MOLECULAR STUDIES ON LIM-DOMAIN PROTEIN CRP1

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Medicine, University of Helsinki, in the Lecture Hall 2, Biomedicum Helsinki, Haartmaninkatu 8, Helsinki on November 30 th, 2011 at 12 noon.

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ISBN 978-952-10-7352-6 (paperback)
ISBN 978-952-10-7353-3 (PDF)

Unigrafia
Helsinki 2011

ABSTRACT

Various intrinsic and external factors are constantly attacking the cells causing damage to DNA and to other cellular structures. Cells in turn, have evolved with different kinds of mechanisms to protect against the attacks and to repair the damage. Ultraviolet radiation (UVR) is one of the major environmental genotoxic carcinogens that causes DNA lesions. At present, 1-10% of radiation reaching the earth is UVB, and over 90% is UVA. However, the proportion of the shorter wavelengths has been in increase due to the depletion of ozone layer. UVR induces inflammation, mutations, immunosuppression, accelerated aging of the skin and skin cancers. Epidermis is the outermost layer of the skin consisting mostly of keratinocytes, whose primary function is to protect the skin against e.g. UV radiation.

LIM domain proteins are a group of proteins involved in regulation of cell growth, damage signalling, cell fate determination and signal transduction. Despite their two zinc fingers, LIM domains do not bind to DNA, but rather mediate protein-protein interactions and function as modular protein binding interfaces. LIM domain proteins may operate through interactions with other proteins by acting as adaptors, competitors, autoinhibitors or localizers.

Csrp1 was initially identified as a UVR-regulated transcript by using expression profiling (Gentile *et al.*, 2003). We have further studied regulation and function of CRP1, a representative of cysteine rich protein- family consisting of two LIM domains, and find that CRP1 is increased by UVB in primary human keratinocytes and by UVC in normal human skin fibroblasts. Ectopic expression of CRP1 protected the cells against UVC and provided a survival advantage for the cells, whereas silencing of CRP1 rendered the cells more photosensitive. Furthermore, in keratinocytes CRP1 was cleaved by caspases preceding apoptosis. Actinic keratosis (AK) is a premalignant lesion in skin caused by excess exposure to sunlight and sunburn which may lead to formation of cutaneous squamous cell carcinoma (SCC). The expression of CRP1 was increased in basal keratinocytes of AK patient specimens suggesting that CRP1 may be increased by constant exposure to UVR and may provide survival advantage for the cells also *in vivo*. In SCC, CRP1 was only expressed in tumor surrounding fibroblasts. Moreover, we found that ectopic expression of CRP1 suppresses cell proliferation.

Transforming growth factor β (TGFβ) is a multifunctional cytokine that regulates several functions in cell including growth, apoptosis and differentiation, and plays important role in pathological disorders like tumorigenesis and fibrosis. We found that TGFβ-signaling pathway regulates CRP1 at protein, but not at transcriptional level. The regulation appeared biphasic, given that CRP1 levels were first rapidly increased within one hour followed by a decrease and a sustained increase then ensued a few hours later and was stable for a few days. The increase was mediated both through Smad and non-Smad signalling pathways involving MAPK/p38. Furthermore, we found that CRP1 increase by TGFβ was associated with myofibroblast differentiation, and that CRP1 was significantly more expressed in idiopathic pulmonary fibrosis (IPF) patient samples as compared to normal lung specimens. As CRP1 is associated with actin cytoskeleton, we also found that CRP1 is required for cell contractility that plays a role in IPF disease.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. Latonen L*, Järvinen PM*, Laiho M. (2008) Cytoskeleton-interacting LIM-domain protein CRP1 suppresses cell proliferation and protects from stress-induced cell death. *Exp Cell Res.* 314:738-47 (* These authors equally contributed to this study)

II. Latonen L, Järvinen PM, Suomela S, Syrjäkari H, Saarialho-Kere U, Laiho M. (2010) Cysteine-rich protein 1 is regulated by UV in human keratinocytes. *Photodermatol Photoimmunol Photomed.* 26:70-7

III. Järvinen PM, Myllärniemi M, Liu H, Moore HM, Leppäranta O, Salmenkivi K, Koli K, Latonen L, Band A, Laiho M. (2011) Cysteine-rich protein 1 is regulated by transforming growth factor-β1 and expressed in lung fibrosis. *J Cell Physiol.* In press.

ABBREVIATIONS

ABLIM	actin-binding LIM
AK	actinic keratosis
ALP	alkaline phosphatase
AP-1	activator protein 1
ATD	actin-target domain
ATR	ATM-related
BCC	basal cell carcinoma
BMP	bone morphogenetic protein
CAF	cancer associated fibroblast
CARP-1	cell cycle and apoptosis regulator protein-1
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
CHK1	CSK-homologous kinase 1
CK1δ	casein kinase 1δ
CPD	cyclobutane pyrimidine dimers
CRIP	cysteine-rich intestinal protein
CRP	cysteine rich protein
ECM	extra-cellular matrix
EMT	epithelial-mesenchymal transition
EPLIN	epithelial protein lost in neoplasm
ER	estrogen receptor
ERK	extra cellular signal-regulated kinase
FA	focal adhesion
FGF	fibroblast growth factor
FHL	four and half LIM domains
βGAL	β galactosidase
GDF	growth and differentiation factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HDAC	histone deacetylase
HGF	hepatocyte growth factor
Hic-5/ARA55	hydrogen peroxide-inducible gene/ androgen receptor associated protein 55
HIPK2	homeobox-interacting protein kinase 2
IL-13	interleukin 13
IPF	idiopathic pulmonary fibrosis
JAM	junctional adhesion molecule
JNK	c-JUN N-terminal kinase
LAP	latent associated protein
LASP	LIM and SH3 protein
LIM	Lin11, Isl-1 & Mec-3
LIMD1	LIM domain-containing protein 1
LIMK	LIM kinase
LMO	LIM-only
LOH	loss of heterozygosity
LTBP	latent transforming growth factor beta-binding protein
MAPK	mitogen activated protein kinase

MDM2	murine double-minute 2
MEKK	mammalian mitogen-activated protein kinase kinase
MICAL	molecule interacting with casl
MLP	muscle LIM protein
MMP	matrix metalloproteinase
MRF	muscle regulatory factor
N-Cor	nuclear receptor corepressor
NEDD4-2	neural precursor cell expressed, developmentally down-regulated
NER	nucleotide excision repair
NHEK	normal human epidermal keratinocyte
NOX-4	NADPH oxidase 4
P300/CBP	p300 / CREB-Binding Protein
PBS	phosphate buffered saline
PDLIM	PDZ and LIM domain
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3-kinase
PINCH	particularly interesting new cysteine- and histidine-rich protein
PKC	protein kinase C
PPAR-γ	peroxisome proliferator-activated receptor
PPM1A	protein phosphatase 1
ROCK	Rho kinase
ROS	reactive oxygen species
SARA	smad anchor for receptor activation
SCC	squamous cell carcinoma
SDF	stromal derived factor
SH3	SRC Homology 3 Domain
SKI	sloan-kettering institute
αSMA	α smooth muscle actin
SMURF	smad ubiquitin ligase
SNON	ski-novel protein
SP-C	surfactant associated protein C
SRF	serum responsive factor
TAK	transforming growth factor-β-activated kinase
TGFβ	transforming growth factor
TGFBR	transforming growth factor receptor
TERC	telomerase RNA template
TERT	telomerase reverse transcriptase
TLP	thymus LIM protein
TMEPAI	transmembrane TGF-beta-inducible protein
TNFα	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
UV(R)	ultraviolet (radiation)
VASP	vasodilator-stimulated phosphoprotein
VHP	villin headpiece domain
XP	xeroderma pigmentosum
ZO	zona occludens

REVIEW OF THE LITERATURE

CANCER REPRESENTS DEREGULATION OF NORMAL CELLULAR FUNCTIONS

Cancer is one of the prevalent causes of deaths in western countries and is a disease of deregulated cell growth. Tumours acquire traits that have been called hallmarks of cancer. These include sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastases. Underlying these hallmarks is the genomic instability that enables the tumors to acquire these properties (Hanahan and Weinberg, 2011).

Same genes that are required to sustain normal cell homeostasis such as cell proliferation, death, angiogenesis and migration, are mutated and dysfunctional in cancers. For example the alterations in genes functioning in DNA damage recognition and repair may lead to the survival of cells with genomic alterations and therefore increase the chance of genomic instability and cancer development (Kastan and Bartek, 2004). Genes affected in tumorigenesis have frequently properties of oncogenes or tumor suppressors. Oncogenes are mutated, expressed in excess or otherwise activated and they have the ability to promote malignant tumor growth, whereas tumor suppressors repress the growth or other traits of the tumors, and are commonly lost or their function is attenuated in cancers.

CELLULAR STRESS

Various intrinsic and external factors are constantly attacking the cells causing damage to DNA and to other cellular structures, and cells in turn have evolved with different kinds of mechanisms to protect against the attacks and to repair the damage. Cells are targets for several different types of stresses including ionizing radiation (IR), ultraviolet radiation (UVR), viruses, mechanical stress, replication errors, heat, free oxygen radicals and hypoxia.

It is important that during cell division DNA is properly replicated and divided without errors so that daughter cells carry the exact copy of DNA of the parental cell. During cell division, cascade of molecules will ensure that DNA is replicated without errors. These are called DNA damage checkpoints (**Fig. 1**). Unrepaired lesions may lead to the formation of cancer.

DNA damage checkpoints occur during the cell cycle in G1 phase in restriction point (R), S-phase and between G2/M-phases. Proteins that are involved in DNA damage checkpoints can be divided into three groups: sensors, transducers and effectors. Sensors will first recognize the damage and signal it to the checkpoint cascade. Signal transducers are often protein kinases and their function is to amplify the signal by phosphorylating downstream protein targets, which are called effector proteins. DNA damage- induced modifications of effector proteins will then lead to the arrest in cell cycle progression, as damaged cell needs to halt the cell cycle to repair the damages before cell division continues. If the damage is unrepairable, cell undergoes apoptosis (Kastan and Bartek, 2004).

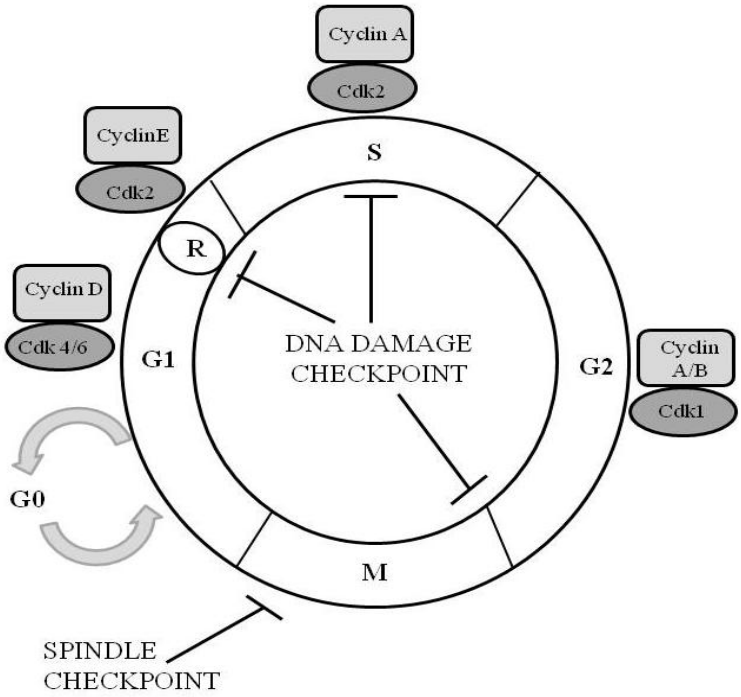


Figure 1. DNA damage checkpoints. Cell cycle consists of four phases G1, S, G2 and M phase, and is driven by different complexes formed between cyclin (box) and cyclin-dependent kinases (circles). Cells may withdraw from the cycle and enter into G0 phase. DNA damage checkpoints control the integrity in DNA in G1 (R), S and between G2/M phases and in M phase (spindle checkpoint).

Ultraviolet radiation (UVR)

The sun emits UV radiation at three different wavelengths: UVA 320-400 nm, UVB 290-320 nm and UVC 200-290 nm (Diffey, 2002). Ozone layer absorbs 97-99% of UVC, and at present 1-10% of earth reaching radiation is UVB, and over 90% is UVA. However, the proportion of the shorter wavelengths on the earth has been in increase due to the depletion of ozone layer (Norval *et al.*, 2011). UVR induces inflammation, mutations, immunosuppression, accelerated aging of the skin and skin cancers (Halliday, 2005).

UVR induced DNA damage

To exert its biological effects, UV must first be absorbed by a cellular chromophore, which transfers the energy into a biochemical signal. The most common chromophore for UVB is DNA (Kulms and Schwarz, 2000).

UVR is one of the major environmental genotoxic carcinogens that cause DNA damage and may lead to the cell death. UVR inflicts several types of lesions on DNA. The most frequent types of lesions caused by UVB and UVC are formation of cyclobutane pyrimidine dimers (CPDs), 6-4 pyrimidine pyrimidone photoproducts (6-4 PP), their Dewar valence photoisomers and DNA strand breaks (Rastogi *et al.*, 2010).

UVA exhibits a poor absorbance to DNA, and it has mainly thought to induce DNA lesions indirectly by inducing reactive oxygen species (ROS) and to result in single-strand breaks in DNA and in DNA–protein crosslinks (Brenner and Hearing, 2008). However, UVA induces CPD-type

mutations in rodents although in lesser extent than UVB or UVC (Pfeifer *et al.*, 2005). A recent publication suggests that different types of lesions show wavelength dependency and that CPDs are the principal type of lesions induced by sun light (Besaratinia *et al.*, 2011) (**Fig. 2**). However, UVA penetrates the skin deeper than UVB or UVC (Narayanan *et al.*, 2010).

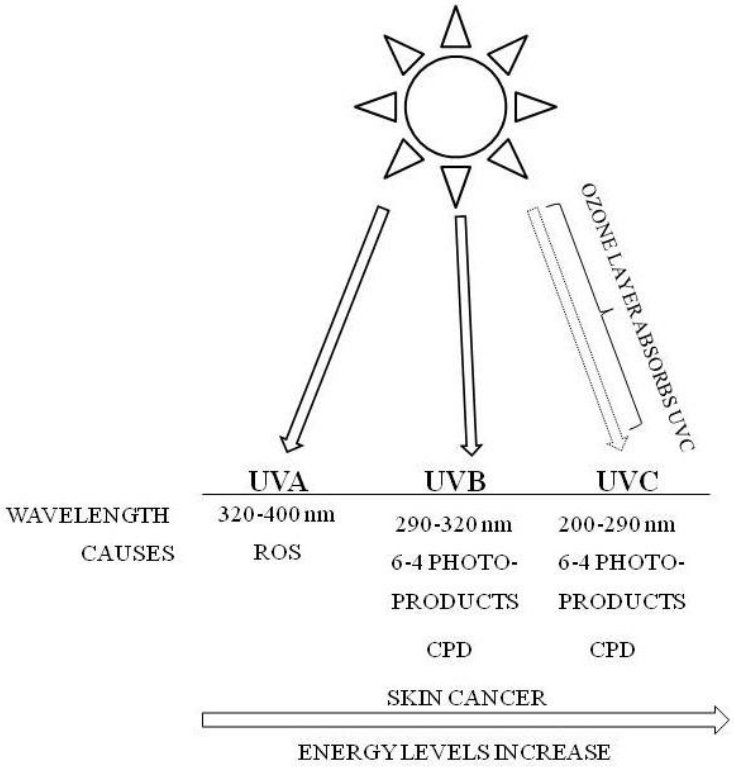


Figure 2. Sun emits ultraviolet radiation at three different wavelengths UVA, UVB and UVC, and the energy levels are negatively correlated with the wavelength. Ozone layer absorbs most of the UVC, and as UVA does not cause directly DNA damage, UVB is the most potent inducer of skin cancer. UVA mainly induces the formation of reactive oxygen species, whereas UVB and UVC causes 6-4 photoproducts and CPD type of DNA lesions. CPD= cyclobutane pyrimidine dimer, ROS= reactive oxygen species

Given that UVR causes damage in DNA, cells have adapted against the attacks by repairing the lesions efficiently. By spreading the signals and orchestrating several signaling pathways, the damage is recognized within seconds, and by activation of DNA damage checkpoints, cell delays cellular processes until the lesions have been repaired (Kastan and Bartek, 2004). ATR (ATM- and Rad3-related) acts as a sensor that recognizes the UVR- induced damage in DNA. ATR is a large protein kinase that binds to ssDNA and phosphorylates critical substrates. This event will initiate the recruitment of several proteins and protein complexes to ssDNA, and will lead to the activation of damage checkpoints. One of the ATR-phosphorylated targets is CHK1, which functions as a transducer of the damage signal by phosphorylating effector protein targets downstream (Bartek and Lukas, 2003). CHK1 targets CDC25A phosphatase (activator of the cyclin E/(A)/CDK2 kinase) for proteasomal degradation, which rapidly delays G1/S transition (Mailand *et al.*, 2000). The phosphorylation of transcription factor p53 is required for sustained G1 arrest by activating CDK inhibitor $p21^{CIP1/WAF1}$. p53 is a key factor in UV-induced cellular stress response, as it halts cell cycle progression until damage has been repaired or provokes the cells to undergo apoptosis in case the damage is too severe to be repaired. *TP53* is one the most commonly mutated genes in human cancers (Kastan and Bartek, 2004). Furthermore, altogether 570 phosphorylated protein targets for UV-induced ATM/ATR have been identified (Stokes *et al.*, 2007).

Different mechanisms have evolved to repair DNA lesions caused by UVR. These include photodamage reversing enzymes (photolyases), base excision repair, nucleotide excision repair (NER) and mismatch repair pathways. Since UVR mainly induces pyrimidine dimer-type of mutations, NER is the main repair system in UVR-induced DNA damages (Rastogi *et al.*, 2010). NER involves damage recognition, local opening of the dsDNA around the lesion, dual incision of the damaged DNA strand, gap repair synthesis, and ligation of the strand. Human genetic disorder Xeroderma pigmentosum (XP) is a disease with dysfunctional NER, and the carriers of the XP mutations are hypersensitive to UV radiation. Seven *XP* (*XPA-XPG*) genes required for NER mediated DNA repair are defective in XP patients and therefore they lack the ability to repair DNA lesions through NER. Hence, they are highly susceptible to all three types of skin cancers (Dworaczek and Xiao, 2007).

UVR also causes damage on *TP53* gene (Brash *et al.*, 1991), and five of the eight *TP53* hot spot mutations carry mutated dipyrimidine nucleotides. These mutations are frequently observed in non-melanoma skin cancers, and are particularly common also in XP skin tumors.

UVR induced damage in the cell

DNA is not the only chromophore for UVB. UVB chromophores also include purine and pyrimidine bases in RNA, protein aromatic amino acids (tryptophan and tyrosine), urocanic acid, melanin, quinones, flavins, 7-dehydrocholesterol, porphyrins and heme groups in catalase and oxidase enzymes (Trautinger, 2001). In addition to damage in DNA, UVR also causes injuries in cell organelles and actin cytoskeleton reorganization. Organelles such as plasma membrane, intercellular contacts, nuclear membranes, Golgi-complex, endoplasmic reticulum, lysosomes, cytoskeleton and mitochondria have been reported to suffer from radiation induced injuries (Somosy, 2000). Moreover, irradiated mouse lymphoma cells revealed that UVB radiation damaged cell structures, resulting in the disappearance of microvilli on the cell surface, destruction of mitochondria and vacuolation of cytoplasm (Maekawa *et al.*, 1996).

In addition, cell morphology and polymerization of filamentous actin are altered (Maekawa *et al.*, 1996). UV-irradiated Chinese hamster ovary cells showed impaired adherence, disruption of actin filaments and stronger F-actin labeling in the center of the cell (Grzanka *et al.*, 2006). Moreover, disruption of the cytoskeleton enhances UV-induced apoptosis (Kulms *et al.*, 2002). Actin cytoskeletal proteins have also been reported to be degraded in response to UVR, as actin mRNA is reduced (Weinreb *et al.*, 2001), and the phosphorylated forms of actin cytoskeleton modulators cofilin-1 and destrin are decreased (Hensbergen *et al.*, 2005).

Cellular responses to UV- induced damage

UVR induces different types of damage to the cells, and the cells may respond either by activating cell death or survival pathways. Severity of the damage is highly dependent on the dose of the UVR, as lower doses will mostly lead to cell cycle arrest during which lesions are repaired and higher doses will lead to apoptosis (Latonen *et al.*, 2001; Gentile *et al.*, 2003).

Several signal transduction pathways are activated in response to UVR. Transcriptional responses have been studied in different cell lines, and the responses vary between the cell types. Responses also show wavelength dependency, as different wavelengths induce different types of DNA lesions and cellular stress responses (Bode and Dong, 2003). Divergent transcriptional responses were detected with doses of UVC either inducing growth arrest or apoptosis in a human skin fibroblast cell line (WS-1) (Gentile *et al.*, 2003). Furthermore, UVB induced expression profiles have been studied in human keratinocytes (Sesto *et al.*, 2002) and in melanocytes (Valery *et al.*, 2001). In

keratinocytes, among the induced transcripts are related to UV-specific inflammatory and stress responses, basal transcription, splicing, translation and to proteasome-mediated degradation. On the other hand, transcripts related to the metabolism and to adhesion were downregulated (Sesto *et al.*, 2002). In melanocytes, among the induced transcripts are genes mostly involved in DNA or RNA binding, synthesis and modification and also ribosomal proteins, transcription factors, receptors, tumor suppressors and oncogenes (Valery *et al.*, 2001).

Apoptosis of the keratinocytes is the major protective mechanism against damage in the skin in response to UV radiation. Through apoptosis the skin will eliminate severely damaged cells and decrease the risk of malignant transformation. Various pathways are involved in UV-induced apoptosis in response to DNA damage: activation of p53, cell death receptors either directly by UV or by death ligands or via mitochondrial damage, and the release of cytochrome C (Kulms and Schwarz, 2000) (**Fig. 3**).

Death receptors are cell surface receptors that transmit cell death signals initiated by binding of their ligands such as Fas, TNFα or TRAIL. Complex formation between death ligands and their receptors leads to a receptor conformational change and triggers the imminent initiation of apoptosis through activation of caspase proteases 8 and 10. This is called "extrinsic" apoptosis. "Intrinsic" apoptosis is initiated by caspase 9 whose activation depends on the release of cytochrome *c* and other pro-apoptotic factors from the mitochondria with the help of the pro-apoptotic proteins like Bid and Bax. Pro-survival protein Bcl-2 antagonizes these effects by inhibiting the release of cytochrome C from mitochondria (van Raam and Salvesen, 2011). Interestingly, UVB and UVC induce different apoptotic pathways in human keratinocytes although the extent of apoptosis is comparable between these wavelengths (Takasawa *et al.*, 2005). 6-4 PPs and CPDs are significantly more frequent following UVC radiation than UVB radiation. Also, the release of cytochrome *c* and Smac/DIABLO and activation of caspase 9 following UVC are greater than after UVB. Importantly, caspase 8 activation occurred only in UVB-radiated cells. Thus, UVB induces apoptosis through both mitochondrial (intrinsic) and caspase 8 activation (extrinsic) pathways, while UVC induces apoptosis only via the intrinsic pathway (Takasawa *et al.*, 2005).

