the TGF−β signaling pathway, the Smads – especially Smad2. The connection between the Smad4 and LIP1 was first discovered by Alan Ashworth/Darrin P.Smith and his group who also suggested that this could somehow be a link between juvenile polyposis syndrome, JPS, and Peutz-Jeghers syndrome, PJS. Both these syndromes result in a drastically increased risk of developing intestinal cancer (Smith, Rayter et al. 2001). If this link is for real it would be a great improvement in understanding these syndromes and maybe result in better diagnosis and treatment.

A/Ds group also found that experiments with LKB1 and LIP1 expression in *Xenopus* embryos showed a phenotype similar to those when the TGF−β signaling was altered (Smith, Rayter et al. 2001). This strengthens the suspicion that LKB1 "crosstalks" with the TGF−β pathway via LIP1.

In a previous experiment done at Ludwig, it was shown that Smad2, 3 and 4 was co-immunoprecipitated together with LIP1. A project was started to further examine the connection between Smads and LIP1. This study is a part of that project where emphasis was put on Smad2 - examining if and how, Smad2 interacted with LIP1 - while other parts of the group examines the relation between the other Smads and LIP1. The project took some twists and turns off the predicted course and the results are far from conclusive but can still be of some help for the further research in the matter.

LIP1 regulates Smad levels

The first observations were promising as LIP1 reduced the Smad2 levels when using pCDNA3 constructs of Smad2 and LIP1 in the 293-T cell line. The effect on Smad2 was less than that on Smad4 but much more pronounced than the effect on Smad3. It was a general thought that this reduction of Smad2 is due to LIP1 destabilizing the protein by ubiquitination or other means. This is supported by the fact that the Smads many times have been found to be regulated by ubiquitination. An easy way to test this would be to see if proteasome inhibitors affect the results in the previous experiments. This was not done in this project but might be in the future.

Some uncertainty began to grow when the magnitude of the reduction decreased or almost disappeared when switching plasmid vector to pDEF – a vector that contains an elongation factor promotor instead of the cytomegalovirus promoter in pcDNA3. If this effect is purely due to some promoter activity or competition can almost surely be ruled out as empty pDEF vectors without LIP1 have no effect. These results may promote the possibility that LIP1 affect Smads already at transcription level. Again, more experiments need to be done in order to find out if this is caused due to how LIP1 regulate Smad levels or by vector incompability. And until the cause is known, there will be a doubt about which result to trust, if LIP1 actually regulates Smad levels in a real cell and whether this will affect other experiments too. However, because of the reproducibility and previous results in the group, the destabilizing effect of LIP1 on Smad2 seems most likely to be true.

Trying the experiments with yet a third and fourth vector could clarify the results but there are better more certain ways. An assay measuring the endogenous levels of the proteins is one of them. None were made in this study, though, but knock-down clones have been made with short interfering RNAs against LIP1 for this purpose. This would also solve the question about if the over-expressing systems fail to emulate the real network of interactions in the cell and give an artificial false result. Hopefully, these clones will soon give a much more reliable and accurate answer of what effect LIP1 have on Smad stability.

Reporter assay

Another enigmatic result came from the reporter assay. The expected result would have been a decrease in the TGF-β response with increasing concentration of LIP1 because of LIP1s destabilizing effect on Smad4. If there is no Smad4 then there can be no increase of the transcription of CAGAC induced genes when stimulating with TGF-β. Still, several CAGAC-reporter assays in and outside this project show no consistent dependency between TGF-β induced transcription and amount of LIP1.

Several factors could be the cause of these strange results. For one, the destabilizing effect on Smad4 has only been seen in overly expressed conditions, maybe the endogenous effects are different. Another factor that could cause this experiment to fail is that not only Smad2 and Smad4 bind the CAGAC sequence but also Smad3, which sometimes seems to be stabilized by LIP1. Now, this touches the fact that the TGF-β pathway is complicated and not yet fully understood. So that to us LIP1 might seem to affect a black box into which we can only see partly, so that the result may seem unpredictable and independent while it really is not. For now, though, the conclusion has to be that no dependency between LIP1 and CAGAC-promoter activity could be detected.

Interaction

The co-immunoprecipitation of Smad2 with LIP1 was a bit tricky as 6xMYC-LIP1 proved to stick quite effortlessly to everything and required stringer washing protocols before the annoying background bands disappeared. There was also a need to increase the levels of Smad2 being expressed, since the amount of Smad2 decreases when co-expressed with LIP1. A much greater problem than Smad2 being destabilized by LIP1, was the difficulty to use Smad4 as a positive control as Smad4 was reduced almost completely when co-expressed with LIP1.

Out of necessity, but reluctantly, the plasmid vector used for transfections was switched to pDEF to stabilize the Smad levels. The repulsion of using the pDEF vector came from the different results and the fact that a weaker effect foretells a weaker interaction - if there is any. It was shown - with the new conditions - that LIP1 pulled down Smad4, as predicted, but not Smad2 or Smad3. There was no time to repeat the last experiments with the pDEF vector plasmid, so there is no strong backup for those results. But since several attempts to pull down Smad2 with LIP1 failed, including the one with Smad4 as positive control to prove the method working, this will be the consensus of this study. But what then is the mechanism by which LIP1 destabilizes Smad2, if it is not by direct interaction as with Smad4?

A puzzle to be continued

A lot of work was put into creating the constructs of Smad2 deletion mutants, which would have been to good help in examining the effect LIP1 has on Smad2, if it hadn't been for the unfortunate enzyme problem. Though the mutants may still prove handy for further research in Ludwig, it would have been nice if they had come to use in this study.

The knowledge about what part of Smad2 that is responsible for the destabilizing effect by LIP1 could give good hints why LIP1 affects Smad2 less than Smad4 and how come Smad3 is more or less unaffected. It could also answer the question about how LIP1 affects Smad2 since it doesn't seem to be by direct interaction as with Smad4. Could it be that LIP1 affects Smad2 through a complex with Smad4? It is well known that Smad2 and Smad4 form a complex with each other. If now LIP1 forms complex with Smad4 it is not too far fetched that LIP1 also could affect Smad2 through this connection. That is, of course, if Smad4 has a possibility to interact with both proteins, i.e. if LIP1 doesn't block Smad4s interaction with Smad2 or vice versa. This is another thing that deletion mutants could have told. If LIP1 fails

to affect a Smad2 mutant lacking the Smad4 binding sites, it would promote the theory of a Smad2-Smad4-LIP1 complex.

There is a lot to speculate about and it feels like that this study has given rise to more questions without answering too many. Even so, in the future when the questions are solved, this study may aid in placing LIP1 into the big puzzle of the TGF-β pathway.

Future work

The near future of this project lies in examining the phenotype of stable clones either with knock down or over-expressing LIP1 genotype and also in yeast-2-hybrid assays, scanning after other proteins that bind LIP1. All this and a lot more will be required until finally LIP1 may be put into its place in the TGF-β pathway. When this connection between LIP1 and Smad is completely understood it will help a lot in understanding not only the pathway itself but also the two syndromes JPS and PJS.

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