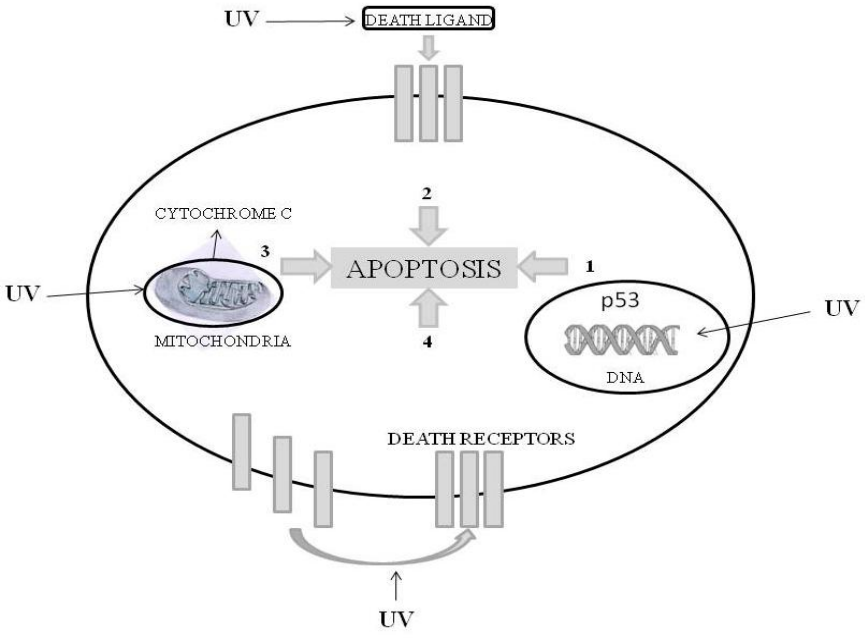


Figure 3. UV-induced cellular pathways. (1) UV causes DNA damage, which activates, among other factors, "the guardian of the genome" p53. (2) UV triggers the binding of death ligands to their receptors. This leads to activation of caspases. (3) UV may also induce the release of cytochrome *c* from mitochondria, and lead to caspase-dependent apoptosis. (4) UV may also induce apoptosis by causing the clustering of the death receptors even in the absence death ligands. Modified from Kulms and Schwarz, 2000.

Several proteins have been shown to promote either death or survival of the cells. The induction of apoptosis by UV radiation involves the activation of protein kinase C (PKC)-pathways. Cleavage and the activation of PKCδ are important upon UV-induced apoptosis in human keratinocytes, since the inactivation of PKCδ promotes the survival of keratinocytes exposed to UV radiation (D'Costa and Denning, 2005). Furthermore, PKCε acts as a photosensitizer, as transgenic mice overexpressing PKCε in the basal epidermal cells and cells of the hair follicle are highly sensitive to UVR-induced cutaneous damage and to development of SCC (Aziz *et al.*, 2007).

Several anti-apoptotic pathways counteract the action of apoptotic pathways, and the end result depends on the balance between these two. Akt/protein kinase B (PKB) pathway protects keratinocytes from UVR, and in contrast, PKC family is involved in keratinocyte death pathways. Activation of PKCδ and PKCε negatively regulate Akt phosphorylation and kinase activity in mouse keratinocytes and modify UVC-induced apoptosis in mouse keratinocytes (Li *et al.*, 2006).

UV-activated signal transduction pathways are primarily mediated by mitogen-activated-protein-kinases (MAPKs), that include extracellular signal regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 kinases. UV-induction of MAPKs is highly dependent on wavelength, dose and cell type used (Bode and Dong, 2003), and both survival and apoptotic functions in UVR- induced cell stress have been suggested for p38. A major effector of the MAP kinase pathways is transcription factor AP-1, which is composed of Jun and Fos family proteins. Transcription of *JUN* and *FOS* genes are induced by a variety of growth factors, cytokines, and environmental stimuli, including UVR (Rittie and Fisher, 2002).

STRUCTURE OF THE SKIN

Skin is composed of three layers: epidermis, dermis and hypodermis. Epidermis is the outermost layer consisting of keratinocytes (95%), melanocytes, Langerhans cells and Merkel cells. The primary function of keratinocytes is to protect the skin against UV irradiation, pathogens, evaporation and heat. Epidermis is composed of five sublayers; stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The epidermis of the skin is under constant renewal, given that undifferentiated basal cells in the stratum basale of the epidermis are continuously dividing, and the daughter cells are migrating towards the upper sublayers of the epidermis while differentiating into keratinocytes and producing keratin. Finally they start to undergo apoptosis and dead cells are then detached from skin surface during a process called cornification. Stratum corneum is then the outermost layer composed of large, flat, polyhedral dead cells filled with keratin, and this layer is the most important for inhibition of water evaporation.

Dermis is connected to epidermis by basal membrane, and consists of two layers; papillary and reticular region. There are different kinds of specialized organelles in dermis such as hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. Collagen, reticular and elastin fibers in this layer provides the skin elasticity, strength and flexibility.

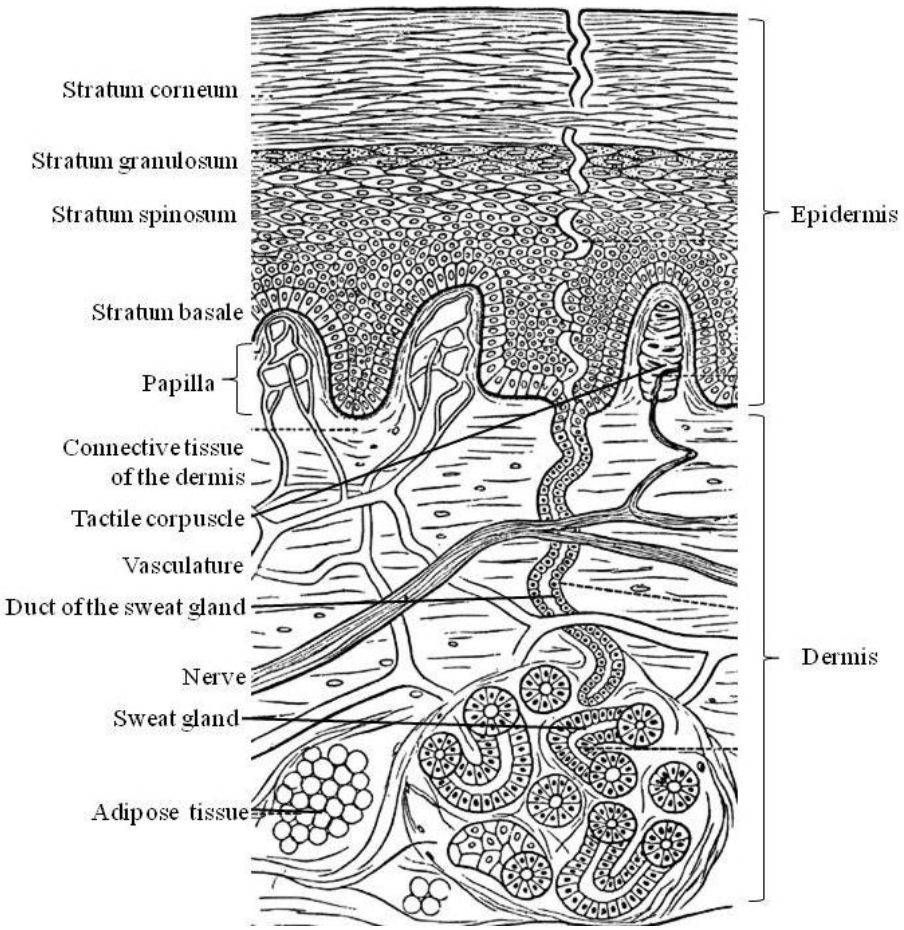


Figure 4. The structure of the skin. Two layers of the skin are represented: epidermis and dermis. Epidermis is mostly composed of keratinocytes in several layers: stratum corneum, stratum granulosum, stratum lucidum, stratum spinosum and stratum basale. Dermis on the other hand is composed of mainly connective tissue, and has several structures, such as sweat gland, nerves, glandula sepacea (not shown in this picture) and tactile corpuscle. Adapted from Florida Center for Instructional Technology.

UV-RELATED SKIN DISEASES

Actinic keratosis

Actinic keratoses (AK) is featured as premalignant lesion on sun-exposed areas of the skin such as the face, lower lip, bald scalp, neck, arms and hand. AK is induced especially by UVB and it is more common in fair skinned Caucasian population. AK lesions are considered as premalignant, since approximately 10% of these lesions will develop into cutaneous squamous cell carcinoma (SCC) with a progression time of approximately 2 years (Fuchs and Marmur, 2007).

Several mutated areas in human genome have been linked with the formation of AK. Mutations in *TP53* are extremely common in cases of AK, as 75-80 % of Caucasian population, 40 % of Korean and 30 % of Japanese will display *TP53* mutations (Park *et al.*, 1996). This suggests that p53 function is needed to protect the cells from UV-induced damage. The expression levels of epidermal p53 were compared with AK, adjacent tissue and non-sun exposed tissue. p53 levels were significantly elevated in AK and adjacent tissue areas suggesting an association with histological evidence of chronic sun damage (Einspahr *et al.*, 1997). Moreover, exposure of human

skin transplants to UVB lead to the induction of AK in 14 and SCC in 3 cases out of 18 in immunodeficient mice (Nomura *et al.*, 1997). Since mutations in *TP53* induced by UV radiation are frequently found (Brash *et al.*, 1991), they may allow a selective pressure for the transformed, damage-resistant keratinocytes, allowing these cells to clonally expand, form AK, and predispose to skin cancer (Ziegler *et al.*, 1994).

Cutaneous squamous cell carcinoma

UVR induced damage contributes to formation of all types of skin cancers including basal cell carcinoma (BCC), cutaneous squamous cell carcinoma (SCC) and cutaneous melanoma. BCC as well as SCCs are originated from keratinocytes, whereas melanomas originate from melanocytes. BCC is the most common type of skin cancers, but usually resides more local and slowly growing than the second most common skin carcinoma SCC. It has been estimated that 2-3 million new cases of BCC and SCC arise each year (Narayanan *et al.*, 2010). It has also been estimated that about 65 % of the SCC cases are initially AKs (Criscione *et al.*, 2009). Whereas AK is located in epidermis, SCC extends more deeply into the dermis.

Consistent epidemiological evidence for a positive association between UV light exposure and SCC risk has been found in 16 of 18 studies (Schmitt *et al.*, 2011). Intact p53 is needed to protect the cells from UV-induced damage, and alterations in this gene may cause the formation of skin carcinomas. Similarly, all *Tp53* knock out mice with chronic exposure to UVB radiation develop skin cancer (Li *et al.*, 1998). After 12 weeks of observation five out of ten mice had invasive SCC, four SCC *in situ* and one AK, whereas none of the mice with wild type *Tp53* developed skin cancer (Li *et al.*, 1998). In humans, *TP53* is also mutated in SCC, and about 90% of the invasive SCC cases carry a mutation in *TP53* (Rass and Reichrath, 2008). Furthermore, both UVA and UVB induced skin carcinoma cases have mutated *TP53*, but the prevalence is fairly different. As about 60% of the UVB induced skin carcinomas cases had a point mutation in their *Tp53* gene in mouse model, only 14% of the UVA induced cases had mutated *Tp53*, suggesting that other gene targets are more important in protecting the cells against UVA (van Kranen *et al.*, 1997).

Immunosuppressed patients develop more aggressive and more numerous SCCs than immunocompetent individuals (Bordea *et al.*, 2004). Furthermore, *XPC* gene required for efficient UVR-induced damage repair is consequently inactivated or lost in almost half of SCCs in non-XPC patients, suggesting that loss or mutation of *XPC* may be an early event during skin carcinogenesis that provides a selective advantage for initiation and progression of SCC (de Feraudy *et al.*, 2010).

TRANSFORMING GROWTH FACTOR β (TGFβ)

TGFβ belongs to a superfamily consisting of over 40 related proteins including TGFβ isoforms, bone morphogenic protein (BMPs), growth and differentiation factors (GDFs), anti-Müllerian hormone (AMH), activin, inhibins and nodal. TGFβ is a pleiotropic cytokine ubiquitously expressed by all cells and tissues in the body and essential for embryogenesis and maintaining the tissue homeostasis. To fulfill these important tasks, TGFβ signaling pathway regulates growth, apoptosis, differentiation, adhesion, invasion and extracellular matrix production of the cell (Meulmeester and Ten Dijke, 2011). The effect of TGFβ is context dependent; in epithelial cells it may act as a growth inhibitor, whereas in fibroblasts it may stimulate the growth. Dysregulation of TGFβ signaling pathway is involved in pathological disorders like cancer, fibrosis, cardiovascular and autoimmune diseases (Rahimi and Leof, 2007).

Mature TGFβ ligands (1, 2, 3) are 25 kDa proteins comprising of two polypeptides. TGFβ is

initially synthesized as a 55 kDa precursor molecule, which is dimerized and proteolytically cleaved to yield N-terminal propeptide and mature TGFβ. A small latent TGFβ complex is then formed between a propeptide called as a latent associated protein (LAP) and mature TGFβ. Usually this small latent TGFβ binds to latent TGFβ binding protein (LTBP) forming a large latent TGFβ complex, which facilitates the secretion of the complex and directs it to the extracellular matrix. Activation of the TGFβ is tightly controlled, and therefore it is produced and secreted as a latent complex preventing the ligand from activation unless certain physiological conditions occur or the target cell is reached. Several enzymes or other proteins, such as plasmin, thrombospondin-1, integrins, matrix metalloproteinases and calpains release TGFβ from latent complexes (Hyytiainen *et al.*, 2004).

TGFβ signaling pathway

TGFβ ligands are first recognized and bound to the TGFβ type II receptor. Binding of the ligand causes the activation of intracellular serine/threonine kinase domain of type II receptor, which phosphorylates the cytoplasmic tail of type I receptor and activates its serine/threonine kinase domain. The activated heterotetrameric receptor complex regulates the downstream of Smad and non-Smad pathways (Ikushima and Miyazono, 2010) (**Fig. 5**). Betaglycan (type III receptor) is TGFβ co-receptor that binds to TGFβ ligands (Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991).

The Smad family consists of eight proteins: Smad 2, 3, 4, 7 are regulated by TGFβ, whereas Smad 1, 5, 6, 8 are involved in BMP pathway. The Smads can be classified into three different fundamental groups; receptor activated- (R-), co- activated- (Co-) and inhibitory (I-) Smads. In TGFβ-signaling pathway Smad2 and Smad3 are receptor activated, Smad4 is co-activated and Smad7 has an inhibitory role. The Smad complex that is able to bind chromatin is a trimer consisting of two R-Smads and one Co-Smad. The binding of TGFβ-ligand on the receptor will ultimately lead to the phosphorylation of receptor-regulated Smads (2, 3) by type I TGFβ-receptor, after which they form heteromeric complex with Smad4, translocate into the nucleus and regulate large transcriptional machinery (Ikushima and Miyazono, 2010).

TGFβ regulates the expression of several target genes. To suppress the cell proliferation, TGFβ represses the expression of Myc and vice versa induces the expression of p15 and p21. Moreover, to enhance the differentiation, TGFβ represses Id1, Id2 and Id3 and to promote apoptosis TGFβ regulates the expression of Bcl-2 family members (Rahimi and Leof, 2007).

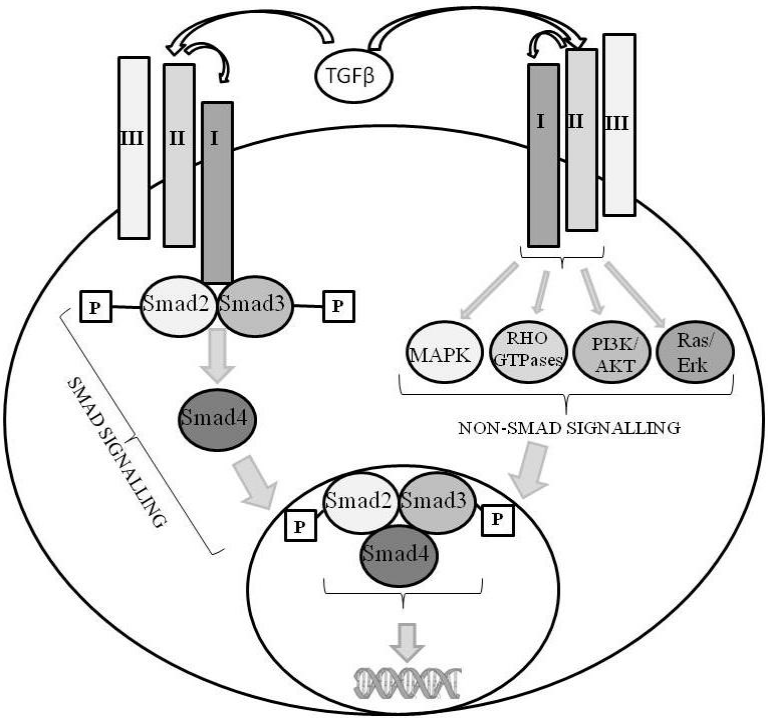


Figure 5. TGFβ-mediated signaling pathways: Smad-signaling and non-Smad signaling pathways. TGFβ activates type II receptor, which in turn phosphorylates type I receptor. Receptor I phosphorylates Smad2/3 which then forms a complex with Smad4 and translocates into the nucleus to regulate transcription. In a non-conventional pathway, the TGFβ-signaling is not mediated through Smads, but receptors I and II directly transduce signals to MAPK, Rho GTPases, PI3K/Akt and Ras/Erk pathways. Crosstalk between the signaling pathways may occur, for example Smads may also activate Rho.

Given that TGFβ has such diverse effects in the cell, it is crucial that the activation is strictly regulated in various ways, including the control of intensity and duration of Smad-signaling. The phoshorylation of receptors play a crucial role in signal activation, and therefore dephosphorylation plays an important role in deactivation of the receptors. Inhibitory Smad7 is transcriptionally induced upon TGFβ-signaling, and represses TGFβ-signaling by competing receptor binding with R-Smads (Yan and Chen, 2011). Furthermore, several factors have been found to positively or negatively regulate the presentation and sequestration of R-Smads to TGFβ-receptor. Smad anchor for receptor activation (SARA) is one of the positive regulators that interacts directly with Smad2/3, facilitates their recruitment to the type I receptor and modifies the balance between Smad2 and Smad3 (Tsukazaki *et al.*, 1998). On the other hand, TMEPAI negatively regulates R-Smad phosphorylation by type I receptor by competing with binding to R-Smads with SARA (Watanabe *et al.*, 2010). Phosphorylation of the Smads is a transient event, and several phosphatases are able to reverse the phosphorylation. PPM1A was found to dephosphorylate and promote the nuclear export of Smad2/3 (Lin *et al.*, 2006), and SCP1, 2, 3 dephosphorylate Smad2/3 at the N-terminus, but not in C-terminus resulting in enhancement of TGFβ signaling (Sapkota *et al.*, 2006).

TGFβ signaling is also regulated through proteasomal degradation of Smads and receptors, and several specific E3 ligases have been identified to target different factors of the signaling cascade. Smad ubiquitin regulator factor 1 and 2 (Smurf1, 2) are E3 ligases that not only target R-Smad and type I receptor for degradation, but also facilitate the inhibitory actions of I-Smads. Smurf2 promotes the degradation of Smad2 (Lin *et al.*, 2000), and both Smurf1 and 2 bind to Smad7 and target type I receptor for degradation (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001). WWP1 and

NEDD4-2 both interact with type I receptor via Smad7 (Komuro *et al.*, 2004; Kuratomi *et al.*, 2005), respectively, and participate in degradation of the receptor. Arkadia is another E3 ligase that targets Smad7 for ubiquitination and degradation, and is therefore an important factor amplifying TGFβ signaling (Koinuma *et al.*, 2003). Also deubiquitinases for Smads exists. One of them is FAM/USP9x that deubiquitinates Smad4, which is monoubiquitinated at lysine 519 thus preventing the interaction with phosphorylated Smad2. As FAM/USP9x is able to revert this ubiquitination, it is able to retain active TGFβ signaling (Dupont *et al.*, 2009).

Ski and SnoN are the members of the Ski family, and important negative regulators of TGFβ-pathway at the transcriptional level. Ski and SnoN regulate TGFβ-pathway by interacting with Smad2/3 and Smad4 and by blocking the ability of the Smad complexes to activate transcription of TGFβ target genes. In addition, Ski or SnoN also prevent the binding of the R-Smads to transcriptional co-activator p300/CBP and actively recruit a transcriptional co-repressor complex containing N-CoR and HDAC to the targeted promoters (Deheuninck and Luo, 2009).

Non-Smad pathways

Besides the canonical Smad-pathway, the functional receptor complex also activates so-called non-Smad mediated pathways. These include MAP kinases (ERK, JNK, p38), Rho-like GTPase signaling and phosphatidylinositol-3-kinase/AKT pathways, which are especially relevant in epithelial to mesenchymal transition (EMT) (Zhang, 2009).

The best characterized non-Smad mediators are JNK and p38 MAPKs, which are rapidly phosphorylated in response to TGFβ signaling by MAP kinase kinases, MEKK4 and MEKK3/6, respectively. Upstream, MEKKs are regulated by TGFβ-activated kinase 1 (TAK1), which is in turn regulated by TRAF6. Smads are not essential for the activation of JNK/p38 pathway, indicating that JNK/p38 are directly regulated by type I and type II receptors. TRAF6-TAK1-MEKK-JNK-p38 pathways play an important role in TGFβ-induced apoptosis and EMT (Zhang, 2009).

TGFβ induces major changes in the cell morphology and actin cytoskeleton during EMT. These are mediated by Smad-dependent and Smad-independent activation of Rho GTPases. Moreover, there seems to be a difference in long-term and short-term induction of EMT. RhoGTPases are first activated directly by type I and type II receptors, but later also activation of Smads seems to be needed. Smad2/3 trigger the activation of RhoA and RhoB GTPases and long-term actin reorganization (Vardouli *et al.*, 2008). However, whereas RhoA is required for TGFβ-induced EMT, Smad signalling is not (Bhowmick *et al.*, 2001).

TGFβ in cancer progression

TGFβ has a dual role in cancer; it acts as a tumor suppressor during the early stages of tumor development by inhibiting growth of endothelial and epithelial cells and by inducing apoptosis and terminal differentiation. In later stages of tumorigenesis TGFβ promotes cell motility and tumor metastases through EMT, suppresses immune system and induces angiogenesis. Tumors may acquire the ability to bypass the growth inhibiting properties of TGFβ, and exploit certain properties to actively promote tumor progression (Derynck and Akhurst, 2007). Different types of tumors such as gliomas, breast and prostate cancer seems to acquire preferential resistance to TGFβ-induced growth arrest, but retain the ability of TGFβ-induced EMT, metastatic dissemination and suppression of the immune system. Tumors with such signature are highly aggressive (Meulmeester and Ten Dijke, 2011).

As the TGFβ signaling cascade is a large network consisting of hundreds of factors, mutations in any of the genes along the pathway may have a severe impact on TGFβ signaling. As TGFβ also promotes tumorigenesis, increased expression by tumor cells correlates with metastatic lesions of breast, colon and prostate cancer progression (Meulmeester and Ten Dijke, 2011). TGFβ type II receptor and Smad4 are frequently inactivated by mutation or by loss of heterozygosity (LOH) in several types of carcinomas. *TGFBR2* is especially affected in gastric tumors, gliomas and colorectal cancer, and *SMAD4* in pancreatic, colorectal and head and neck cancers, suggesting that they are tumor suppressors (Levy and Hill, 2006; Meulmeester and Ten Dijke, 2011). In contrast, the inhibitory factors like Smad7, Smurf1/2 and SnoN/Ski are often overexpressed (Luo, 2004) in various carcinomas (Levy and Hill, 2006). High levels of Smurf2 inversely correlate to Smad2 levels with higher invasion rate, lymph node metastases and with poor prognosis of esophageal SCC patients (Fukuchi *et al.*, 2002). Smad7 was found to be more frequently lost than amplified in colorectal cancer, and loss of Smad7 was thought to sensitize the cells to tumor suppressor activity of TGFβ, whereas its amplification is thought to have an adverse effect. Interestingly, patients with deleted *SMAD7* gene have better prognosis than patients with amplified *SMAD7*, implicating that resistance to TGFβ-mediated apoptosis is important in colorectal tumorigenesis (Boulay *et al.*, 2003). Ski/SnoN are thought to act as a oncogenes, as their overexpression transforms cells, and they are found overexpressed in several cancer cell lines (Luo, 2004). Moreover, in breast carcinoma high SnoN levels together with ERα+ -signature correlate with poor prognosis (Zhang *et al.*, 2003). Betaglycan itself represses proliferation, migration and adhesion (Lambert *et al.*, 2011), and the loss of betaglycan in several types of tumors supports its role as a tumor suppressor (Gatza *et al.*, 2010).

Although carcinomas are originated from adjacent epithelial tissue, the tumor surrounding tissue, called tumor microenvironment, including ECM, fibroblasts, immune cells (macrophages, lymphocytes, mast cells) and vasculature (endothelial cells, pericytes and smooth muscle cells) largely affects the tumorigenesis of the epithelial cells (Bhowmick *et al.*, 2004). TGFβ is one of the paracrine factors that mediate the interaction between the tumor and the host tissue. TGFβ regulates non-cell-autonomous signaling, epithelial-stromal interactions, immune system evasion and angiogenesis in the tumor microenviroment (Bhowmick *et al.*, 2004). TGFβ also regulates angiogenesis as high levels of TGFβ correlate with high level of vasculature in prostate cancer (Wikstrom *et al.*, 1998). Also, high levels of TGFβ in plasma positively correlate with tumor vascularity in hepatocellular carcinoma (Ito *et al.*, 1995).

As excess TGFβ promotes tumor progression and apparently plays a major role in tumorigenesis of different types of tumors, several approaches have been taken to target and block TGFβ-signaling. These include: blocking the synthesis of TGFβ by antisense molecules, ligand traps (antibodies and soluble receptors) and small molecule inhibitors (Meulmeester and Ten Dijke, 2011). Antisense molecules have been developed against TGFβ-ligand and type II receptor, ligand traps include monoclonal antibodies and soluble type II and III receptor molecules, whereas small molecule compounds target the receptor kinase activities. One of the compounds, SB431542, (used in study III) blocks totally TGFβ-signaling by preventing Smad phosphorylation by type I receptor. Currently, several of these are under investigation in clinical trials, but although promising results exist, concerns in inhibiting TGFβ-signaling have arisen due to its role as a tumor suppressor (Meulmeester and Ten Dijke, 2011).

TGFβ in epithelial-mesenchymal transdifferentiation

Epithelial cells act as a protective barrier that line both the outer (skin) and inner (alveoli in lungs, gastrointestine) body compartments, and also function in secretory and glandular tissues. Epithelial

tissue has several functions depending on the organ where they are located ranging from nutrient absorption in intestines to gaseous exchange in lungs or lactation in mammary gland. Epithelial cells are under constant repair and renewal, and about 90% of the cancers are of epithelial origin (McCaffrey *et al.* 2011).

Epithelial cells are immotile and polar cells, and are characterized by strong adhesions between neighboring cells. These cell-cell contacts include tight and adherens junctions, which are connected to the circumferential actin belt. A key component of the adherens junctions is E-cadherin, which forms homophilic trans-interactions at sites of cell-cell contacts in the presence of Ca^{2+}. This is vital for initiating and maintaining epithelial architecture *in vitro* and *in vivo* (Yilmaz and Christofori, 2009). E-cadherin is linked to the underlying actin cytoskeleton through α and β catenins.

During epithelial-mesenchymal transdifferentiation (EMT), epithelial junctions are disintegrated and cells become more motile and apolar fibroblast-cell like. EMT is a normal process during embryo development and wound healing, but occurs also during pathological conditions like carcinogenesis and fibrotic diseases. Molecular characteristics of EMT are loss of epithelial markers such as E-cadherin, zona occludens-1 (ZO-1), and the increase in mesenchymal markers like N-cadherin, α smooth muscle actin (αSMA), matrix metalloproteinases and ECM components including collagen and fibronectin (Rahimi and Leof, 2007).

TGFβ plays an important role in EMT (Xu et al., 2009). TGFβ regulate the expression of well known EMT- inducer transcription factor families Snail, ZEB and bHLH (Xu et al., 2009; Micalizzi *et al.*, 2010). In addition to the canonical TGFβ-signaling pathway, also non-canonical TGFβ-induced signalling pathways induce EMT. Rho family of Small GTPases is important for cell adhesion, morphology and migration. RhoA promotes TGFβ-induced EMT in mammary epithelial cells (MEC), and inhibition of RhoA or its downstream effector ROCK leads to the inhibition of EMT (Bhowmick *et al.*, 2001). Furthermore, miRNAs represent post-transcriptional regulators that bind complementary sequences on target mRNA. MiR-200 and miR-205 were found repressed during TGFβ-induced EMT. These miRNAs negatively regulate expression of E-cadherin transcriptional repressors ZEB1 and ZEB2. Expression of these miRNAs were found diminished in invasive breast cancer cell lines with mesenchymal properties implicating that downregulation of the miRNAs may be an important step during tumorigenesis (Gregory *et al.*, 2008).

Myofibroblasts in pathological conditions (in fibrotic diseases and in tumor progression)

Myofibroblasts are cells with features of both fibroblasts and smooth muscle cells. Hallmarks of the myofibroblasts are extracellular matrix protein production, development of supermature focal adhesions and formation of contractile bundles composed of actin and myosin (Hinz *et al.*, 2007). Myofibroblasts may differentiate from several origins; mesenchymal cells such as fibroblasts, hepatic stellate or smooth muscle cells, epithelial or endothelial cells through EMT, bone marrow cells or fibrocytes. Cells first differentiate transiently into promyofibroblast and then fully maturate into myofibroblasts that are characterized by *de novo* expression of αSMA and of fibronectin splice variant ED-A (Hinz *et al.*, 2007).

TGFβ is an inducer of myofibroblast transdifferentiation as it increases the transdifferentiation of fibroblasts to myofibroblasts *in vitro* and *in vivo* (Desmouliere *et al.*, 1993; Sime *et al.*, 1997), respectively. TGFβ also promotes the myofibroblast differentiation of hepatic stellate cells (Gressner *et al.*, 2002) and causes myofibroblast differentiation through EMT (Zavadil and Bottinger, 2005). TGFβ regulates αSMA expression directly via Smad3 (Hu *et al.*, 2003), and the regulation is mediated by two CArG elements (Hautmann *et al.*, 1997). αSMA has further been

shown to increase the contractility of the fibroblasts, and it is much more potent inducer of the contractility than cardiac α or γ-cytoplasmic actin (Hinz *et al.*, 2001).

Myofibroblasts are needed for normal wound repair. Activated myofibroblasts help the wound closure by secretion of matrix metalloproteinases, collagen and other ECM components and by contraction. After wound healing myofibroblasts normally disappear through apoptosis. However, repair process may become pathogenic, if they are not appropriately controlled. In fibrotic lesions activated myofibroblasts persist, and are responsible for excessive production of ECM that alters the tissue architecture leading to the organ failure (Hinz *et al.*, 2007). Chronic inflammation and dysfunctional repair can trigger the excessive accumulation of ECM and lead to the formation of a permanent fibrotic scar (Wynn, 2008).

Fibrosis is a pathological condition that can occur in various organs, such as lung, liver, kidney and cardiovasculature, and can be characterized by overgrowth, stiffening, scarring and excess production of ECM. It is usually caused by chronic inflammatory reactions induced by various stimuli including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation and tissue injury (Wynn, 2008). TGFβ has implicated to play a major role in fibrosis formation in many tissues, including the lung (Sime *et al.*, 1997).

Myofibroblast also contributes to tumor progression (Hinz, 2010). During tumor development, tissue homeostasis between different cell types is disturbed. The invasive nature of tumor cells is an end result of interaction between epithelial cells and the tumor stroma. The tumor stroma includes cancer associated fibroblasts (CAFs), immune cells, vasculature and extracellular matrix (De Wever and Mareel, 2003). CAFs are often present in carcinomas, and they play a role in tumorigenesis by secreting paracrine growth factors and in this way promote tumor growth, angiogenesis and invasion (Rasanen and Vaheri, 2010).

Idiopathic pulmonary fibrosis (IPF)

IPF is an interstitial lung disease characterized by uncontrolled production of extracellular matrix, formation of scar tissue and disruption of normal lung architecture. IPF has a poor prognosis and the 5-year survival is only 20% (Scotton *et al.* 2007). Chronic inflammation and accumulation of myofibroblasts as fibroblastic foci is observed in the lung leading to decreased alveolar gas exchange and pulmonary volume restriction (American Thoracic Society 2000).

Usually, injury in the lung will lead to damage in epithelial or endothelial cells together with vascular leakage and edema. This will lead to the recruitment of inflammatory cells, activation of local coagulation pathways, ECM deposition and myofibroblast recruitment to promote wound contraction. To maintain the normal alveolar structure of the lung, the mesenchymal and inflammatory cells are then dismissed through apoptosis or phagocytosis. In IPF these processes are dysfunctional, and although the etiology of IPF disease is largely unknown persistent lung injury, inflammation and inefficient wound repair contribute to the disease. Furthermore, acquired or hereditary genetic alterations may predispose to IPF (Hardie *et al.*, 2010). Pulmonary surfactant SP-C is a lipoprotein complex that maintains alveolar stability during respiration and is needed for normal lung function. Several mutations have been found in SP-C coding gene *SFTPC* in individuals with family history of lung fibrosis (Hardie *et al.*, 2010). Furthermore, mutations in telomerase reverse transcriptase TERT and RNA component of telomerase TERC have also been found in IPF families. Telomere shortening confers a dramatic increase in susceptibility to IPF (Tsakiri *et al.* 2007).

A key process of repairing lung injury is the activation of TGFβ, and its uncontrolled activity contributes to IPF. An emerging concept has been that IPF is a disease of deregulated EMT

crosstalk (Coward *et al.*, 2010). IPF can be triggered by alveolar injury that leads to the activation of TGFβ and alveolar basement membrane disruption. Activated TGFβ can then lead to enhanced epithelial apoptosis and EMT, as well as fibroblast and fibrocyte differentiation into myofibroblasts. Deposition of excess extracellular matrix by the myofibroblasts may then lead to the development of IPF (Coward *et al.*, 2010). Elevated levels of TGFβ have been detected in IPF lung specimens as compared to the controls (Khalil *et al.*, 1991; Bergeron *et al.*, 2003), and the presence of TGFβ1 in the lung epithelium indicates chronic injury (Khalil *et al.*, 1996). Polymorphism in the *TGFB1* gene have been found in codons 10 and 25, and while they do not predispose to the development of IPF, they may affect the progression of the disease (Xaubet *et al.*, 2003). Moreover, specific inhibitors of the type I receptor reduced myofibroblast transformation and collagen gel contraction in a rat bleomycin-induced lung fibrosis model (Kapoun *et al.*, 2006).

As TGFβ has been linked with the formation of IPF, several genes are reported to mediate the TGFβ- induced effect. NAPDH/oxidase 4 (NOX-4) belongs to a group of enzymes that catalyze the O^2 to ROS and is required for TGFβ-induced myofibroblast differentiation, ECM production and contractility. Furthermore, silencing or pharmacological targeting of NOX-4 abrogated the formation of fibrosis in two murine models with lung injury (Hecker *et al.*, 2009). Peroxisome proliferator-activated receptor-γ (PPAR-γ) has been shown to repress TGFβ-promoted myofibroblast differentiation via Smad-independent manner by affecting two TGFβ-dependent pro-survival pathways involved in myofibroblast differentiation (Kulkarni *et al.*, 2011). MiRNAs have been associated with formation of lung fibrosis/ IPF (Pandit *et al.*, 2011). MiRNA-21 was found to be up-regulated in fibrotic bleomycin-induced mice and in IPF patients, and mediated the fibrogenic activity of TGFβ. Sequestration of miRNA-21 led to the abrogation of bleomycin-induced lung fibrosis (Liu *et al.*, 2010). Another miRNA associated with IPF formation is let-7d, which is down-regulated by TGFβ. Inhibition of Let-7d leads to the increase in mesenchymal markers such as N-cadherin 2, vimentin and αSMA, and is significantly reduced in IPF patient lungs (Pandit *et al.*, 2010).

LIM- DOMAIN PROTEINS

LIM domain was first characterized as a cysteine-rich sequence from a cDNA encoded by *Caenorhabditis elegans MEC-3* gene which is required for the specification of the mechanosensory neurons. At that time there were no similar sequences found in databases until cloning of two other genes, LIN-11 (*C. elegans* lineage protein) and Isl1 (the rat insulin gene-enhancer-binding protein), which led to the identification of a new protein domain then named as LIM after LIN-11, Isl1 and MEC-3, respectively (Kadrmas and Beckerle, 2004). Individual LIM domains were characterized by sequence $CX_2CX_{16–23}HX_2CX_2CX_2CX_{16–21}CX_2$(C/H/D) (where X denotes any amino acid) (Schmeichel and Beckerle, 1994), although human LIM domains were later noticed to show slightly a broader consensus sequence (Kadrmas and Beckerle, 2004). In general, LIM domains consist of 50-60 amino acids and form two zinc fingers, which are separated by two amino acids as shown in **Fig. 6** (Zheng and Zhao, 2007). Although zinc fingers are known as a DNA binding domains, no evidence exists for LIM domains with direct DNA-binding properties.

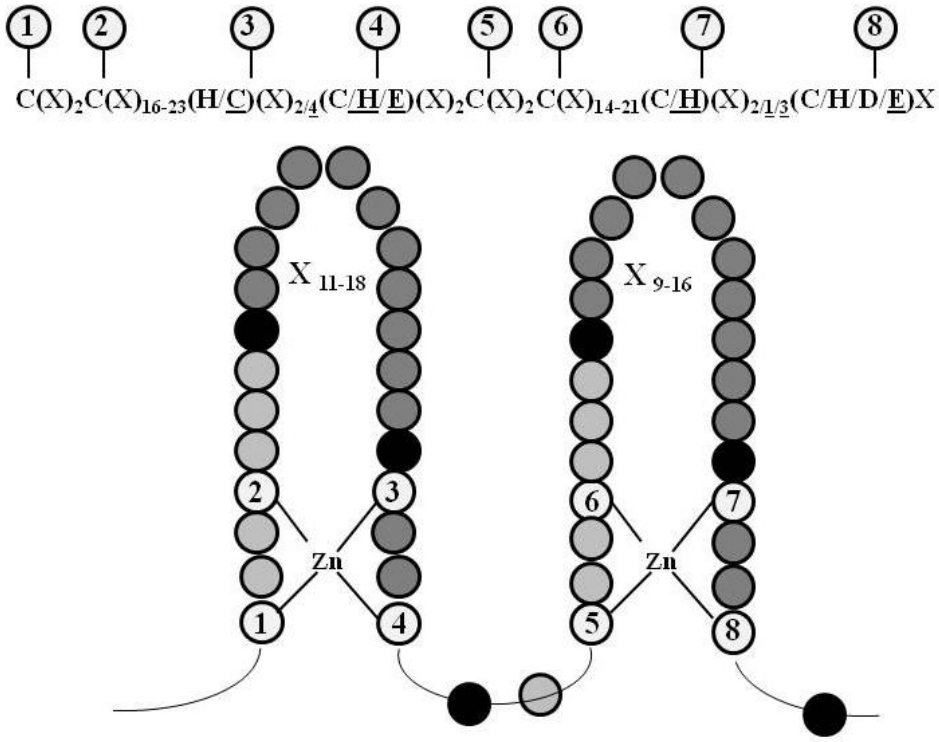


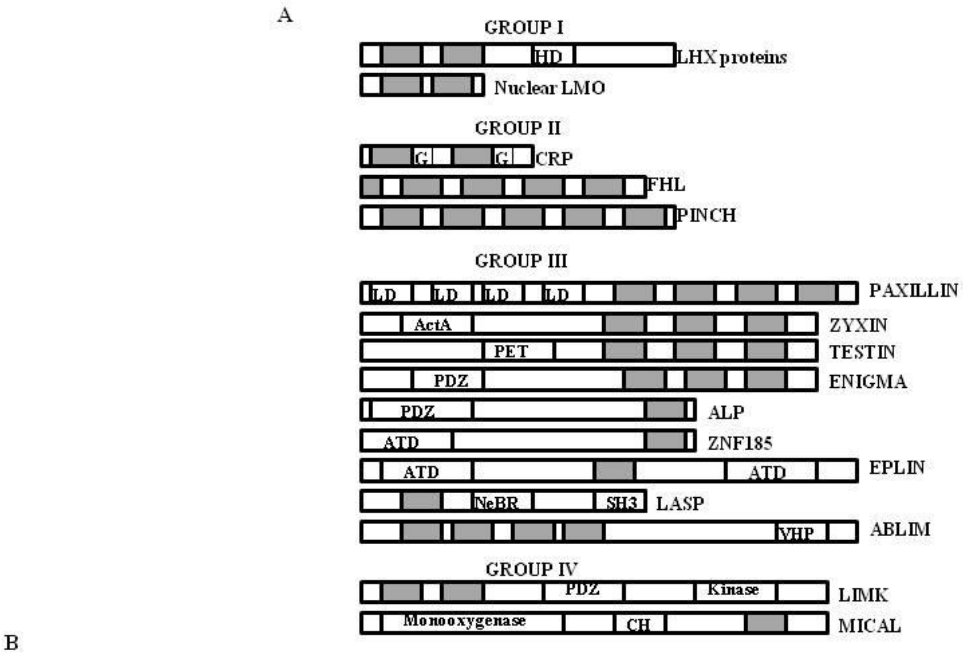
Figure 6. LIM domain. Eight zinc-binding residues (1-8) were identified and positioned based on analyzing 135 human LIM sequences. Infrequently observed amino acids (<10% of cases) are underlined and X represents any amino acid. Below the sequence, the topology of zinc coordination is shown. Numbered circles indicate the zinc-binding residues, and the semi-conserved residues are shown as black circles. Non-conserved residues are represented as grey circles. Dark grey circles indicate a variable number of residues (X) that are possible within the sequence. Modified from Kadrmas and Beckerle, 2004.

LIM domains have been found in a wide variety of eukaryotes, and 135 LIM domains have been identified within 58 genes (Kadrmas and Beckerle, 2004). Some human LIM domain containing proteins comprise only of LIM domains, whereas some proteins carry other functional domains in addition to LIM domain, including homeodomains, catalytic domains, actin cytoskeleton binding domains or other protein-binding modules such as SH3, LD or PDZ domains. LIM domain proteins have been categorized into four groups (**Fig. 7**); the first group consists of N-terminal tandem LIM domain proteins such as LHX and nuclear LMO proteins, which are found in the nucleus and act as a transcription factors or cofactors. Second group of proteins consist of LIM-only proteins that can be found both from nucleus and cytoplasm, and these protein families include CRP, FHL and PINCH. The third and the fourth groups include proteins also carrying other functional domains. The third group proteins usually have a C-terminal LIM domain in addition to various other protein-protein interaction motifs such as PDZ, LD (leucine-aspartate repeat) and ATD (actin-target domain) and these proteins include protein families Paxillin, Zyxin, Testin, Enigma, ALP, ZNF185, EPLIN, LASP and ABLIM. The fourth group of proteins carries in addition to LIM and protein-protein interaction domains also mono-oxygenase or kinase catalytic motifs, like LIMK and MICAL proteins (Zheng and Zhao, 2007).

LIM domain proteins have been identified both in cytoplasm and in nucleus. Whereas some are located solely in the nucleus and act as a transcription factors, most are associated with actin cytoskeleton in the cytoplasm. Several are able to shuttle between cytoplasm and nucleus, and extracellular stimulation, e.g. by UV, has been shown to trigger the translocation of zyxin to the nucleus (Hervy *et al.*, 2010). Furthermore, zyxin responds to cell stretching by translocating from

focal adhesions into nucleus to regulate gene expression (Wojtowicz *et al.*, 2010). Thus LIM domain proteins have been suggested to mediate signals between the nucleus and cytoplasm (Kadrmas and Beckerle, 2004).

LIM domain mediates protein-protein interactions. The proteins may operate through interacting with other proteins by acting as adaptors, competitors, autoinhibitors or localizers. Diverse roles for LIM domain proteins have been suggested, such as development, gene expression, cytoarchitecture, cell adhesion and motility, signal transduction, cell fate determination and tumor formation (Kadrmas and Beckerle, 2004; Zheng and Zhao, 2007). These activities are mediated by protein-protein interactions, and depending on their interacting protein partners a LIM domain protein may have several different functions (Zheng and Zhao, 2007).



B

GROUP I		GROUP II			GROUP III							GROUP IV	
LHX	LMO	CRP	FHL	PINCH	PAXILLIN	ZYXIN	TESTIN	ENIGMA	ALP	LASP	OTHER	MICAL	LIMK
LMX1a-b	LMO1-2,4	CRP1-3	FHL1-3	PINCH1-2	PAXILLIN	AJUBA	RILP	CYPHER	ALP	NRAP	ABLIM	MICAL-Like	LIMK1-2
ISL1-2	LMOX		ACT		HC-5	LIMD1	TESTIN	ENH	CLP-36	LASP	EPLIN	MICAL	
LHX1-9					LEUPAXIN	LPP	LMO6	ENIGMA	RIL	LIM-NEBU-LETTE	LMO7	MIRAB	
						MIGFILIN	DYXIN		MYSTI-QUE		SCIELIN		
						WTIP					ZNF185		
						TRIP6							
						ZYXIN							

Figure 7. LIM domain family proteins as grouped based on their sequence and their function. (A) Group I consists of nuclear LHX proteins and LMO (LIM only) proteins. Group II consists of CRP, FHL and PINCH proteins, which only have LIM domains, but associate with actin cytoskeleton. Group III represents proteins that have other functional domains in addition to the LIM domains. Group IV consists of proteins that have catalytic activity in addition to LIM domain. Grey boxes represent LIM domain. Other domains are LD, ActA, PDZ, ATD, NeBR, SH3 and VHP. Figure (B) represents the protein families and their members grouped from I-IV. G=glycine rich repeat. Modified from Zheng and Zhao, 2007 and Kadrmas and Beckerle, 2004.

LIM domain proteins in actin cytoskeleton

Actin cytoskeleton is required for various cellular processes, and actin rich structures include focal adhesions, lamellipodia, filopodia, actin stress fibers, the cleavage furrow and the mitotic apparatus.

Actin may be present as a monomeric G-actin or form filamentous F-actin structures (Schmidt and Hall, 1998). Several LIM domain proteins regulate actin cytoskeleton linked processes, like actin bundling, stress fiber organization, migration and adhesion.

Zyxin, a protein with five LIM domains, is a component of focal adhesions and regulates actin stress fiber reorganization and cell migration during TGFβ-induced EMT (Mori *et al.*, 2009). Zyxin plays an important role if the stress fibers are damaged, as it promotes the recruitment of α-actinin to the sites of local stress fiber damage to restore actin integrity, and α-actinin together with VASP to promote repair and stabilization of the stress fiber (Smith *et al.*, 2010). Furthermore, mechanical force may also translocate zyxin from focal adhesions to actin filaments (Yoshigi *et al.*, 2005).

RhoGTPases are the major regulators of actin cytoskeleton. Rho regulates stress fiber and focal adhesion assembly, Rac regulates the formation of lamellipodia and membrane ruffles and Cdc42 triggers filopodial extensions (Schmidt and Hall, 1998). Several LIM domain proteins are directly regulated by RhoGTPases. EPLIN displays two distinctly regulated transcripts, and EPLINα is regulated by RhoA (Chen *et al.*, 2000). LIMK 1/ 2, are serine protein kinases involved in the regulation of actin polymerisation and microtubule disassembly. Rho kinase (ROCK) regulates the activity of LIMKs by phosphorylation, and on the other hand LIMKs phosphorylate and inactivate the actin depolymerizing factors ADF/cofilin leading to the increase in filamentous actin (Bernard, 2007). LIMK1 is involved in cancer metastasis (Wang *et al.*, 2006), while LIMK2 activation promotes cells cycle progression (Sumi *et al.*, 2006).

Migration is a process facilitated by actin cytoskeleton, and needed during normal development, chemotactic migration of inflammatory cells and wound healing, but it may also be involved in pathological processes by providing the possibility for cancer cells to metastasize (Hall, 2009). Proteins localized in focal adhesions or actin cytoskeleton often participates in cell migration. PINCH1 is a protein with five tandem LIM domains and required for cell-ECM adhesion. PINCH1 regulates cell adhesion and cell spreading via two distinct interactions. Interaction between its LIM domain and integrin-linked kinase is needed for cellular adhesion, whereas interaction between its C-terminal region and Ras suppressor protein 1 is needed for cellular spreading (Ito *et al.*, 2010). Ajuba is localized into cellular adhesive complexes, and silencing of Ajuba leads to abnormal migration due to the abnormal formation of lamellipodia. Ajuba regulates the cell migration by recruiting p130Cas, a major Src substrate, to nascent adhesion sites. This is needed for activation of Rac and to formation of lamellipodia (Pratt *et al.*, 2005).

LASP-1, a protein with LIM and SH3 domains, binds actin and regulates cell migration (Lin *et al.*, 2004). LASP-1 interacts with chemokine receptor CXCR2 and this interaction is critical for CXCR2-mediated chemotaxis in neutrophils, macrophages and endothelial cells (Raman *et al.*, 2010). FHL2 is another LIM domain protein involved in inflammatory cell migration as it regulates chemotactic factor-induced dendritic cell migration (Konig *et al.*, 2010). Also LIM-domain proteins FHL3, Mystique and LMO7 participate in the regulation of cell motility. FHL3 enhances cell spreading by inhibiting the actin bundling properties of α-actinin and thereby promotes actin disassembly (Coghill *et al.*, 2003). Mystique (PDLIM2) is required for cellular adhesion and migration (Loughran *et al.*, 2005) and LMO7 mediates cell-specific activation of Rho-MRTF-SRF pathway and promotes the breast cancer cell migration (Hu *et al.*, 2011).

LIM domain proteins in growth and tumorigenesis

LIM domain proteins display various functions in the cell and have also been associated with cell growth and tumorigenesis. LIMK2 has been linked with regulation of actin cytoskeleton, but is also involved in cell division and required for the formation of a proper mitotic spindle (Po'uha *et al.*, 2010). Moreover, ectopic expression of splice variant LIMK2b has been shown to inhibit

cytokinesis by inactivating the function of cofilin leading to the formation of multinucleated cells (Hsu *et al.*, 2010). Overall, LIMK2b has been implicated as a tumor suppressor as it is downregulated in esophageal and thyroid cancers and in number of established cancer cell lines (Hsu *et al.*, 2010). Zyxin is specifically phosphorylated during mitosis, and localizes and interacts with h-warts/LATS1 complex in the mitotic apparatus. Inhibiting the mitotic localization of zyxin prolongs the duration of mitosis significantly (Hirota *et al.*, 2000). Furthermore, zyxin shows tumor suppressor properties in Ewing sarcoma cells, since re-expression in the cells reduces the migration, inhibits anchorage-independent growth and impaires tumour formation in mice (Amsellem et al., 2005).

RIL is LIM domain protein that suppresses cell growth. Its gene is localized on chromosome 5q31, a region commonly deleted in acute myelogenous leukemia and myelodysplastic syndrome, and is silenced by methylation in several cancer cell lines. Furthermore, methylation of RIL is a marker for poor prognosis in myelodysplastic syndrome (Boumber *et al.*, 2007). LIM domain-containing protein 1 (LIMD1) functions through interaction with retinoblastoma protein (pRB), and therefore inhibits E2F-mediated transcription and suppresses cell growth (Sharp *et al.*, 2004). LIMD1 was also identified as a tumor suppressor, as it is frequently deleted in many solid malignancies. FHL 1-3 inhibited anchorage dependent and independent growth of human hepatoma cell line *in vitro* and tumour formation *in vivo* (Ding *et al.*, 2009).

Mystique (PDLIM2) suppresses anchorage-independent growth and tumor formation by inhibiting the function of NFκB, which becomes constitutively activated during colon tumorigenesis. Mystique is repressed in various human colorectal cancer cell lines and the repression involves promoter methylation. As restoring the mystique expression resulted in growth arrest, it has been suggested to act as a tumor suppressor in colorectal (Qu *et al.*, 2010b) and breast cancer (Qu *et al.*, 2010a).

Although several LIM domain proteins function or are putative tumor suppressors, also oncogenic LIM domain proteins exist. LMO proteins are transcription factors and have frequently been associated with cancer development. LMO1 and LMO2 act as oncogenic proteins in acute T-cell lymphoblastic leukemia, and both LMO1 (Wang *et al.*, 2011) and LMO3 (Aoyama *et al.*, 2005) are identified as oncogenes in neuroblastoma. Moreover, LMO4 contributes to the development of breast cancer (Zheng and Zhao, 2007) and is overexpressed in late stage pancreatic cancer (Yu *et al.*, 2008). Silencing studies of LMO4 revealed that LMO4 may promote cell growth and survival of both normal mammary epithelial and breast cancer cells (Visvader *et al.*, 2001; Tian *et al.*, 2010).

LIM domain proteins in cellular stress and fate decisions

Several LIM domain proteins are phosphorylated or activated in response to DNA damage and cell stress, and play a role in cell fate decision between survival or apoptosis. As p53 has a crucial role in repair, several LIM domain proteins have been shown to be p53 targets and/or to modulate p53 dependent pathway through protein-protein interactions.

Zyxin is phosphorylated and accumulates into the nucleus from focal adhesions in response to UVC-irradiation (Hervy *et al.*, 2010). Zyxin interacts with cell cycle and apoptosis regulator protein-1 (CARP-1) in response to UVC and promotes apoptosis of cells (Hervy *et al.*, 2010). Zyxin also regulates apoptosis in response to DNA damage via HIPK2-p53 pathway (Crone *et al.*, 2011). HIPK2 activates the apoptotic response of the cells in response to DNA damage by phosphorylating p53 at serine 46. Normally, levels of HIPK2 are kept low by siah-1 and WSB1, however, DNA damage causes zyxin to regulate siah1 activity by interfering with its dimerization. As silencing of zyxin results in attenuated HIPK2 protein levels and reduces DNA damage-induced p53 Ser46

phosphorylation and caspase activation, zyxin is implicated to regulate HIPK2-p53 signaling and to contribute apoptosis in response to DNA damage (Crone *et al.*, 2011).

LIMK2 is a direct p53 target gene and promotes the survival of the cells in response to damage (Croft *et al.,* 2011). Also the splice variant LIMK2b has been described as a p53 target and shown to be needed for G2/M arrest after DNA damage. Furthermore, it was suggested that LIMK2b regulates G2/M arrest through phosphorylation of cofilin and thus by modulating the dynamics of actin polymerization (Hsu *et al.*, 2010). As expression of LMO3 suppresses the expression of p53 dependent target genes, but still facilitates p53 binding to its response elements, LMO3 has been implicated to act as a co-repressor of p53 (Larsen *et al.*, 2010). Moreover, enigma negatively regulates p53 through MDM2 by inhibiting MDM2 self-ubiquitination and increasing its ubiquitin ligase activity towards p53 in cells. Furthermore, enigma promotes cell survival and chemoresistance by suppressing p53-mediated apoptosis in cell lines and in mice (Jung *et al.*, 2010).

RIL expression sensitizes cancer cells to apoptosis, as RIL expression in colon cancer cells results in reduced cell growth and clonogenicity and an approximately 2.0-fold increase in apoptosis following UV exposure (Boumber *et al.*, 2007).

FHL2/ DRAL is a transcriptional target of p53, and five potential p53 target sites have been identified in human *FHL2/ DRAL* gene. Furthermore, FHL2/ DRAL expression efficiently triggers apoptosis in three cell lines of different origin (Scholl *et al.*, 2000). The transcription factor E4F1 is one of the key players in controlling mammalian embryonic and somatic cell proliferation and survival, and its antiproliferative effects have been shown to depend on its capacity to repress transcription and to interact with pRb and p53. FHL2 is negative regulator of E4F1 thus inhibiting its ability to promote cell proliferation. E4F1-FHL2 complexes form upon UVR-induced nuclear accumulation of FHL2 (Paul *et al.*, 2006). Besides the damage- induced cell death, LIM domain proteins participate in apoptosis e.g. during development. Sonic hedgehog (SHH) and its main receptor, Patched (PTC), are implicated in both neural development and tumorigenesis. SHH is a survival factor, whereas PTC induces apoptosis in the absence of SHH. PTC triggers caspase 9 activation and promotes cell death through a caspase 9-dependent mechanism. In the absence of SHH, PTC serves as an anchor for a caspase-activating complex that includes FHL2/ DRAL and caspase 9. FHL2/ DRAL is required for the pro-apoptotic activity of PTC both in immortalized cells and during neural tube development in chick embryos (Mille *et al.*, 2009).

Table 1. Summary of LIM- domain proteins playing a role in cell proliferation and/ or in tumour formation.

PROTEIN	GROUP	AFFECTS THE CELL PROLIFERATION	INVOLVED IN FORMATION OF CANCER	INVOLVED IN CELLULAR STRESS
LMO1 **LMO2** **LMO3** **LMO4**	I	act as oncogenes LMO4 promotes cell growth and survival of both normal mammary epithelial and breast cancer cells	LMO1/2 in acute T-cell lymphoblastic leukemia, LMO 1/3 in neuroblastoma, LMO4 in breast carcinoma and pancreatic cancer	LMO3 binds p53, and inhibits its transcriptional activity
FHL 1-3	II	inhibit anchorage dependent and independent growth of human hepatoma cell line *in vitro* and tumor formation *in vivo*	FHL proteins are often downregulated in hepatocellular carcinomas	FHL2/ DRAL is a transcriptional target of p53, expression triggers apoptosis
zyxin	III	inhibition prolongs the duration of mitosis significantly	tumor suppressor activity in Ewing tumor cells	is phosphorylated and accumulates in nucleus in response to UVC-irradiation and promotes apoptosis of cells regulates apoptosis via HIPK2-p53 pathway
LIMD1	III	interacts with retinoblastoma protein (pRB), and inhibits E2F-mediated transcription and suppresses cell growth	frequently deleted in many solid malignancies	
RIL	III	suppresses cell proliferation	commonly deleted in AML and myelodysplastic syndrome, methylation of RIL is a marker for poor prognosis in myelodysplastic syndrome	RIL expression sensitizes cancer cells to apoptosis in response to UV-irradiation
Mystique= **PDLIM2**	III	suppresses anchorage-independent growth and tumor formation	repressed in various human colorectal cancer cell lines by promoter methylation, acts as a tumor suppressor in colorectal and breast cancer	
EPLIN	III	depletion enhances EMT, proliferation, migration and invasion, and its downregulation correlates with metastases	SCC of head and neck cancers	
LIMK2	IV	required for formation of proper spindle		direct p53-target gene, promotes survival
LIMK2b	IV	overexpression inhibits cytokinesis and leads to multinucleated cells	downregulated in esophageal and thyroid cancers and in several cancer cell lines	novel p53 target and shown to be needed for G2/M arrest after DNA damage

LIM domain proteins in TGFβ-signaling pathway

Several LIM domain proteins are involved in TGFβ-signaling pathway, and activation of TGFβ1 induces several LIM domain proteins. The expression of CRP2 is regulated by TGFβ in vascular smooth muscle cells (VSMC) and in hepatic stellate cells. TGFβ transcriptionally induces CSRP2 via type I receptor (Herrmann *et al.*, 2006) and activating transcription factor 2 (ATF2). Silencing of CRP2 leads to the increased migration of VSMCs, and the migration is further enhanced by TGFβ-treatment (Lin *et al.*, 2008).

Hydrogen peroxide-inducible clone 5 (Hic-5/ARA55) is a coactivator of androgen receptor (AR). It is expressed in prostate stroma and has four LIM domains. It modulates TGF-β-signaling by interaction with Smads. Hic-5 interacts with Smad3, Smad4 (Wang *et al.*, 2005) and Smad7 (Wang *et al.*, 2008), but not with Smad2 (Wang *et al.*, 2005). Hic-5 suppresses Smad7 by physically interacting with Smad7 through its third LIM domain inducing Smad7 loss (Wang *et al.*, 2008). The same third LIM domain is also needed for interaction between Hic-5 and Smad3, which leads to the downregulation of Smad3 mediated signaling (Wang *et al.*, 2005). Furthermore, suppressive activity of Hic-5 on Smad3 and Smad7 enhances Smad2 activity, thus suggesting that TGFβ responses depend on the balance on Smad3- and Smad2- dependent signals (Wang *et al.*, 2008).

LMO1 is a nuclear LIM domain protein with no binding ability to DNA, and it regulates Gasdermin in TGFβ-dependent manner and functions in TGFβ-induced apoptosis in the gastric epithelium (Saeki *et al.*, 2007). Zyxin is another target of TGFβ signaling cascade, and is required for stress fiber reorganization and cell migration during EMT in mouse mammary gland epithelial cells (NMuMG) and in endocardial cells through transcription factor Twist1 (Mori *et al.*, 2009). LIMK together with Rho kinase (ROCK) is required for TGFβ-stimulated transcriptional activity of SRF and actin stress fiber reorganization during EMT. Furthermore, LIMK is not required for TGFβ-induced 2D motility, but is needed for TGFβ-induced cell invasion in matrix (Morin *et al.*, 2011).

FHL proteins (FHL1, FHL2, FHL3) interact with Smads (Smad2, Smad3, Smad4) and regulate TGFβ-responsive transcription by enhancing Smad2/3 phosphorylation, Smad2/3 and Smad4 interaction and nuclear accumulation via casein kinase 1δ (CK1δ) independently of TGFβ-receptor signaling. In hepatocellular carcinomas, FHL proteins are often downregulated, and the levels correlate with decreased TGFβ-like responses in the clinical samples (Ding *et al.*, 2009).

THE FAMILY OF CYSTEINE RICH PROTEINS

CRP family consists of three members: CRP1, CRP2 and CRP3/MLP (Weiskirchen *et al.*, 1995). TLP (thymus LIM protein) is closely related, and sometimes referred as a fourth member of the family (Kirchner *et al.*, 2001). CRPs are small proteins, 22 kDa of size, and comprise of two functional LIM domains, each of them linked to glycine-rich repeat.

Several related proteins have been found to share common features with the CRP vertebrate counterparts, suggesting that these proteins are evolutionarily conserved. CRP like LIM domain proteins have been identified in arthropods, protozoas and plants. Two members of CRP counterpart in *Drosophila melanogaster* have been found and referred to as MLP (Weiskirchen and Günther, 2003). DdLIM is considered as CRP counterpart in *Dictyostelium discoideum*, and although it has similarities to CRP proteins, DdLIM only contains one LIM domain, it has no putative nuclear targeting signal and a very diffuse glycine-rich repeat (Prassler *et al.*, 1998).

It is noteworthy that most plant LIM domains actually are related to CRP family (Thomas *et al.*, 2007a). Although plant LIM proteins share similar structure with two LIM domains, they also show distinct features. Plant LIM domain proteins lack glycine rich repeats, and have impaired second

LIM domain. WLIM has two LIM domains, binds actin and bundle actin filaments and has two different isoforms (Thomas *et al.*, 2006; Thomas *et al.*, 2007b).

CRPs exhibit a differential expression pattern in chicken; CRP1 expression is detected in most of the tissues, especially enriched in smooth muscle cells, whereas CRP2 is restricted to arteries and fibroblasts and CRP3/mlp to striated muscle (Louis *et al.*, 1997). However, when expressed in fibroblasts, a common feature for these proteins is association with actin cytoskeleton, and interaction with α-actinin and zyxin (Louis *et al.*, 1997). The carboxyl-terminus of CRPs (LIM2) is predicted to have at least one protein partner and CRP3/mlp also interacts with β1-spectrin (Flick and Konieczny, 2000). Thus it has been suggested that although CRPs have common interacting protein partners, binding proteins may also differ from one another allowing the different functions in the cell.

Csrp1/ CRP1

Human CRP coding gene (*Csrp1*) was first cloned 1990 (Liebhaber *et al.,* 1990). The cDNA was described to be 1778 bp in length, and to carry duplicated domains each with two putative zinc fingers and glycine-rich repeats. It seems to be very well conserved in evolution from yeast to human. It is represented as a single copy in human genome, and localizes to chromosome 1q24-1q32 (Wang *et al.*, 1992; Erdel and Weiskirchen, 1998). CRP1 has also been cloned from chicken (Crawford *et al.*, 1994).

Significant structural similarities exist between CRP1 and cysteine-rich intestinal protein (CRIP). CRP1 contains four zinc fingers consisting of a 25 amino acid domain, whereas CRIP only consists of two zinc fingers. Based on the sequence similarities between zinc fingers 1-3 and 2-4, it has been suggested that CRP1 might have evolved as duplication of *CRIP* gene (Liebhaber *et al.*, 1990). Four human transcripts have been found. As CRP1 and CRP2 are highly identical, CRP genes are predicted to have arisen of the common ancestor probably through gene duplication (Lilly *et al.*, 2001).

The three dimensional structure of chicken CRP1 has been determined by multidimensional nuclear magnetic resonance (NMR). The structure analysis showed that two LIM domain components are spatially separated with no interaction between one another, suggesting that they may function as independent units as an adapters or linkers. CRP1 has also been suggested to occur as monomers (Yao *et al.*, 1999). CRP1 also carries a putative nuclear localization signal in its sequence (Weiskirchen *et al.*, 1995).

Regulation of CRP1 expression

CRP1 is expressed both in vascular and nonvascular tissues containing smooth muscle cells (Yet *et al.,* 2008) and it has been suggested to act as a smooth muscle marker (Henderson *et al.*, 1999). In chicken, CRP1 protein is most prominently expressed in intestine, stomach and gizzard tissue which are enriched with smooth muscle cells (Crawford *et al.*, 1994). Furthermore, *Csrp1* mRNA was found by northern blotting to be strongly expressed in brain, lung and kidney and with weaker staining in heart, spleen, skeletal muscle and testis in mice (Henderson *et al.*, 1999). Under more careful investigation, CRP1 was found expressed prominently in smooth muscle cells in different mouse tissues. Hybridization of *Csrp1* probe revealed that *Csrp1* transcript was found specifically in smooth muscle cells of adult murine cardiac arteries, but not in cardiac muscle tissue itself. CRP1 is also expressed abundantly in stromal compartment of the human prostate, where CRP1 is strongly expressed in cytoplasm, and with no significant staining in the secretory epithelium (Dube *et al.*, 1998).

SRF regulates transcriptionally many smooth muscle specific genes containing CArG box enhancer elements in response to numerous stimuli causing reorganization of the actin cytoskeleton (Olson and Nordheim, 2010). SRF regulates the transcription of smooth muscle specific genes by binding to CArG-box in the promoter region. In *Csrp1* the CArG-box is located in the 5.0 kb enhancer, and binding of SRF in this element is needed to direct the expression in arterial but not in venous or visceral smooth muscle cells (Lilly *et al.*, 2001).

Ca^{2+} signaling promotes the activation of many downstream effectors, including Ca^{2+}/calmodulin (CaM)-dependent protein kinases consisting of three members: CaMKI, CaMKII, and CaMKIV. CaMKIV, but not CaMKII can induce CRP1 expression through the CRE and CArG box regions in the promoter (Najwer and Lilly, 2005).

Function of CRP1

Serum induction after starvation provokes the cells to switch from arrested to proliferative state of growth. The genes upregulated during the switch are called early immediate response genes and include transcription factors and proto-oncogenes (Bravo, 1990; Lau and Nathans, 1985). *Csrp1* is one of the genes induced in response to growth factor stimulation in parallel with Myc-oncogene, and is therefore considered to play a role in cell growth and differentiation. Furthermore, early studies showed that CRP1 is dramatically reduced in transformed avian fibroblasts, emphasizing the possible role of CRP1 in cell transformation (Wang *et al.*, 1992).

Both CRP1 and CRP2 have been implicated to act as a potential smooth muscle differentiation co-factors, because they are highly expressed in smooth muscle cells and able to interact with SRF and GATA6 transcription factors and to enhance the transcription of the αSMA-reporter (Chang *et al.*, 2003). However, *Csrp1* knock out mice, as well as *Csrp1/ Csrp2* double knock out mice, were viable and fertile, and showed no distinguishable phenotype as compared to wild type counterparts (Lilly *et al.*, 2010). Interestingly, the knock out of *Csrp1* leads to the attenuated (Lilly *et al.*, 2010) and knock out of *Csrp2* to increased neointima formation after balloon surgery and in double knock out mice the formation of neointima is comparable to wild type. Therefore, it was suggested that smooth muscle-associated CRP1 and CRP2 are not essential for normal smooth muscle differentiation during development, but they may modulate the smooth muscle response during pathophysiological stress (Lilly *et al.*, 2010).

In zebrafish model, CRP1 acts as an important factor during gastrulation and cell movement of the mesoderm and cardiac mesoderm. Convergent extension is the process that plays a crucial role in organization of the cells during embryogenesis and during it the tissue is restructured to narrow along one axis and elongate along another axis by cellular movement. Wnt signalling pathway is essential for convergent extension during morphogenesis and CRP1 interacts with Wnt signalling pathway components Dishevelled and Diversin. CRP1 inhibition leads to abnormal cell movement in convergent extension resulting in deformities in midline structures (Miyasaka *et al.*, 2007).

CRP1 exhibits dual localization patterns in cell; it is found both in nucleus and in cytoplasm. In nucleus, CRP1 functions as a transcriptional co-regulator (Chang *et al.*, 2003). CRP1 interacts with actin cytoskeleton in two ways; indirectly via interaction with known actin binding proteins, like α-actinin (Pomies *et al.*, 1997) and zyxin (Sadler *et al.*, 1992). Both LIM domains of CRP1 and the first of the three LIM domain of zyxin are needed for this interaction (Schmeichel and Beckerle, 1998). However, LIM domains are not needed for alpha-actinin binding, but lysine 65 in N-terminal glycine-rich repeat is (Harper *et al.*, 2000). CRP1 also directly interacts with actin. CRP1 may play a role in actin cytoskeleton remodeling by bundling the stress fibers by crossing actin filaments and by stabilizing the interactions of α-actinin with actin filament bundles (Tran *et al.*, 2005; Jang and Greenwood, 2009).

LIM domains of different LIM domain proteins have also been noticed to interact with PKC, as was the case with CRP1 as well (Kuroda *et al.,* 1996). However, CRP1 does not affect the activation of PKC (Maturana *et al.*, 2011).

Csrp2/ CRP2

Csrp2 gene was first cloned from rat (Okano *et al.*, 1993) and later also from human and localized to chromosome 12q21.1 (Weiskirchen *et al.*, 1997).

CRP2 localizes to actin cytoskeleton and in addition to its interaction with α-actinin and zyxin, CRP2 also binds actin directly (Grubinger and Gimona, 2004). CRP2 also interacts with STAT1-protein through its C-terminal LIM domain (Weiskirchen *et al.*, 2001) and with cysteine rich protein 2 binding protein (CRP2BP), although the relevance of these interactions remains unclear (Weiskirchen and Gressner, 2000). Like several other LIM domain proteins, CRP2 is also regulated by TGFβ, and expressed under TGFβ-regulated control in hepatic stellate cells and in vascular smooth muscle cells (Herrmann *et al.*, 2006; Lin *et al.*, 2008).

CRP2 is mostly expressed in smooth muscle cells, especially in vasculature (Yet *et al.*, 1998). Moreover, the expression of CRP2 in adult vasculature is mediated through intronic CArG box (Chen *et al.*, 2010). The role of CRP2 in vascular development was studied in *Csrp2* knock out mice and it had no clear effect on vascular development (Wei *et al.*, 2005). This was further supported by the data from *Csrp1/ Csrp2* knock out study, where double knock out showed no difference in smooth muscle tissue (Lilly *et al.*, 2010). Silencing of CRP2 enhanced vascular smooth muscle cell migration and lead to the increased neointima formation following arterial injury (Wei *et al.*, 2005).

However, CRP2 is transiently expressed in heart during embryogenesis and has a role in cardiac muscle differentiation. Cardiomyocyte-specific expression of transgenic CRP2 switches on smooth muscle gene expression in cardiac myocytes in mice (Chang *et al.*, 2007). In this study CRP2 was suggested to function as a transcriptional co-adaptor protein (Chang *et al.*, 2007). Moreover, targeted disruption of *Csrp2* gene resulted in subtle changes in cardiac ultrastructure, although mice with non-functional CRP2 were otherwise viable and fertile. The thickness of the cardiomyocytes was increased and the cells were hypertrophic (Sagave *et al.*, 2008).

Csrp3/ CRP3/ MLP

CRP3/MLP was first isolated as a cDNA from rat skeletal muscle (Arber *et al.*, 1994). CRP3 has turned out to be the most extensively studied member of CRP family, and it is expressed in both cardiac and skeletal striated muscle cells.

Skeletal muscle differentiation starts by embryonic stem cells developing into muscle precursor cells known as myoblasts, which then withdraw from cell cycle, fuse into multinucleated cells and start to express a large number of muscle-specific genes. Myogenesis is a complex event regulated by multiple signaling pathways, one of them being muscle regulated factors (MRF) including helix-loop-helix transcription factors MyoD, MRF4, myogenin and Myf-5 which interact with E proteins. CRP3 is a positive regulator of myogenic differentiation, and overexpression of CRP3 in myoblasts enhances skeletal myogenesis. On the other hand, myoblasts with silenced *Csrp3* fail to exit cell cycle and block terminal differentiation. CRP3 accumulates into nuclei at the beginning of muscle differentiation and later during the development it is observed in the cytoplasm (Arber *et al.*, 1994). Furthermore, CRP3 has been identified as a cofactor in myogenesis inducing complex, which

interacts in nucleus with MyoD, myogenin and MRF4 through its first LIM domain and the helix-loop-helix motifs of the MRFs (Kong *et al.*, 1997).

The *Csrp3* knock out mice are viable and born in expected ratio, but already at birth, the hearts are abnormally soft, and have alterations in actin cytoskeleton. Subsequently, the disruption of *Csrp3* leads to the development of dilated cardiomyopathy with hypertrophy and heart failure. Further analyses of the cardiomyocytes show disruption of cytoarchitecture (Arber *et al.*, 1997). The phenotype of *Csrp3* knock out mice resemble the appearance of hypertrophic cardiomyopathy, which in humans is a hereditary disease of myocardium, where for unknown reason the myocardium is thickened. It is the most common cause of sudden cardiac death in young individuals. *Csrp3* knock out mice were the first genetically altered mouse model that mimicked the disease, and consequently, CRP3 has been widely used experimental model in cardiology (Buyandelger *et al.*, 2011). Moreover, mutations in *Csrp3* gene have been found in families with hypertrophic cardiomyopathy (Geier *et al.*, 2003), and CRP3 was found to be down-regulated in humans with heart failure (Zolk *et al.*, 2000).

CRP3 appears to be needed for adaptation of adult heart to the hemodynamic changes after birth (Buyandelger *et al.*, 2011). CRP3 has been associated with stress in striated muscle, and to be induced during several different *in vivo* and *in vitro* experimental settings such as cell stretch (Campos *et al.*, 2009), mechanical stimulation after Botulinum neurotoxin-A injection (Velders *et al.*, 2008), fatiguing exercise (Lehti *et al.*, 2009) and lengthening and shortening contractions (Kostek *et al.*, 2007).

Like all CRPs, CRP3 has been associated with actin cytoskeleton. It interacts with cofilin, and regulates F-actin dynamics in cardiac and skeletal muscle (Papalouka *et al.*, 2009). Furthermore, CRP3 interacts and co-localizes with β-spectrin at the sarcolemma overlying the Z- and M-lines of myofibrils in both cardiac and skeletal muscle tissue (Flick and Konieczny, 2000).

Table 2. This table summarizes the literature of CRP-family including the tissues where particular protein of the family is expressed, binding partners and knock-out/ knock-in mice phenotypes. VSMC= vascular smooth muscle cell, SMC=smooth muscle cell.

PROTEIN	EXPRESSED IN TISSUE (ADULT)	BINDING PROTEINS	KNOCK-OUT/ KNOCK-IN PHENOTYPE
CRP1	both in vascular and nonvascular tissues containing smooth muscle cells	α-actinin zyxin, actin, SRF, GATA6, PKC, Dishevelled, Diversin	knock out mice: viable, fertile no effect on smooth muscle differentiation attenuated neointima formation after balloon surgery knock out zebra fish: abnormal cell movement during development
CRP2	especially in smooth muscle cells in vasculature	α-actinin, zyxin, actin, STAT1, SRF, GATA6, CRP2BP	knock out mice: viable, fertile no effect on smooth muscle differentiation knock out mice: no effect on vascular development enhanced VSMC migration increased neointima formation following arterial injury knock out mice: subtle changes in cardiac ultrastructure knock in mice: transgenic CRP2 switches on SMC-gene expression in cardiac myocytes
CRP3/mlp	cardiac muscle and skeletal muscle	α-actinin, zyxin, cofilin, MyoD, myogenin, MRF4, β-spectrin	knock out mice: viable and born in expected ratio, hearts abnormally soft, cardiomyocytes show disruption of cytoarchitecture, leads to the development of dilated cardiomyopathy mutations found in families with hypertrophic cardiomyopathy

AIMS OF THE STUDY

This study is based on a microarray study detailing UV-regulated transcriptome aiming at identifying transcripts induced with low and high dose of UVC in normal human skin fibroblasts (Gentile *et al.*, 2003). The initial aim of this thesis was to identify and functionally characterize novel UV-regulated genes. *Csrp1* was found to be induced by low and high dose of UVC. We set a hypothesis that CRP1 is needed during UVR-induced cell stress. The aims of this study were:

1. To study UV-induction of *Csrp1* and the functional role of CRP1 in UVR-induced cell stress.
2. To study CRP1 regulation by UVR using physiologically relevant wavelengths and doses in human keratinocytes and in the human skin.
3. To study the function and regulation of CRP1.

MATERIALS AND METHODS

MATERIALS

Cell lines

Several cell lines were used in this study including normal human skin fibroblasts (WS1), spontaneously immortalized mouse fibroblasts (NIH3T3), human cervical cancer cell line (HeLa), human lung adenocarcinoma cell line (A549), human epidermoid carcinoma cell line (A431), embryonic kidney cells (293-T), spontaneously immortalized keratinocytes (HaCat) and fibroblasts isolated from human IPF patients. All the cell lines were maintained in DMEM supplemented with 10 % FCS, except NIH3T3 which were supplemented with new born calf serum. Additionally, WS1 cells were supplemented with non-essential amino acids and HaCaT cells with L-glutamine. All cells were maintained in +37°C atmosphere containing 5 % CO_2.

UV-treatment of the cells were carried out by using UVB (312 nm) or UVC (254 nm) with Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA).

Antibodies

Primary antibodies used were monoclonal anti-CRP1 antibody (BD Transduction Laboratories, Franklin Lakes, NJ), polyclonal anti-CRP1 (Abcam), rabbit polyclonal antibody raised against GST-CRP1 fusion protein in New Zealand rabbits (I), monoclonal anti-GAPDH (clone 9.B.88, Europa Bioproducts Ltd, Cambridge, UK), monoclonal anti-Smad2/3 (BD Transduction Laboratories), phosphospecific antibody for phospho-Smad2/3 (kindly provided by Dr. C. H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), phosphospecific antibody for phospho- p38/MAPK Thr 180/Tyr 182 (Cell Signaling Technology, Danvers, MA), mouse monoclonal αSMA (Sigma Aldrich, St Louis, MO), polyclonal anti-PARP (Cell Signaling Technology), monoclonal anti-Myc (clone 9E10, Nordic Biosite AB), monoclonal α-actinin (H-2 Santa Cruz), anti-p53 (DO-1), β-tubulin (BD Pharmingen) and anti-Flag (M2 Sigma Aldrich).

Plasmids

Human *Csrp1* cDNA was cloned from cDNA clone 690545 (ATCC). *Csrp1* was subcloned to pAMC pC1-neo (Promega, Madison, WI) for Myc-tag, Flag-CMV-2 (Stratagene) for Flag-tag and to pEGFP-C2 (Clontech, Palo Alto) for EGFP-fusion protein. All tags were cloned in N-terminus of CRP1. CMV-promoter driven β-galactosidase plasmid was used as a transfection control. p53 and p53 mutant (p53R273H) were cloned in pcDNA3. ShRNA constructs for silencing of CRP1 were cloned in pENTR^{TM} (Invitrogen) or in pDSL_hpUGIH (Invitrogen).

Small molecule inhibitors

Inhibitors used in this study were staurosporine (LC Laboratories, Woburn, MA), actinomycin D (Sigma Aldrich), 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma Aldrich), cyclohexamide (Calbiochem, Darmstadt, Germany), Smad3 inhibitor (SIS3, Calbiochem), p38 inhibitor (SB203580, Tocris, Bristol, UK), TGF-β type I receptor inhibitor (SB431542, Sigma Aldrich) and caspase inhibitor VI (z-VAD-FMK, Calbiochem).

Growth factors

TGFβ was purchased from Peprotech EC (London, UK).

Tissue samples

Human tissue samples, fixed in formalin and embedded in paraffin, were obtained as collaboration either from Department of Dermatopathology, University of Helsinki (II) or from Department of Medicine, Division of Pulmonary Medicine, University of Helsinki (III). The use of all samples was approved by the Helsinki University Central Hospital Ethics Committee.

METHODS

Preparation of cellular extracts and immunoblotting (I, II, III)

Monolayered cells were washed with phosphate buffered saline (PBS), scraped from the plates and lysed in EBC buffer (25 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin and 10 μg/ml leupeptin). The samples were then incubated on ice for at least 20 minutes followed by centrifuging the cells for 15 minutes at 14 000 rpm. Protein concentration was measured by using Bio-Rad D_C protein assay kit, protein samples were normalized and boiled in LSB-DTT for 5 minutes.

Lysates were separated by using 10-12.5 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of proteins to nitrocellulose membrane (Trans-Blot, Transfer Medium, Bio-Rad) by using wet blot-method. The membranes were then blocked using 5 % milk in Tris-buffered saline (TBS) and immunoblotting was carried out with specific antibodies diluted in 1 % BSA. After the wash with TBS containing 0.05 % Tween 20 (Amersham Biosciences) the primary antibodies were followed by secondary antibodies and again after the washes the secondary antibodies coupled to horseradish peroxidase (Dako Cytomation, Denmark) was used. The signal was then detected with enhanced chemiluminescence ECL (Amersham Life Sciences or Millipore).

Immunofluorescence (I, II, III)

Monolayer cells were cultured on cover slips, washed with PBS and fixed with 3.5 % paraformaldehyde (PFA). Fixation was followed by permeabilization of the cells with 0.5 % NP-40

and blocking with 3 % BSA. The cover slips were incubated at 37° C for 45 minutes with primary antibodies diluted in 3 % BSA, after which they were washed with PBS. Secondary antibodies (conjugated Alexa fluorochromes 488 nm or 595 nm from Molecular Probes) were incubated on cells for 45 minutes at 37° C followed by washes with PBS. DNA was stained using DAPI (Molecular Probes) or Hoechst33522 and cover slips were mounted on microscopy slides. Rhodamine phalloidin was used to stain actin filaments (I).

The fluorochromes were visualized by Zeiss Axioplan 2 Imaging MOT (Jena, Germany) and imaged with Zeiss Axiocam CCD-videocamera and AxioVision program.

For 5BrdU-incorporation assay (I), the cells were first incubated in the presence of 5BrdU for indicated times followed by fixation of the cells with 3.5 % PFA and permeabilization in 1.5 M HCl for 20 minutes. 5BrdU-labeling was then detected using anti-5BrdU- antibody (Amersham) and fluorescent secondary antibodies.

Northern blotting (I)

Total RNA was purified from the cells by using RNeasy kit (Qiagen, Valencia, CA). Extracted RNA was separated in 1 % agarose gels containing formaldehyde followed by a transfer to the nylon membrane (Hybond-N+, Amersham) using 20x standard sodium citrate (SSC). cDNA insert was probed with [α-^{32}P] dCTP by random priming (Ready-To-Go, Pharmacia) using ExpressHyb solution (Clontech). Autoradiograms were exposed on film and quantitated by Fujifilm BAS-2500 Image Analyzer. RNA levels were normalized against 18 S RNA.

Flow cytometry (I)

Cells were trypsinized, and either fixed in 3.5 % PFA or in 70 % cold ethanol followed by washes with PBS. Cells were then permeabilized with 0.5% NP-40 in PBS and incubated with indicated antibodies diluted in 1% BSA containing 0.1% Tween-20. After washes primary antibodies were conjugated with secondary antibodies by using fluorescent Alexa-488 (Molecular Probes). DNA was stained with propidium iodide (Molecular Probes) or with DAPI. Analysis of the cells was carried out with LSR flow cytometer and Cell Quest program (Becton Dickinson, Franklin Lakes, NJ).

Immunohistochemistry (II, III)

Paraffin embedded tissue sections were deparaffinized using xylene and rehydrated in decreasing percentages of alcohol. Antigens were retrieved by heating the samples in citrate buffer for 20 minutes (pH 6.0). Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxidase and Vectastain Elite ABC kit was used (Vector Laboratories) for immunohistochemistry. Primary antibodies were incubated on the samples overnight, and the detection was carried out using biotinylated antibodies, horseradish peroxisidase complex and AEC chromogen. The nuclei were stained using Mayers' hematoxylin and mounted on glass slides. The control sections were treated with goat IgG to rule out unspecific staining.

Quantitative PCR (III)

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was used to study the transcriptional regulation of CRP1 by TGFβ. For this purpose, total RNA was extracted using Trizol isolation method and the RNA concentration was measured using Nanodrop. 1.35 μg of RNA was used for reverse transcription reaction performed with First-strand Super Script kit (Invitrogen). Each sample was used as a triplicate to perform qPCR reaction using SYBR GREEN I master mix (Atila Biosystem, Mountain View, CA). qPCR (ABI PRISM 7900HT) used in this study was from Applied Biosystems, Foster City, CA.

Collagen contractility (III)

Collagen contractility was used to study the ability of the cells to contract and the role of CRP1 in this event. The cells were trypsinized and counted. Type I rat tail collagen (2.2 mg/ml in 0.6% acetic acid) was mixed with concentrated medium (10x DMEM, L-glutamine and sodium bicarbonate 7.5%) and the pH of the mixture was neutralized with sterile 1 M NaOH. 50,000 WS1 cells were resuspended in FCS, and the cells were plated inside the collagen latice on 24-well plate. After polymerization DMEM containing 10 % FCS was added on the wells, and the gels were released from the edges. After 24 hours, the diameter of the contracted gels was measured.

Transfection (I, II, III)

Several methods to transfect the cells have used in the study. For electroporation cells were first trypsinized, suspended in Optimem, mixed with DNA and then electropulsed with Gene Pulser II (Bio-Rad Laboratories, Inc., Hercules, CA). Commercial transfection kits were used according to the manufacturers protocol: JetPEI (Polyplus-transfection Inc. New York, NY), Lipofectamine (Invitrogene) and Fugene HD (Roche).

Measurement of the metabolic activity (I)

Cells were first transfected with indicated plasmids, and 48 hours post transfection, cells were trypsinized and replated in similar densities. Metabolic activity was measured using WST-1 cell proliferation assay (Roche) according to the manufacturers protocol. Absorbance was determined by Titertek Multiskan at 450 nm using 690 nm as a reference.

Silencing by using lentiviral shRNA vectors (I, III)

To silence CRP1 by lentiviral transduction using shRNA vectors, 293T cells were first transfected with gag, pol, env coding plasmids plp1, plp2 and plp-VSVG, respectively either with scrambled vector cloned in pDSL_phUGIH or CRP1 targeting shRNA cloned in pDSL_phUGIH. Cells were incubated for two days followed by collection of the medium containing the virus particles. Cells were then transduced with the viral supernatant in the presence of polybrene overnight and incubated for two days.

SiRNA (III)

siRNA oligos against Smad2 (#4392420) and negative control (# 4390843) were from Ambion (Austin, TX), and were transfected to the cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers protocol.

RESULTS AND DISCUSSION

LOCALIZATION OF CRP1 TO THE ACTIN CYTOSKELETON (I, III)

To get overview of the function of CRP1 in the cell, we studied its localization. Like several other LIM domain proteins, CRP1 also localizes to actin-rich structures such as focal adhesions, actin stress fibers, protrusion, cleavage furrow and midbody (**Fig. 8A, 9A**).

CRP1 localizes to stress fibers (**Fig. 8A**), and in myofibroblasts it also co-localizes with αSMA (III). Stress fibers may display a periodical staining pattern, as is the case with α-actinin and myosin II (Naumanen *et al.*, 2008), but CRP1 localizes along the whole stress fiber (**Fig. 8**). WLIM1 (tobacco) (Thomas *et al.*, 2007b) and hhLIM (human heart LIM) (Zheng *et al.*, 2008) are examples of LIM domain proteins that are able to bind actin and bundle stress fibers. Also CRP1 has been shown to bundle monomeric actin into filamentous form and ectopic expression to result in thicker actin bundles (Tran *et al.*, 2005). Our unpublished data supports the observation that ectopic expression of CRP1 promotes the formation of thicker actin stress fibers.

Latrunculin A is a chemical that disrupts the actin cytoskeleton by sequestering monomeric actin and preventing its polymerization. CRP2 has the ability to bind both G and F forms of actin, and to localize to stress fibers in differentiating smooth muscle cells and to nucleus in response to actin cytoskeleton disruption (Kihara *et al.*, 2011). Furthermore, overexpression of WLIM1 delayed the actin depolymerization by latrunculin B by promoting the bundling (Thomas *et al.*, 2006). In our studies, latrunculin A increased the levels of CRP1 in 4 hours, suggesting that CRP1 also responds to disruption of the actin cytoskeleton (**Fig. 8B**). This implicates that it may be needed to stabilize the actin stress fibers or to delay the disruption similarly to WLIM1. The ratio of G actin/ F actin affects the activation of SRF, and the monomeric actin retains its coactivator MAL in cytoplasm (Miralles *et al.* 2003). As CRP1 is transcriptionally regulated by SRF (Lilly *et al.* 2001), but is not a target of MAL (Selvaraj & Prywes, 2004), the increase of CRP1 by latrunculin A is probably not mediated by SRF-MAL pathway, but the increase may rather be the consequence of the stress response in actin cytoskeleton. Furthermore, inhibition of Rho effector ROCK also disrupts the filamentous actin. Interestingly, ROCK inhibitor (Y27632) effect on CRP1 expression level is opposite to the one detected with Latrunculin A, since ROCK inhibitor decreases its levels. The results implicate that CRP1 acts directly under regulation of Rho- pathway (**Fig. 8 C**).

Like several other LIM domain- proteins, CRP3 and zyxin are also essential for actin cytoskeleton structure and stress fiber reorganization. CRP3 regulates the actin stress fiber depolymerization in a complex with cofilin (Papalouka *et al.*, 2009), and CRP3 deficient mice display disrupted cytoskeleton (Arber *et al.*, 1997). Moreover, zyxin is required for actin stress fiber formation (Hoffman *et al.*, 2006) and also during TGFβ- induced EMT (Mori *et al.*, 2009). However, murine *Csrp1* knock out smooth muscle cells did not show any detectable changes in actin cytoskeleton organization (Lilly *et al.* 2010). This was further supported by our own data by using CRP1 silencing and actin cytoskeleton staining (unpublished data).

Focal adhesions are structures that are required for cell attachment to the surface, and provide a link between actin cytoskeleton and the extracellular matrix. Cell adhesion is also a required for cell growth, since cells do not grow in anchorage independent manner. CRP1 localizes to focal adhesions and interacts with α-actinin (Pomies *et al.*, 1997) and zyxin (Sadler *et al.*, 1992), which can both be found from focal adhesions as well. The formation and maturation of focal adhesions involves significant changes in protein composition and requires acto-myosin contractility. Two

recent studies have enlightened the composition of the focal adhesion proteome and revealed differences in it after inhibition of myosin II which is needed for maturation of focal adhesions (Schiller *et al.*, 2011; Kuo *et al.*, 2011). Interestingly, LIM domain proteins were represented as a large group of proteins lost from focal adhesions in response to inhibition of myosin II activity (Schiller *et al.*, 2011). CRP1 was identified in both studies as one of them suggesting that several LIM domain proteins, like CRP1, are recruited to focal adhesions in response actomyosin contractility.

Lamellipodias and protrusions are actin-rich structure in the mobile edge of the cell. Several LIM domain proteins are known to regulate cell migration and spreading, including LASP-1 (Raman *et al.*, 2010), PINCH1 (Ito *et al.*, 2010), PINCH2 (Zhang *et al.*, 2002), zyxin (Mori *et al.*, 2009), CRP2 (Lin *et al.*, 2008) and FHL3 (Coghill *et al.*, 2003). As CRP1 is strongly localized in protrusions of the cell, it is intriguing to speculate that CRP1 could either function in cell migration or polarization. Furthermore, in a zebrafish model *Csrp1* was identified as a regulator of dynamic cell movements of the mesendoderm and cardiac during morphogenesis of tissues and organs (Miyasaka *et al.*, 2007). However, we found no clear evidence for its function in migration in human epithelial or fibroblast cells (unpublished results). It is possible that CRP1 is only required for cell migration during embryonal development or that the discrepancy between the results is due to the cell or organism specificity.

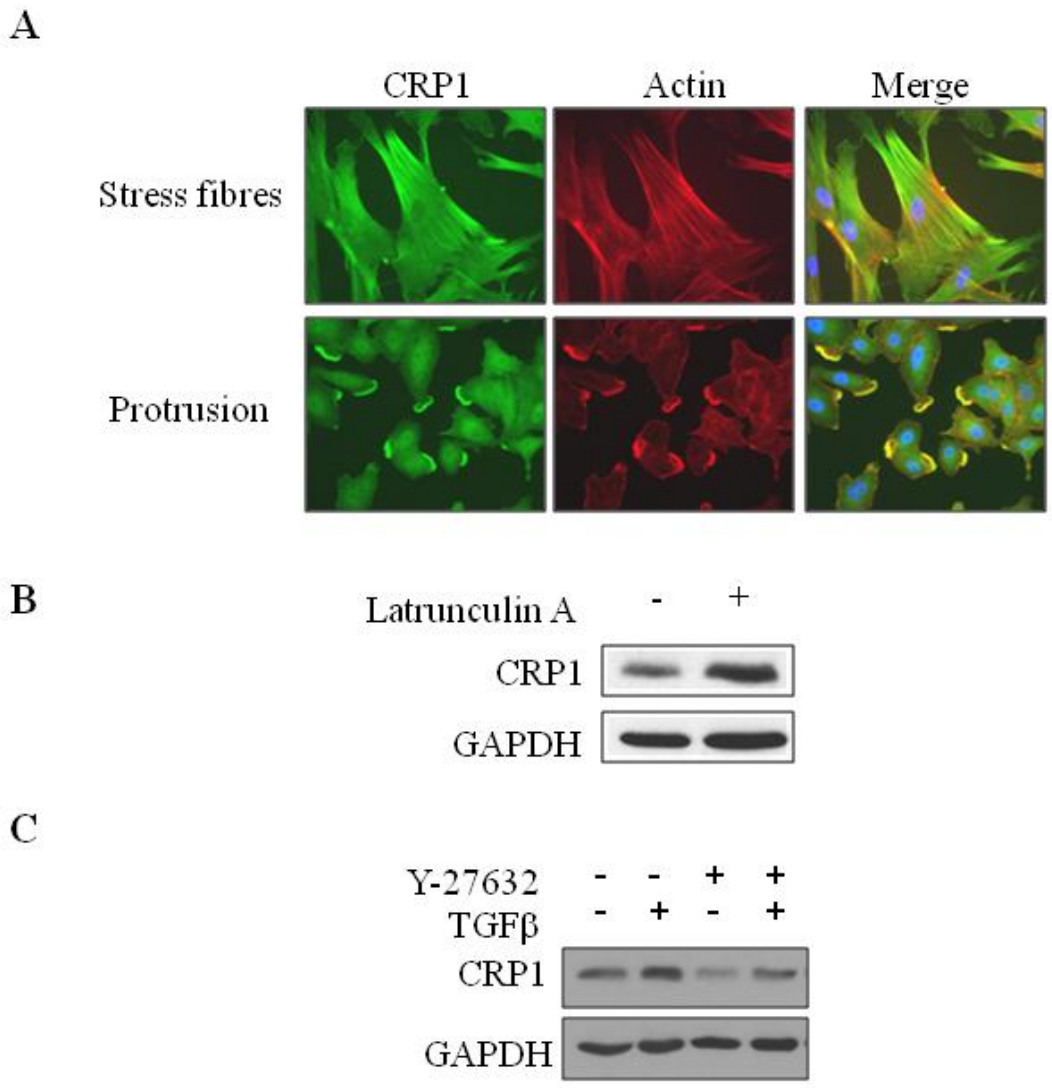


Figure 8. CRP1 localizes to actin rich-structures, and is regulated by actin cytoskeleton disrupting chemicals. Human lung adenocarcinoma cells (A549) were stained for CRP1 (green), actin cytoskeleton (rhodamine phalloidin, red) and nuclei (DAPI, blue) and imaged using fluorescent microscopy (A). Normal human skin fibroblasts were treated with latrunculin A and cells were lysed 4 hours post treatment. Western analysis of CRP1 is shown. GADPH used as a loading control (B). A549 cells were treated with ROCK inhibitor (Y27632) for 24 hours. Western analysis was performed as in B (C).

EFFECTS OF CRP1 ON CELL PROLIFERATION (I, unpublished data)

Already in early studies, CRP1 was noticed to be decreased under starvation in resting cells, and to be rapidly induced in response to serum addition. Furthermore, it was also downregulated in transformed cells (Wang *et al.*, 1992).

When *Csrp1* was identified as a UVC-induced gene in microarray study, it was chosen due to its potential impact on cell growth and differentiation. During the course of our studies we have collected evidence that CRP1 affects the cell growth. Our attempts to generate ectopic CRP1 expressing cell clones turned out to be difficult, since overexpression of CRP1 was not achieved despite repeated attempts. Only 2 mouse fibroblast (NIH3T3) cell clones transfected with Myc tagged CRP1 out of 21 expressed CRP1 at a very weak level. The level of CRP1 was increased by

treating the cells with proteosome inhibitor, MG132, implicating that excess amount of CRP1 was constantly degraded (unpublished observations).

Ectopic Myc-CRP1 suppressed cell proliferation based on 5-BrdU analysis (I). Ectopic expression of CRP1 also suppressed cell proliferation of human fibroblasts (WS1) as determined by cell counting experiments (I). Furthermore, CRP1 levels were increased by cell confluency (I), but later on dramatically decreased, when the cells were withdrawn from cell cycle. It is possible that CRP1 is involved in signaling which is needed to stop the cells from multiplication in response to cell confluency.

We have observed that CRP1 is increased in mitotic cells (**Fig. 9A**). Because changes in protein levels may be challenging to determine in mitotic cells under the microscope, we wanted to verify these results using western blotting. For that purpose we used nocodazole to inhibit microtubule polymerization and to arrest the cells in G2 phase. We detected stabilization of CRP1 after nocodazole treatment, suggesting that CRP1 is indeed increased in cells undergoing mitosis (**Fig. 9B**).

CRP1 is localized in the actin-rich midbody, which is needed during the last phase of cell division when the parental cell divides into two daughter cells (**Fig. 9A**). Several actin-associated and LIM domain proteins are localized in cleavage furrow/midbody region including CRP1 binding partners zyxin (Hirota *et al.*, 2000) and α-actinin (Mukhina *et al.*, 2007), which have both been reported to be required for proper cytokinesis. Both of these proteins localized to the cleavage furrow, and silencing/ overexpression of these proteins lead to a failure in cytokinesis. Zyxin was reported to function through interaction with tumor suppressor hwarts/ LATS1 (Hirota *et al.*, 2000). Although several other LIM domain proteins are also localized in midbody and cleavage furrow, and interfere with cytokinesis causing mitotic defects such as bi- or multinucleated cells, we did not detect apparent distinct phenotype changes of mitotic *Csrp1* knock down cells (unpublished data). However, it is possible that CRP1 reduces cell proliferation and delays cell cycle progression, interefering with cell division through other mechanisms than causing mitotic defects (I).

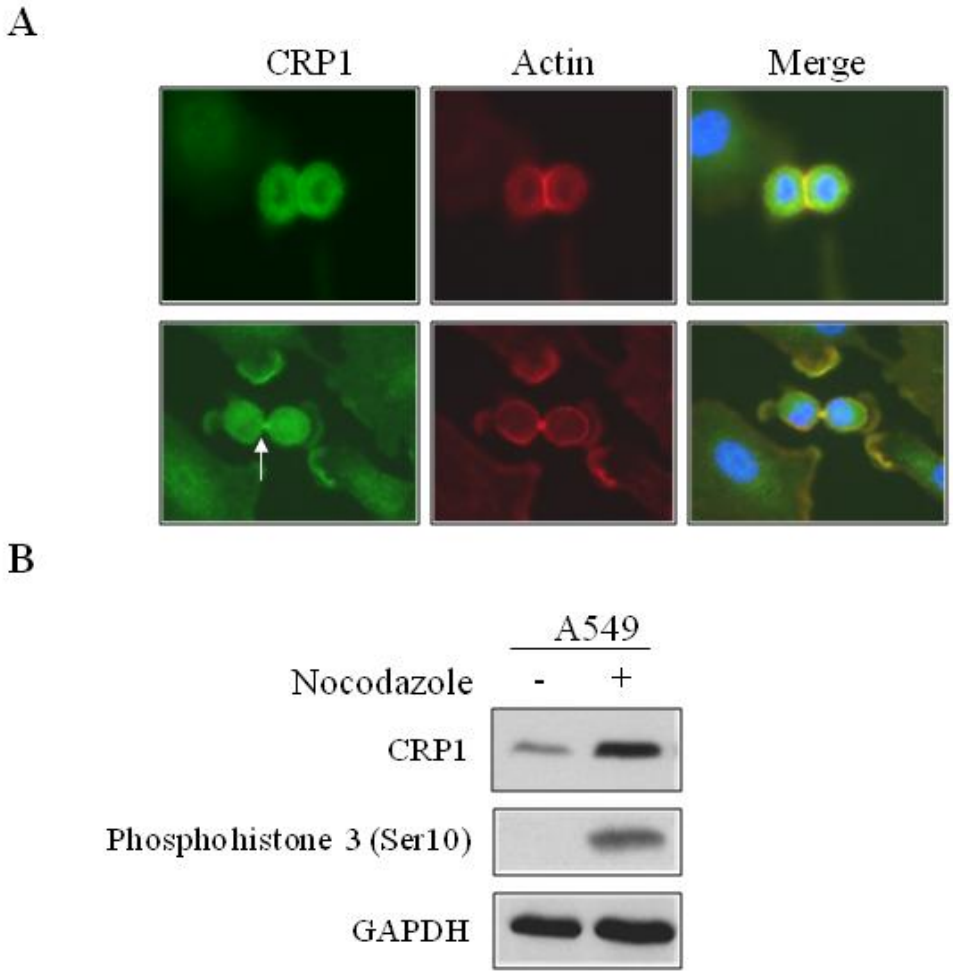


Figure 9. CRP1 localizes to midbody and cleavage furrow, and its levels are increased in G2/M arrested cells. Human lung adenocarcinoma cells (A549) were stained for CRP1 (green), actin (red) and nuclei (DAPI, blue) and imaged using fluorescent microscopy (A). A549 and human cervical cancer cells (Hela) were treated with nocodazole for 24 hours and CRP1 and phosphorylated histone 3 (mitosis marker) were detected in western blotting. GAPDH was used as a loading control.

REGULATION OF CRP1 BY UV RADIATION (I, II)

Csrp1 gene was induced by UVC in a microarray study performed in our lab (Gentile *et al.*, 2003) aiming at identification of UVC regulated genes in WS1 cells. The cells were treated either with low (10 J/m^2) or high (50 J/m^2) dose of UVC for 6, 12, 18, 24 hours. *Csrp1* underwent sustained transcriptional induction by high dose with all time points after UVR, and transient transcriptional induction by a low dose. As a cellular response, the low dose induces a transient cell cycle arrest and high dose induces apoptosis (Gentile *et al.*, 2003). Assuming that highly stressed cells would most probably not consume energy on producing transcripts with no relevance, we studied the function of CRP1 in UVC-induced cell stress. We showed using several methods that CRP1 provides a survival advantage in response to UVC. Cells expressing ectopic CRP1 supported cellular attachment and metabolic activity and were less apoptotic after UVC irradiation and staurosporine treated cells. Furthermore, silencing of CRP1 resulted in increased cell death in irradiated and staurosporine treated cells (I).

Keratinocytes comprise the majority of the cells in the skin that are exposed to solar radiation. Given that the microarray study was performed in normal human fibroblasts, and *Csrp1* induction by UVC was verified in the same cells, we wanted to study the UV responsiveness of CRP1 in keratinocytes with physiologically relevant doses and wavelength (UVB). We found that CRP1 was induced by UVB in normal human epithelial keratinocytes (NHEK), but the induction was impaired in transformed keratinocytes (HaCaT, A431) (II). Out of several cancer cell lines tested, only A549 cells show induction of CRP1 in response to UV irradiation. We observed that the expression of CRP1 was affected by cell growth conditions, and that the induction of CRP1 did not occur unless the cells were in the actively growing phase (I). It seems possible that some UVC-regulated pathways are impaired or not functional in transformed keratinocytes and CRP1 is mainly regulated by UV in normal untransformed cells. Furthermore, we found that CRP1 was cleaved as a caspase-dependent manner during the UVB-induced apoptosis. This may suggest general protein degradation, or that the cleavage products have more special functions (II).

Although UVR causes DNA damage and UVC largely models pure DNA damage, we found no evidence that CRP1 would be directly induced by DNA damage itself. The primary types of UVR induced DNA damage are CPDs and pyrimidine dimers. These are mimicked by 4-nitroquinoline 1-oxide (*4NQO*), which was used to treat the cells. However, 4NQO did not increase the levels of CRP1 (unpublished data). Furthermore, ionizing radiation mainly causes double strand breaks in DNA, but did not increase the levels of CRP1 either (I). Since UVR causes other types of damage in the cell, like disruption of the actin cytoskeleton, we hypothesize that this may be one possible explanation of CRP1 induction by UVR. Interestingly, CRP1 has been proposed to act as an actin cytoskeleton bundling factor (Tran *et al.*, 2005) and we have seen that CRP1 is enhanced by latrunculin A, which disrupts the actin cytoskeleton by inhibiting actin polymerization (**Fig. 8B**). Therefore, it is possible that one way to promote cell survival in response to UVR is to induce the expression of CRP1 in order to maintain cell morphology by stabilizing the structure of actin cytoskeleton.

Interestingly, CRP1 was increased by UVC in human skin fibroblasts (I), but not in primary human keratinocytes (II). On the other hand, CRP1 was increased by UVB in NHEK (II), but not in WS1 cells (II). UVA slightly increased the levels (1.5) of CRP1 in NHEK. MAPK genes are an example of genes, whose induction by UVR is highly dependent on wavelength, doses and cell lines used (Bode and Dong, 2003). The induction of CRP1 by UVR also seems to vary by different wavelengths, cell types used and doses.

In addition to CRP1, several other LIM domain proteins are also regulated by UV-treatment including zyxin, RIL, FHL2 and enigma. Although zyxin, the interacting protein partner of CRP1, was phosphorylated in response to UVC-irradiation and translocated into nucleus (Hervy *et al.*, 2010), no phosphorylation nor localization changes of CRP1 was detected. Several LIM domain proteins have been shown to participate in cell fate decision between survival and apoptosis, although the effects differ from one another. Similarly to the finding that CRP1 promoted cell survival in response to UVR and staurosporine treatment, enigma also promoted tumor cell survival in mice by suppressing p53 dependent apoptosis (Jung *et al.*, 2010). FHL2 accumulated in the nucleus after UV-irradiation, where it was bound to E4F1 and negatively regulated its ability to inhibit cell proliferation (Paul *et al.*, 2006). However, also cell death promoting LIM domain proteins exists, like zyxin, which promotes apoptosis by interacting with CARP-1 (Hervy *et al.*, 2010) and RIL, which sensitizes cells to apoptosis in response to UVR (Boumber *et al.*, 2007).

LIM domain proteins modulate signal transduction pathways, such as PKC and TGFβ. PKCs are serine threonine kinases that participate in several signal transduction pathways and are activated upon signals, such as Ca^{2+} or diacylglycerol. PKC has been found to interact with LIM domains of several different LIM domain proteins (Kuroda *et al.*, 1996). Enigma homolog (ENH1) interacts

with N-terminus of PKCβ1 via its LIM domains, translocates PKCβ1 from cytoplasm to plasma membrane and activates it. LIM domain proteins cypher1 and enigma operated similarly, whereas many other LIM domain proteins were able to interact, but not to activate PKCβ1. NH1 activated PKC in isoform dependent manner; increasing the activation of PKCα and PKCγ, and decreasing the activity of PKCζ (Maturana *et al.*, 2011). We found that PKC pathway mediates the induction of CRP1 by UVC (I). CRP1 was one of the interacting LIM domain proteins, although not capable of activation (Kuroda *et al.*, 1996). We also showed that staurosporine, which is a PKC inhibitor and a potent apoptosis inducer, increased the expression of CRP1 (I). The increase in CRP1 by staurosporine was posttranscriptional. Further experimentation would be needed to resolve whether apoptosis or PKC pathway inhibition trigger CRP1 protein increases in the cell.

CRP1 IN SKIN DISEASES (II)

Given that solar exposure primarily affects the skin and causes skin diseases, we studied CRP1 expression in normal and in sun exposed human skin. CRP1 was found expressed in several normal skin structures such as smooth muscle cells, vasculature, hair root sheat, sebaceous gland and sweat gland. However, very little CRP1 was expressed in normal keratinocytes (II).

We also studied the expression of CRP1 in AK and SCC. We analyzed four AK human skin samples, and found that three out of four samples had epidermal expression of CRP1 in basal keratinocytes in dysplastic area (II). On the other hand CRP1 was not expressed in transformed cells in SCC, but was expressed in cancer-associated myofibroblasts surrounding the tumor area in 2 out of 4 samples. These findings were further supported by publicly available microarray-data, which suggested that *Csrp1* expression was increased in AK, and decreased in SCC. The results suggest that UVR also induces CRP1 in keratinocytes as the levels of CRP1 are increased in AK skin sections. It is also possible that CRP1 participates in the survival of damaged keratinocytes, therefore promoting the formation of the disease. Interestingly, differences in expression of death receptors (FAS and TRAIL receptor) between non-irradiated, AK and SCC skin have observed (Erb *et al.*, 2005). Apoptotic pathways are commonly dysfunctional in cancers, and the expression of death receptor expression is present in AK, but absent in SCC. Because our data showed that CRP1 may act as an anti-apoptotic factor upon UVR and staurosporine induced apoptosis, it is possible that in AK CRP1 expression is high because of still functional apoptotic pathways, but impaired apoptosis pathways in SCC do not support the expression of CRP1 even in the presence of cellular damage.

Furthermore, several LIM domain proteins affect cell growth and are involved in tumorigenesis. Interestingly, *Csrp1* has also been found aberrantly methylated, and subsequently downregulated, in approximately half of hepatocellular carcinomas (Hirasawa *et al.*, 2006). EPLIN (epithelial protein lost in neoplasm) is another LIM domain protein that is frequently silenced in several carcinomas, including SCC of head and neck cancers. Depletion of EPLIN enhanced EMT, proliferation, migration and invasion, and its downregulation correlated with metastases of prostate, breast, colon and head and neck SCC (Zhang *et al.*, 2011). We did not find CRP1 to be required for EMT (III) or migration (unpublished data), but given that we observed using several approaches that CRP1 inhibits cell proliferation, the silencing of CRP1 might be advantageous in some cases to promote the tumor characteristics. Whether CRP1 is downregulated in other carcinomas as well, and for what reason would that be beneficial for the tumors, remains to be determined.

REGULATION OF CRP1 BY TGFβ SIGNALING PATHWAY (III)

We have shown that CRP1 is expressed at higher levels in myofibroblasts than in human skin

fibroblasts, suggesting that its levels may be regulated by the differentiation status of the cells (I, II, III). Given that TGFβ causes the transdifferentiation of myofibroblasts and regulates several LIM domain proteins, we studied the levels and regulation of CRP1 by TGFβ1.

We found that TGFβ1 upregulated CRP1 at protein level in biphasic appearing manner. We treated the cells with two different TGFβ concentrations, 3 and 5 ng /ml, and observed that although magnitude in increase of CRP1 was similar, the increase was more rapid with the higher concentration (5ng/ml) (III). CRP1 levels increased 4 hours after TGFβ-treatment using the lower concentration, and already within 1 hour using the higher concentration. The increase in CRP1 was sustained for several days.

Interestingly, both low and high doses of TGFβ cause rapid transient (15-45 min) increase in the levels of CRP1, parallel to SMAD2/3 phosphorylation. Using the low dose of TGFβ, the increase in CRP1 was consistenly diminished around one hour, whereas this decrease was less prominent using the higher dose, possibly because the sustained increase of CRP1 started approximately at this time. The biphasic response was abrogated when the cells were incubated with an inhibitor of proteasomal activity (MG132), suggesting that the increased levels of CRP1 are actively degraded by proteasome (III).

Sustained induction was blocked with the treatment with SMAD3 phosphorylation inhibitor and using TGFβ1 type I receptor inhibitor (SB431542) (III). Given that *Csrp1* mRNA levels were largely unaffected following TGFβ treatment, the increase of CRP1 seemed to be independent of TGFβ1-induced transcriptional regulation. Regulation of CRP1 on protein level was further supported by observation that ectopic CRP1 is also stabilized by TGFβ. The kinetics of induction of CRP2 by TGFβ1 are similar, as CRP2 protein were increased after two hours of TGFβ1 peaking at 24 hours post treatment (Lin *et al.*, 2008), and CRP2 induction was also dependent on type I receptor (Herrmann *et al.*, 2006). However, in contrast to CRP1, CRP2 is regulated by TGFβ1 at a transcriptional level (Lin *et al.*, 2008).

Although the sustained increase of CRP1 appeared dependent on type I receptor and activated Smad-pathway, the rapid induction was independent of both (III). However, these pathways are not fully differently regulated, as both are dependent on p38 signaling pathway (**Fig. 10**). Interestingly, p38/MAPKs have been reported to mediate the signaling from TGFβ receptor III independently of Smads (Margulis *et al*. 2008). Furthermore, p38 MAPK has been shown to mediate TGF-β regulated apoptosis and EMT independently of Smads (Yu et al., 2002), and p38 MAPK pathway is activated by TGF-β type III receptor (betaglycan) (Santander *et al*., 2006; You *et al.,* 2007). Given that the type I receptor inhibitor used here was without effect on the rapid response, it is possible that the type III receptor mediates, via p38 MAPK signaling, the rapid CRP1 response.

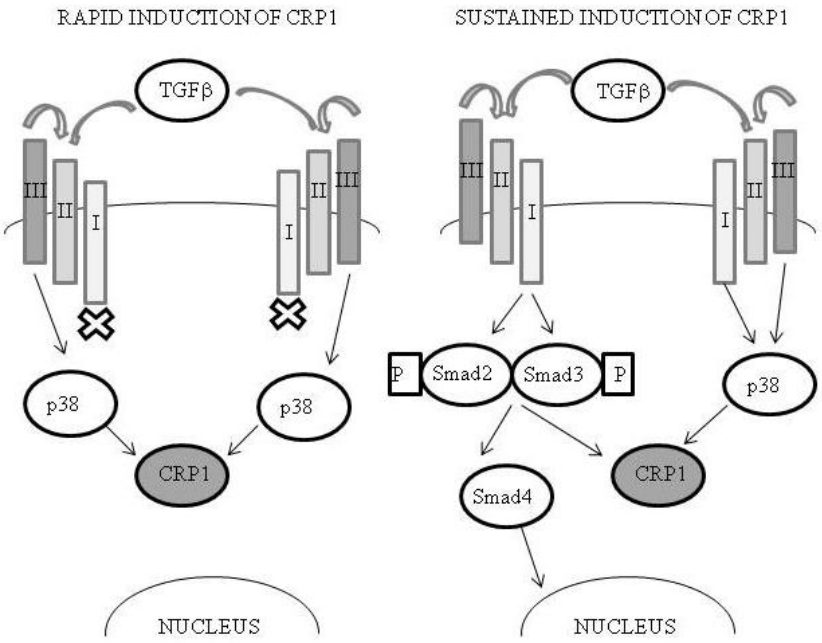


Figure 10. CRP1 is regulated by TGFβ in biphasic-appearing manner. Rapid increase of CRP1 occurs within one hour and is independent on receptor I-mediated signaling, but dependent on p38/MAPK pathway. Sustained induction of CRP1 by TGFβ is dependent on receptor I-mediated signaling and p38-mediated signaling. CRP1 increase does not occur at transcriptional level.

Furthermore, dyxin is another LIM domain protein that is regulated by p38/ MAPKs. Dyxin may participate in regulating hypertrophic process, since the expression of dyxin was rapidly upregulated in response to mechanical load, and this increase being at least partially mediated by p38 MAPK (Luosujarvi *et al.*, 2010).

LIM domain proteins modulate TGFβ-signaling pathway. Hic-5/ARA55 (Wang *et al.*, 2005, Wang *et al.*, 2008) and FHL proteins (Ding *et al.*, 2009) interact with Smads. Given that the sustained increase of CRP1 by TGFβ was dependent on Smad-signaling and that the rapid induction paralleled Smad phosphorylation, we tested whether CRP1 could interact with Smad3 or Smad7. However, no interaction between CRP1 and Smad3 or Smad7 was detected (III). We were also interested whether CRP1 participates in the phosphorylation of Smads. However, we could find no evidence supporting this (III).

CRP1 IN MYOFIBROBLAST DIFFERENTIATION (I, II, III) AND IN CONTRACTILITY (III)

Given the wide variety of different functions of TGFβ and that several LIM domain proteins are involved in TGFβ-pathway, we tested whether CRP1 mediates TGFβ-regulated functions. We noticed that CRP1 levels were elevated in normal human skin fibroblasts co-expressing α-SMA. TGFβ regulates the expression of α-SMA, and induces myofibroblast differentiation. Since CRP1 has previously been shown to regulate α-SMA expression together with SRF and GATA6 (Chang *et al.*, 2003), and is regulated by TGFβ (III), we tested whether CRP1 regulates myofibroblast differentiation. We expressed CRP1 in NIH3T3, and double-stained the cells for FLAG-CRP1 and α-SMA. Ectopic CRP1 could not induce α-SMA expression in the cells (III). Because CRP1, at least alone, could not induce myofibroblast differentiation, we also tested whether it acts as a limiting factor. However, silencing of CRP1 in WS1 cells confirmed our results that CRP1 did not

affect myofibroblast differentiation (unpublished data, III). This observation was further supported by a mouse knock out study, which it showed that CRP1 is not required for α-SMA expression in the absence or presence of TGFβ (Lilly *et al.*, 2010).

TGFβ promotes EMT, and myofibroblasts can originate from epithelial cells as suggested regarding IPF. Moreover, LIM domain proteins EPLIN (Zhang *et al.*, 2011) and zyxin (Mori *et al.*, 2009) negatively and positively, respectively, regulate EMT. Given that CRP1 is associated with myofibroblasts and localizes to actin cytoskeleton, we studied whether CRP1 has a role in EMT. We silenced CRP1 in A549 cells, and treated the cells with TGFβ for 48 hours. However, silencing of CRP1 did not seem to have an impact on EMT markers (III).

RhoA is one of RhoGTPases that regulates actin cytoskeleton by inducing the formation of actin stress fibers and cell contractility. Inhibition of ROCK significantly reduced the endogenous basal levels of CRP1, implicating that CRP1 is at least partially under regulation of RhoA pathway (**Fig. 8C**). Since RhoA pathway also mediates TGFβ- signaling independently of Smads, we also tested whether RhoA mediates the CRP1 increase by TGFβ. CRP1 levels were elevated by TGFβ even in the presence of ROCK inhibitor indicating that RhoA pathway is not needed for CRP1 increase (**Fig. 8C**). Furthermore, we found that CRP1 was localized to αSMA positive stress fibers needed for contraction (III). In addition to CRP1, other LIM domain proteins, such as EPLINα (Chen *et al.*, 2000), LMO7 (Hu *et al.*, 2011) and LIMK 1/2 (Bernard, 2007), are also regulated by RhoA.

Given that cell contractility is a distinct feature of myofibroblasts, and that CRP1 is associated with actin cytoskeleton and myofibroblasts, we studied whether CRP1 is needed for cell contraction. Our data using CRP1 silencing showed that CRP1 was needed for the contractility of the fibroblasts (III). In contrast to our results, *Csrp1* knock out smooth muscle cells did not show difference in contractility as compared to the wild type cells (Lilly *et al.,* 2010). Difference between the results may be due to the difference in cell type used or difference in method used.

CRP1 IN IDIOPATHIC PULMONARY FIBROSIS (III) AND IN CANCER- ASSOCIATED FIBROBLASTS (II)

Injury in alveolar epithelial cells together with TGFβ may cause the accumulation of myofibroblasts in the lung and promote the formation of fibrosis. Because CRP1 is expressed at higher level in myofibroblasts, we studied the expression of CRP1 in normal human and in IPF lungs *in vivo*. No expression of CRP1 was detected in normal alveolar epithelial cells, whereas strong expression of CRP1 was observed in fibroblastic foci in IPF patient lungs (III). The expression of CRP1 co-localized with αSMA in the fibroblastic foci. A significant difference in CRP1 expression between control and IPF patients was detected (lung tissues derived from normal (n = 5) and IPF (n = 10)) (III). Given that CRP1 expression levels are increased in IPF, and TGFβ is one of the factors promoting the formation of IPF, it is possible that CRP1 is elevated due to the increased production of TGFβ.

Smad and p38/MAPK have also a role in IPF, which may implicate that CRP1 increase is mediated via these pathways. Several studies have shown that myofibroblasts in IPF disease may have transdifferentiated from alveolar epithelial cells through EMT (Willis *et al.* 2005). Both canonical and non-canonical TGF-β-induced pathways are involved in EMT in pulmonary epithelial cells, although they exhibit differential roles during it (Kolosova *et al*., 2011). Smad2/3 are needed for collagen production during EMT, but not for loss of E-cadherin or typical morphological changes seen upon EMT. However, p38 MAPK is required for morphological and actin cytoskeleton changes during EMT (Kolosova *et al.*, 2011). MAP kinase pathways, including p38, are elevated in IPF patient lung samples (Yoshida *et al*., 2002) and p38 kinase substrate MK2 is involved in TGF-β

induction α-SMA expression and in myofibroblast differentiation (Sousa *et al*., 2007). Interestingly, p38 inhibitor (Esbriet) was recently approved for treatment of IPF in Europe (Moran, 2011).

Other LIM-domain proteins have also been identified in fibrosis given that CRP2 levels were also increased in activated hepatic stellate cells, when they were transdifferentiating into myofibroblasts (Weiskirchen *et al.*, 2001). CRP2 was also induced during liver fibrogenesis (Herrmann *et al.*, 2006). Furthermore, PINCH 1 and 2 double knock-out mice exhibited severe dilated cardiomyopathy and died of heart failure within 4 weeks. Mutated cardiomyocytes were significantly altered and suffered from abnormal adhesion, cell growth, and cell death. Ventricules were thinner and fibrotic causing heart failure (Liang *et al.*, 2009) suggesting PINCH proteins may also play a role in fibrotic processes.

Interestingly, several LIM-domain proteins, including FHL2, PINCH and CRP1, have been found expressed in CAFs surrounding the tumour tissue. FHL2 was found co-expressed with αSMA in myofibroblasts of the tumour invasive front in sporadic colon and in hereditary non-polyposis colon-rectal cancers. In fibroblasts FHL2 expression was regulated by TGFβ- signalling pathway, and required for TGFβ- induced migration. The results implicated that tumour-derived TGFβ via FHL2 induces the migration of the peritumoural fibroblasts and increases the tight connection between tumour cells and myofibroblasts (Gullotti *et al*., 2011). Furthermore, the expression of PINCH was increased in stroma of normal mucosa, to colorectal adenocarcinoma to metastasis and is higher in invasive front than in intratumoural stroma. Expression of PINCH is primarily seen in fibroblasts, myofibroblasts and proportion of endothelial cells and correlates with worse prognosis, implicating that PINCH may affect the tumour-stroma association promoting progression of the tumour (Gao *et al*., 2004). CRP1 was also found expressed in CAFs in cutaneous squamous cell carcinoma (II). Interestingly, CAFs have been reported to add to the tumorigenic potential of epidermal cells of SCC in a study that compared the properties of normal dermal fibroblasts to CAFs (Commandeur *et al.*, 2011). CAFs decreased the proliferation and differentiation of the epidermal cells, and further increased the invasive potential and dermal-epidermal detachment of two SCC cell lines, implicating that CAFs play an important role during the formation of SCC (Commandeur *et al.*, 2011).

Suprisingly, all the LIM-domain proteins associated with fibrotic myofibroblasts and CAFs can be categorized into LIM-only proteins. Whether the structural and functional similarity of these proteins actually plays a role in formation of fibrosis and whether they have the capacity to transform the normal fibroblasts into CAFs remain to be seen. Moreover, the exact role of CRP1 in fibrosis formation and in transformation of normal fibroblasts to CAFs remains to be answered in future.

CONCLUSIONS

Ultraviolet radiation (UVR) is one of the major environmental genotoxic carcinogens that cause DNA lesions. UVR induced damage in the cell contributes to formation of all types of skin cancers including basal cell carcinoma (BCC), cutaneous squamous cell carcinoma (SCC) and cutaneous melanoma. It has been estimated that 2-3 million new cases of BCC and SCC occur worldwide each year (Narayanan *et al.*, 2010). The original aim of this study was to identify and functionally chracterize novel UVR regulated genes based on a microarray study detailing UV-regulated transcriptome.

LIM domain proteins are involved in multiple functions in cell including cell growth, damage signalling, cell fate determination and signal transduction. We studied the regulation and function of LIM domain protein, cysteine rich protein 1 (CRP1) upon UV irradiation and found *Csrp1* induced by UVC in normal human skin fibroblasts with lethal dose. CRP1 was also increased in normal human keratinocytes by UVB. Furthermore, we find that CRP1 is cleaved in caspase-dependent manner prior to UVB-induced apoptosis and provides a survival advantage for the cells when ectopically expressed by decreasing cellular death and increasing cellular metabolic activity and attachment. Silencing of CRP1 predisposed the cells to apoptosis. Actinic keratosis (AK) is a disease associated with excess expose to sunlight and to sunburn, and may lead to cutaneous squamous cell carcinoma (SCC). We studied the levels of CRP1 in normal, AK and SCC samples. Very little expression of CRP1 was detected in normal keratinocytes whereas CRP1 levels were increased in basal keratinocytes in AK. In SCC, CRP1 was only expressed in cancer-associated fibroblasts. These results implicate that CRP1 is increased in keratinocytes in response to excess exposure to sunlight and may protect the cells from sunburn induced apoptosis. However, the role of CRP1 in AK and SCC lesions needs further validation.

CRP1 is affected by cellular growth conditions, as the UV-induction was only detected in actively growing normal, untransformed cells. Furthermore, CRP1 levels increase by proliferation suppressing signals such as cell confluency, apoptosis inducing staurosporine-treatment and it also suppresses the cell proliferation. The function and mechanism of how CRP1 affects the cell proliferation still remains unclear and needs further experimentation.

CRP1 was also found to be regulated by TGFβ in biphasic appearing manner. TGFβ treatment caused first rapid increase in CRP1 which was then diminished within an hour and later sustained increase in CRP1 which lasts for several days. Elevation occurred in transcription independent manner via Smad and non-Smad signaling pathways. The regulation of CRP1 by TGFβ was associated with myofibroblast differentiation and fibrosis, and CRP1 expression was significantly increased in idiopathic pulmonary fibrosis (IPF) specimens as compared to the normal lung. CRP1 was also required for cell contractility. However, the function of CRP1 in TGFβ signaling needs further exploration.

As a conclusion, we have studied LIM domain protein CRP1 in UVR induced cell stress and in cell proliferation. We find CRP1 as a growth suppressive protein regulated by UVR which protects the cells against UVR-induced cell death. Furthermore, we find CRP1 levels elevated in sunlight exposed keratinocytes in AK samples. CRP1 was also found regulated by TGFβ via Smad and non-Smad pathways and to be significantly elevated in idiopathic pulmonary fibrosis.

These studies identify CRP1 as a stress responsive and cytokine regulated cytoskeletal protein that participates in pathological processes involved in fibrotic diseases and cancer.

ACKNOWLEDGEMENTS

This work has been carried out in the Molecular Cancer Biology Program, Biomedicum Helsinki, and Department of Virology, Haartman Institute, University of Helsinki. I wish to thank Prof. Kari Alitalo, the head of the Program, for providing the great facilities to make research. I also wish to thank Prof. Jorma Keski-Oja for his kind help with various things.

I'm very grateful for my supervisor Prof. Marikki Laiho who gave me the opportunity to work in Laiholab. When I entered the lab year 2001 to do my Master's thesis, I had no idea that the journey would last ten years and end in a PhD degree. The years have past, and I've been introduced to a fascinating field of molecular cancer biology. I thank you for your patience with the project and your willingness to carry the study to this point.

Thesis reviewers Maria Vartiainen and Olli Carpén are warmly thanked for their work to improve the quality of this book. Their valuable comments and flexibility are greatly appreciated!

Marjukka Myllärniemi and Kati Koli are thanked for their collaboration, help, support and discussions on the last article. I thank also Outi Leppäranta for sharing the protocols and helping me with the statistics.

I feel deep gratitude to people who are and have been working in the Laiholab during the years: Arja, Anni-Helena, Henna, Kaisa, Karita, Leena, Maija, Mari-Lotta, Max, Mia, Runar, Sari J., Sari T., Stina, Taija and Virpi. For years the lab has almost felt like a second home, and you have created warm and pleasant atmosphere to work. I realized it how impossible this all would have been without you, when I have been writing this thesis inside home alone during warm summer days. I wish you all the best in the future! Leena has had a substantial role in the CRP1-study: thank you for pushing the projects forward! I also wish to thank Maija, Anni-Helena, Mari-Lotta and Kaisa for helping me in the lab. Thanks go also to our neighbouring Holmberg lab to Carina, Olli and Leena for scientific collaboration as well as to funny company!

I also wish to acknowledge the family and friends. I want to thank my family being there for me whenever needed: my mother Onerva and my father Lasse for their endless support and encouragement and my sisters Outi and Minna, and her family Tuomas, Ellen and Oliver, for their existence and company. During the years, there have been numerous funny occations with the friends: trips in Finland and abroad, dinners, dog walks, chats and jogging, you name it :). Annukka and Pete; Heidi; Piia, Kati, Hanna and Mari; Marja, Salla and Veera: I thank each one of you for your friendship and brilliant company! Jani: I like your positive and energetic attitude and it feels like with you my dreams can come true! And how could I forget to mention my little friend Väinö Koiranen who makes me smile every single day :).

I thank Graduate school of Biotechnology and Molecular Biology for travel grants and educational scientific events. This work has also been supported by K. Albin Johanssons Foundation.

Helsinki, November 2011

REFERENCES

American Thoracic Society. (2000) Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am J Respir Crit Care Med* **161**: 646-664.

Amsellem V, Kryszke MH, Hervy M, Subra F, Athman R, Leh H, Brachet-Ducos C, Auclair C. (2005) The actin cytoskeleton-associated protein zyxin acts as a tumour suppressor in Ewing tumor cells. *Exp Cell Res* **304:** 443-56.

Aoyama M, Ozaki T, Inuzuka H, Tomotsune D, Hirato J, Okamoto Y, Tokita H, Ohira M, Nakagawara A. (2005) LMO3 interacts with neuronal transcription factor, HEN2, and acts as an oncogene in neuroblastoma. *Cancer Res* **65:** 4587-4597.

Arber S, Halder G, Caroni P. (1994) Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* **79:** 221-231.

Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Borg J. (1997) MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* **88:** 393-403.

Aziz MH, Manoharan HT, Verma AK. (2007) Protein kinase C epsilon, which sensitizes skin to sun's UV radiation-induced cutaneous damage and development of squamous cell carcinomas, associates with Stat3. *Cancer Res* **67:** 1385-1394.

Bergeron A, Soler P, Kambouchner M, Loiseau P, Milleron B, Valeyre D, Hance AJ, Tazi A. (2003) Cytokine profiles in idiopathic pulmonary fibrosis suggest an important role for TGF-beta and IL-10. *Eur Respir J* **22:** 69-76.

Bernard O. (2007) Lim kinases, regulators of actin dynamics. *Int J Biochem Cell Biol* **39:** 1071-1076.

Besaratinia A, Yoon JI, Schroeder C, Bradforth SE, Cockburn M, Pfeifer GP. (2011) Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight. *FASEB J* **25**: 3079-91.

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. (2001) Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12:** 27-36.

Bhowmick NA, Neilson EG, Moses HL. (2004) Stromal fibroblasts in cancer initiation and progression. *Nature* **432:** 332-337.

Bode AM, Dong Z. (2003) Mitogen-activated protein kinase activation in UV-induced signal transduction. *Sci STKE 2003***:** RE2.

Bordea C, Wojnarowska F, Millard PR, Doll H, Welsh K, Morris PJ. (2004) Skin cancers in renal-transplant recipients occur more frequently than previously recognized in a temperate climate. *Transplantation* **77:** 574-579.

Boulay JL, Mild G, Lowy A, Reuter J, Lagrange M, Terracciano L, Laffer U, Herrmann R, Rochlitz C. (2003) SMAD7 is a prognostic marker in patients with colorectal cancer. *Int J Cancer* **104:** 446-449.

Boumber YA, Kondo Y, Chen X, Shen L, Gharibyan V, Konishi K, Estey E, Kantarjian H, Garcia-Manero G, Issa JP. (2007) RIL, a LIM gene on 5q31, is silenced by methylation in cancer and sensitizes cancer cells to apoptosis. *Cancer Res* **67:** 1997-2005.

Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, Halperin AJ, Pontén J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A* **88:** 10124-10128.

Bravo R. (1990) Growth factor-responsive genes in fibroblasts. *Cell Growth Differ* **1:** 305-309.

Brenner M, Hearing VJ. (2008) The protective role of melanin against UV damage in human skin. *Photochem Photobiol* **84:** 539-549.

Buyandelger B, Ng KE, Miocic S, Piotrowska I, Gunkel S, Ku CH, Knöll R. (2011) MLP (muscle LIM protein) as a stress sensor in the heart. *Pflugers Arch* **462:** 135-42.

Campos LC, Miyakawa AA, Barauna VG, Cardoso L, Borin TF, Dallan LA, Krieger JE. (2009) Induction of CRP3/MLP expression during vein arterialization is dependent on stretch rather than shear stress. *Cardiovasc Res* **83:** 140-147.

Chang DF, Belaguli NS, Chang J, Schwartz RJ. (2007) LIM-only protein, CRP2, switched on smooth muscle gene activity in adult cardiac myocytes. *Proc Natl Acad Sci U S A* **104:** 157-162.

Chang DF, Belaguli NS, Iyer D, Roberts WB, Wu SP, Dong XR, Marx JG, Moore MS, Beckerle MC, Majesky MW, Schwartz RJ. (2003) Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. *Dev Cell* **4:** 107-118.

Chen CH, Wu ML, Lee YC, Layne MD, Yet SF. (2010) Intronic CArG box regulates cysteine-rich protein 2 expression in the adult but not in developing vasculature. *Arterioscler Thromb Vasc Biol* **30:** 835-842.

Chen S, Maul RS, Kim HR, Chang DD. (2000) Characterization of the human EPLIN (epithelial protein lost in neoplasm) gene reveals distinct promoters for the two EPLIN isoforms. *Gene* **248:** 69-76.

Coghill ID, Brown S, Cottle DL, McGrath MJ, Robinson PA, Nandurkar HH, Dyson JM, Mitchell CA. (2003) FHL3 is an actin-binding protein that regulates alpha-actinin-mediated actin bundling: FHL3 localizes to actin stress fibers and enhances cell spreading and stress fiber disassembly. *J Biol Chem* **278:** 24139-24152.

Commandeur S, Ho SH, de Gruijl FR, Willemze R, Tensen CP, El Ghalbzouri A. (2011) Functional characterization of cancer-associated fibroblasts of human cutaneous squamous cell carcinoma. *Exp Dermatol* **20:**737-42

Coward WR, Saini G, Jenkins G. (2010) The pathogenesis of idiopathic pulmonary fibrosis. *Ther Adv Respir Dis* **4:** 367-388.

Crawford AW, Pino JD, Beckerle MC. (1994) Biochemical and molecular characterization of the chicken cysteine-rich protein, a developmentally regulated LIM-domain protein that is associated with the actin cytoskeleton. *J Cell Biol* **124:** 117-127.

Criscione VD, Weinstock MA, Naylor MF, Luque C, Eide MJ, Bingham SF (2009) Actinic keratoses: Natural history and risk of malignant transformation in the veterans affairs topical tretinoin chemoprevention trial. *Cancer* **115:** 2523-2530.

Croft DR, Crighton D, Samuel MS, Lourenco FC, Munro J, Wood J, Bensaad K, Vousden KH, Sansom OJ, Ryan KM, Olson MF. (2011) p53-mediated transcriptional regulation and activation of the actin cytoskeleton regulatory Rho C to LIMK2 signaling pathway promotes cell survival. *Cell Res.* **21**: 666-82.

Crone J, Glas C, Schultheiss K, Moehlenbrink J, Krieghoff-Henning E, Hofmann TG. (2011) Zyxin is a critical regulator of the apoptotic HIPK2-p53 signaling axis. *Cancer Res* **71:** 2350-2359.

D'Costa AM, Denning MF. (2005) A caspase-resistant mutant of PKC-delta protects keratinocytes from UV-induced apoptosis. *Cell Death Differ.* **12**: 224-32

de Feraudy S, Ridd K, Richards LM, Kwok PY, Revet I, Oh D, Feeney L, Cleaver JE. (2010) The DNA damage-binding protein XPC is a frequent target for inactivation in squamous cell carcinomas. *Am J Pathol* **177:** 555-562.

Deheuninck J, Luo K. (2009) Ski and SnoN, potent negative regulators of TGF-beta signaling. *Cell Res* **19:** 47-57.

Denning MF, Wang Y, Nickoloff BJ, Wrone-Smith T. (1998) Protein kinase cdelta is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J Biol Chem* **273:** 29995-30002.

Derynck R, Akhurst RJ. (2007) Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nat Cell Biol* **9:** 1000-1004.

Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* **122:** 103-111.

De Wever O, Mareel M. (2003) Role of tissue stroma in cancer cell invasion. *J Pathol* **200:** 429-447.

Diffey BL. (2002) Sources and measurement of ultraviolet radiation. *Methods* **28:** 4-13.

Ding L, Wang Z, Yan J, Yang X, Liu A, Qiu W, Zhu J, Han J, Zhang H, Lin J, Cheng L, Qin X, Niu C, Yuan B, Wang X, Zhu C, Zhou Y, Li J, Song H, Huang C, Ye Q. (2009) Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-beta-like signaling pathway. *J Clin Invest* **119:** 349-361.

Dube JY, Chapdelaine P, Trahan PL, Deperthes D, Frenette G, Tremblay RR. (1998) Abundant cysteine-rich protein-1 is localized in the stromal compartment of the human prostate. *Arch Androl* **40:** 109-115.

Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, Martello G, Stinchfield MJ, Soligo S, Morsut L, Inui M, Moro S, Modena N, Argenton F, Newfeld SJ, Piccolo S. (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. *Cell* **136:** 123-135.

Dworaczek H, Xiao W. (2007) Xeroderma pigmentosum: A glimpse into nucleotide excision repair, genetic instability, and cancer. *Crit Rev Oncog* **13:** 159-177.

Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, Miyazono K. (2001) Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* **276:** 12477-12480

Einspahr J, Alberts DS, Aickin M, Welch K, Bozzo P, Grogan T, Nelson M. (1997) Expression of p53 protein in actinic keratosis, adjacent, normal-appearing, and non-sun-exposed human skin. *Cancer Epidemiol Biomarkers Prev* **6:** 583-587.

Erb P, Ji J, Wernli M, Kump E, Glaser A, Buchner SA. (2005) Role of apoptosis in basal cell and squamous cell carcinoma formation. *Immunol Lett* **100:** 68-72.

Erdel M, Weiskirchen R. (1998) Assignment of CSRP1 encoding the LIM domain protein CRP1 to human chromosome 1q32 by fluorescence in situ hybridization. *Cytogenet Cell Genet* **83:** 10-11.

Flick MJ, Konieczny SF. (2000) The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of betaI-spectrin. *J Cell Sci* **113:** 1553-156

Fuchs A, Marmur E. (2007) The kinetics of skin cancer: Progression of actinic keratosis to squamous cell carcinoma. *Dermatol Surg* **33:** 1099-1101.

Fukuchi M, Fukai Y, Masuda N, Miyazaki T, Nakajima M, Sohda M, Manda R, Tsukada K, Kato H, Kuwano H. (2002) High-level expression of the smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal squamous cell carcinoma. *Cancer Res* **62:** 7162-7165.

Gao J, Arbman G, Rearden A, Sun XF. (2004) *Neoplasia* **6:** 796-801.

Gatza CE, Oh SY, Blobe GC. (2010) Roles for the type III TGF-beta receptor in human cancer. *Cell Signal* **22:** 1163-1174.

Geier C, Perrot A, Ozcelik C, Binner P, Counsell D, Hoffmann K, Pilz B, Martiniak Y, Gemlich K, van der Ven PF, Fürst Do, Vornwald A, von Hodenberg E, Nürnberg P, Scheffold T, Dietz R, Osterziel KJ. (2003) Mutations in the human muscle LIM protein gene in families with hypertrophic cardiomyopathy. *Circulation* **107:** 1390-1395.

Gentile M, Latonen L, Laiho M. (2003) Cell cycle arrest and apoptosis provoked by UV radiation-induced DNA damage are transcriptionally highly divergent responses. *Nucleic Acids Res* **31:** 4779-4790.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodal Y, Goodal GJ. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10:** 593-601.

Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. (2002) Roles of TGF-beta in hepatic fibrosis. *Front Biosci* **7:** 793-807.

Grubinger M, Gimona M. (2004) CRP2 is an autonomous actin-binding protein. *FEBS Lett* **557:** 88-92.

Grzanka D, Domaniewski J, Grzanka A, Zuryn A. (2006) Ultraviolet radiation (UV) induces reorganization of actin cytoskeleton in CHOAA8 cells. *Neoplasma* **53:** 328-332.

Gullotti L, Czerwitzki J, Kirfel J, Propping P, Rahner N, Steinke V, Kahl P, Engel C, Schüle R, Buettner R, Friedrichs N. (2011) FHL2 expression in peritumoural fibroblasts correlates with lymphatic metastasis in sporadic but not in HNPCC-associated colon cancer. *Lab Invest.* Aug. 8. Epub ahead of print

Hall A. (2009) The cytoskeleton and cancer. *Cancer Metastasis Rev* **28**: 5-14.

Halliday GM. (2005) Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat Res* **571:** 107-120.

Hanahan D, Weinberg RA. (2011) Hallmarks of cancer: The next generation. *Cell* **144:** 646-674.

Hardie WD, Hagood JS, Dave V, Perl AK, Whitsett JA, Korfhagen TR, Glasser S. (2010) Signaling pathways in the epithelial origins of pulmonary fibrosis. *Cell Cycle* **9:** 2769-2776.

Harper BD, Beckerle MC, Pomies P. (2000) Fine mapping of the alpha-actinin binding site within cysteine-rich protein. *Biochem J* **350:** 269-274.

Hautmann MB, Madsen CS, Owens GK. (1997) A transforming growth factor beta (TGFbeta) control element drives TGFbeta-induced stimulation of smooth muscle alpha-actin gene expression in concert with two CArG elements. *J Biol Chem* **272:** 10948-10956.

Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, Pennathur S, Martinez FJ, Thannickal VJ. (2009) NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat Med* **15:** 1077-1081.

Henderson JR, Macalma T, Brown D, Richardson JA, Olson EN, Beckerle MC. (1999) The LIM protein, CRP1, is a smooth muscle marker. *Dev Dyn* **214:** 229-238.

Hensbergen P, Alewijnse A, Kempenaar J, van der Schors RC, Balog CA, Deelder A, Beumer G, Ponec M, Tensen CP. (2005) Proteomic profiling identifies an UV-induced activation of cofilin-1 and destrin in human epidermis. *J Invest Dermatol* **124:** 818-824.

Herrmann J, Borkham-Kamphorst E, Haas U, Van de Leur E, Fraga MF, Esteller M, Gressner AM, Weiskirchen R. (2006) The expression of CSRP2 encoding the LIM domain protein CRP2 is mediated by TGF-beta in smooth muscle and hepatic stellate cells. *Biochem Biophys Res Commun* **345:** 1526-1535.

Hervy M, Hoffman LM, Jensen CC, Smith M, Beckerle MC. (2010) The LIM protein zyxin binds CARP-1 and promotes apoptosis. *Genes Cancer* **1:** 506-515.

Hinz B. (2010) The myofibroblast: paradigm for a mechanically active cell. *J Biomech* **43**: 146-55

Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. (2001) Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* **12:** 2730-2741.

Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. (2007) The myofibroblast: One function, multiple origins. *Am J Pathol* **170:** 1807-1816.

Hirasawa Y, Arai M, Imazeki F, Tada M, Mikata R, Fukai K, Miyazaki M, Ochiai T, Saisho H, Yokosuka O. (2006) Methylation status of genes upregulated by demethylating agent 5-aza-2'-deoxycytidine in hepatocellular carcinoma. *Oncology* **71:** 77-85.

Hirota T, Morisaki T, Nishiyama Y, Marumoto T, Tada K, Hara T, Masuko N, Inagaki M, Hatakeyama K, Saya H. (2000) Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. *J Cell Biol* **149:** 1073-1086.

Hoffman LM, Jensen CC, Kloeker S, Wang CL, Yoshigi M, Beckerle MC. (2006) Genetic ablation of zyxin causes Mena/VASP mislocalization, increased motility, and deficits in actin remodeling. *J Cell Biol* **172:** 771-782.

Hsu FF, Lin TY, Chen JY, Shieh SY. (2010) p53-mediated transactivation of LIMK2b links actin dynamics to cell cycle checkpoint control. *Oncogene* **29:** 2864-2876.

Hu B, Wu Z, Phan SH. (2003) Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am J Respir Cell Mol Biol* **29:** 397-404.

Hu Q, Guo C, Li Y, Aronow BJ, Zhang J. (2011) LMO7 mediates cell-specific activation of rho-MRTF-SRF pathway and plays an important role in breast cancer cell migration. *Mol Cell Biol* **31:** 3223-40.

Hyytiainen M, Penttinen C, Keski-Oja J. (2004) Latent TGF-beta binding proteins: Extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* **41:** 233-264.

Ikushima H, Miyazono K. (2010) TGFbeta signalling: A complex web in cancer progression. *Nat Rev Cancer* **10:** 415-424.

Ito N, Kawata S, Tamura S, Shirai Y, Kiso S, Tsushima H, Matsuzawa Y. (1995) Positive correlation of plasma transforming growth factor-beta 1 levels with tumor vascularity in hepatocellular carcinoma. *Cancer Lett* **89:** 45-48.

Ito S, Takahara Y, Hyodo T, Hasegawa H, Asano E, Hamaguchi M, Senga T. (2010) The roles of two distinct regions of PINCH-1 in the regulation of cell attachment and spreading. *Mol Biol Cell* **21:** 4120-4129.

Jang HS, Greenwood JA. (2009) Glycine-rich region regulates cysteine-rich protein 1 binding to actin cytoskeleton. *Biochem Biophys Res Commun* **380:** 484-488.

Jung CR, Lim JH, Choi Y, Kim DG, Kang KJ, Noh SM, Im DS. (2010) Enigma negatively regulates p53 through MDM2 and promotes tumor cell survival in mice. *J Clin Invest* **120:** 4493-4506.

Kadrmas JL, Beckerle MC. (2004) The LIM domain: From the cytoskeleton to the nucleus. *Nat Rev Mol Cell Biol* **5:** 920-931.

Kapoun AM, Gaspar NJ, Wang Y, Damm D, Liu YW, O'young G, Quon D, Lam A, Munson K, Tran TT, Ma JY, Murphy A, Dugar S, Chakravarty S, Protter AA, Wen FQ, Liu X, Rennard SI, Higgins LS. (2006) Transforming growth factor-beta receptor type 1 (TGFbetaRI) kinase activity but not p38 activation is required for TGFbetaRI-induced myofibroblast differentiation and profibrotic gene expression. *Mol Pharmacol* **70:** 518-531.

Kastan MB, Bartek J. (2004) Cell-cycle checkpoints and cancer. *Nature* **432:** 316-323.

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* **6:** 1365-1375.

Khalil N, O'Connor RN, Flanders KC, Unruh H. (1996) TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: An immunohistochemical study. *Am J Respir Cell Mol Biol* **14:** 131-138.

Khalil N, O'Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Bereznay OH, Greenberg AH. (1991) Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* **5:** 155-162.

Kihara T, Shinohara S, Fujikawa R, Sugimoto Y, Murata M, Miyake J. (2011) Regulation of cysteine-rich protein 2 localization by the development of actin fibers during smooth muscle cell differentiation. *Biochem Biophys Res Commun* **411**: 96-101

Kirchner J, Forbush KA, Bevan MJ. (2001) Identification and characterization of thymus LIM protein: Targeted disruption reduces thymus cellularity. *Mol Cell Biol* **21:** 8592-8604.

Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, Nukiwa T, Miyazawa K, Imamura T, Miyazono K. (2003) Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. *EMBO J* **22:** 6458-6470.

Kolosova I, Nethery D, Kern JA. (2011) Role of Smad2/3 and p38 MAP kinase in TGF-β1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J Cell Physiol* **226**: 1248-1254.

Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K, Miyazawa K. (2004) Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). *Oncogene* **23:** 6914-6923.

Kong Y, Flick MJ, Kudla AJ, Konieczny SF. (1997) Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. *Mol Cell Biol* **17:** 4750-4760.

Konig K, Diehl L, Rommerscheidt-Fuss U, Golletz C, Quast T, Kahl P, Kolanus W, Knolle P, Buettner R, Heukamp LC. (2010) Four-and-a-half LIM domain protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. *J Immunol* **185:** 1466-1475.

Kostek MC, Chen YW, Cuthbertson DJ, Shi R, Fedele MJ, Esser KA, Rennie MJ. (2007) Gene expression responses over 24 h to lengthening and shortening contractions in human muscle: Major changes in CSRP3, MUSTN1, SIX1, and FBXO32. *Physiol Genomics* **31:** 42-52.

Kulkarni AA, Thatcher TH, Olsen KC, Maggirwar SB, Phipps RP, Sime PJ. (2011) PPAR-gamma ligands repress TGFbeta-induced myofibroblast differentiation by targeting the PI3K/Akt pathway: Implications for therapy of fibrosis. *PLoS One* **6:** e15909.

Kulms D, Dussmann H, Poppelmann B, Stander S, Schwarz A, Schwarz T. (2002) Apoptosis induced by disruption of the actin cytoskeleton is mediated via activation of CD95 (Fas/APO-1). *Cell Death Differ* **9:** 598-608.

Kulms D, Schwarz T. (2000) Molecular mechanisms of UV-induced apoptosis. *Photodermatol Photoimmunol Photomed* **16:** 195-201.

Kuo JC, Han X, Hsiao CT, Yates JR 3rd, Waterman CM. (2011) Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for beta-pix in negative regulation of focal adhesion maturation. *Nat Cell Biol* **13:** 383-393.

Kuratomi G, Komuro A, Goto K, Shinozaki M, Miyazawa K, Miyazono K, Imamura T. (2005) NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. *Biochem J* **386:** 461-470.

Kuroda S, Tokunaga C, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, Gill GN, Kikkawa U. (1996) Protein-protein interaction of zinc finger LIM domains with protein kinase C. *J Biol Chem* **271:** 31029-31032.

Lambert KE, Huang H, Mythreye K, Blobe GC. (2011) The type III transforming growth factor-{beta} receptor inhibits proliferation, migration, and adhesion in human myeloma cells. *Mol Biol Cell* **22:** 1463-1472.

Larsen S, Yokochi T, Isogai E, Nakamura Y, Ozaki T, Nakagawara A. (2010) LMO3 interacts with p53 and inhibits its transcriptional activity. *Biochem Biophys Res Commun* **392:** 252-257

Latonen L, Taya Y, Laiho M. (2001) UV-radiation induces dose-dependent regulation of p53 response and modulates p53-HDM2 interaction in human fibroblasts. *Oncogene* **20:** 6784-6793.

Lau LF, Nathans D. (1985) Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO J* **4:** 3145-3151.

Lehti M, Kivela R, Komi P, Komulainen J, Kainulainen H, Kyrolainen H. (2009) Effects of fatiguing jumping exercise on mRNA expression of titin-complex proteins and calpains. *J Appl Physiol* **106:** 1419-1424.

Levy L, Hill CS. (2006) Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* **17:** 41-58.

Liang X, Sun Y, Ye M, Scimia MC, Cheng H, Martin J, Wang G, Rearden A, Wu C, Peterson KL, Powell HC, Evans SM, Chen J. (2009) Targeted ablation of PINCH1 and PINCH2 from murine myocardium results in dilated cardiomyopathy and early postnatal lethality. *Circulation* **120:** 568-76.

Liebhaber SA, Emery JG, Urbanek M, Wang XK, Cooke NE. (1990) Characterization of a human cDNA encoding a widely expressed and highly conserved cysteine-rich protein with an unusual zinc-finger motif. *Nucleic Acids Res* **18:** 3871-3879.

Li G, Tron V, Ho V. (1998) Induction of squamous cell carcinoma in p53-deficient mice after ultraviolet irradiation. *J Invest Dermatol* **110:** 72-75.

Lilly B, Clark KA, Yoshigi M, Pronovost S, Wu ML, Periasamy M, Chi M, Paul RJ, Yet SF, Beckerle MC. (2010) Loss of the serum response factor cofactor, cysteine-rich protein 1, attenuates neointima formation in the mouse. *Arterioscler Thromb Vasc Biol* **30:** 694-701.

Lilly B, Olson EN, Beckerle MC. (2001) Identification of a CArG box-dependent enhancer within the cysteine-rich protein 1 gene that directs expression in arterial but not venous or visceral smooth muscle cells. *Dev Biol* **240:** 531-547.

Li L, Sampat K, Hu N, Zakari J, Yuspa SH. (2006) Protein kinase C negatively regulates akt activity and modifies UVC-induced apoptosis in mouse keratinocytes. *J Biol Chem* **281:** 3237-3243.

Lin DW, Chang IC, Tseng A, Wu ML, Chen CH, Patenaude CA, Layne MD, Yet SF. (2008) Transforming growth factor beta up-regulates cysteine-rich protein 2 in vascular smooth muscle cells via activating transcription factor 2. *J Biol Chem* **283:** 15003-15014.

Lin YH, Park ZY, Lin D, Brahmbhatt AA, Rio MC, Yates JR 3rd, Klemke RL. (2004) Regulation of cell migration and survival by focal adhesion targeting of lasp-1. *J Cell Biol* **165:** 421-432.

Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC, Shi Y, Chen YG, Meng A, Feng XH. (2006) PPM1A functions as a smad phosphatase to terminate TGFbeta signaling. *Cell* **125:** 915-928.

Lin X, Liang M, Feng XH. (2000) Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. *J Biol Chem* **275:** 36818-36822.

Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, Abraham E. (2010) miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med* **207:** 1589-1597.

Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J. (1991) Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* **67:** 785-795.

Loughran G, Healy NC, Kiely PA, Huigsloot M, Kedersha NL, O'Connor R. (2005) Mystique is a new insulin-like growth factor-I-regulated PDZ-LIM domain protein that promotes cell attachment and migration and suppresses anchorage-independent growth. *Mol Biol Cell* **16:** 1811-1822.

Louis HA, Pino JD, Schmeichel KL, Pomies P, Beckerle MC. (1997) Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. *J Biol Chem* **272:** 27484-27491.

Luo K. (2004) Ski and SnoN: Negative regulators of TGF-beta signaling. *Curr Opin Genet Dev* **14:** 65-70.

Luosujarvi H, Aro J, Tokola H, Leskinen H, Tenhunen O, Skoumal R, Szokodi I, Ruskoaho H, Rysä J. (2010) A novel p38 MAPK target dyxin is rapidly induced by mechanical load in the heart. *Blood Press* **19:** 54-63.

Maekawa TL, Takahashi TA, Fujihara M, Akasaka J, Fujikawa S, Minami M, Sekiquchi S. (1996) Effects of ultraviolet B irradiation on cell-cell interaction; implication of morphological changes and actin filaments in irradiated cells. *Clin Exp Immunol* **105:** 389-396.

Mailand N, Falck J, Lukas C, Syljuasen RG, Welcker M, Bartek J, Lukas J. (2000) Rapid destruction of human Cdc25A in response to DNA damage. *Science* **288:** 1425-1429.

Margulis V, Maity T, Zhang XY, Cooper SJ, Copland JA, Wood CG. (2008) Type III transforming growth factor-beta (TGF-beta) receptor mediates apoptosis in renal cell carcinoma independent of the canonical TGF-beta signaling pathway. *Clin Cancer Res.* **14**: 5722-30.

Maturana AD, Nakagawa N, Yoshimoto N, Tatematsu K, Hoshijima M, Tanizawa K, Kuroda S. (2011) LIM domains regulate protein kinase C activity: A novel molecular function. *Cell Signal* **23:** 928-934.

McCaffrey LM, Macara IG. (2011) Epithelial organization, cell polarity, and tumorigenesis. *Trends Cell Biol* Jul 20. Epub ahead of print

Meulmeester E, Ten Dijke P. (2011) The dynamic roles of TGF-beta in cancer. *J Pathol* **223:** 205-218.

Micalizzi DS, Farabaugh SM, Ford HL. (2010) Epithelial-mesenchymal transition in cancer: Parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia* **15:** 117-134.

Mille F, Thibert C, Fombonne J, Rama N, Guix C, Hayashi H, Corset V, Reed JC, Mehlen P. (2009) The patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. *Nat Cell Biol* **11:** 739-746.

Miralles F, Posern G, Zaromytidou AI, Treisman R. (2003) Actin dynamics control SRF activity by regulation of its cofactor MAL. Cell. **113:** 329-42.

Miyasaka KY, Kida YS, Sato T, Minami M, Ogura T. (2007) Csrp1 regulates dynamic cell movements of the mesendoderm and cardiac mesoderm through interactions with dishevelled and diversin. *Proc Natl Acad Sci U S A* **104:** 11274-11279.

Moran N. (2009) p38 kinase inhibitor approved for idiopathic pulmonary fibrosis. Nat Biotechnol. **29**: 301

Mori M, Nakagami H, Koibuchi N, Miura K, Takami Y, Koriyama H, Hayashi H, Sabe H, Mochizuki N, Morishita R, Kaneda Y. (2009) Zyxin mediates actin fiber reorganization in epithelial-mesenchymal transition and contributes to endocardial morphogenesis. *Mol Biol Cell* **20:** 3115-3124.

Morin P, Wickman G, Munro J, Inman GJ, Olson MF. (2011) Differing contributions of LIMK and ROCK to TGFbeta-induced transcription, motility and invasion. *Eur J Cell Biol* **90:** 13-25.

Mukhina S, Wang YL, Murata-Hori M. (2007) Alpha-actinin is required for tightly regulated remodeling of the actin cortical network during cytokinesis. *Dev Cell* **13:** 554-565.

Najwer I, Lilly B. (2005) Ca2+/calmodulin-dependent protein kinase IV activates cysteine-rich protein 1 through adjacent CRE and CArG elements. *Am J Physiol Cell Physiol* **289:** C785-93.

Narayanan DL, Saladi RN, Fox JL. (2010) Ultraviolet radiation and skin cancer. *Int J Dermatol* **49:** 978-986.

Naumanen P, Lappalainen P, Hotulainen P. (2008) Mechanisms of actin stress fibre assembly. *J Microsc* **231:** 446-454.

Nomura T, Nakajima H, Hongyo T, Taniguchi E, Fukuda K, Li LY, Kurooka M, Sutoh K, Hande PM, Kawaguchi T, Ueda M, Takatera H. (1997) Induction of cancer, actinic keratosis, and specific p53 mutations by UVB light in human skin maintained in severe combined immunodeficient mice. *Cancer Res* **57:** 2081-2084.

Norval M, Lucas RM, Cullen AP, de Gruijl FR, Longstreth J, Takizawa Y, van der Leun JC. (2011) The human health effects of ozone depletion and interactions with climate change. *Photochem Photobiol Sci* **10:** 199-225.

Okano I, Yamamoto T, Kaji A, Kimura T, Mizuno K, Nakamura T. (1993) Cloning of CRP2, a novel member of the cysteine-rich protein family with two repeats of an unusual LIM/double zinc-finger motif. *FEBS Lett* **333:** 51-55.

Olson EN, Nordheim A. (2010) Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol* **11:** 353-365.

Pandit KV, Corcoran D, Yousef H, Yarlagadda M, Tzouvelekis A, Gibson KF, Konishi K, Yousem SA, Singh M, Handley, D, Richards T, Selman M, Watkins SC, Pardo A, Ben-Yehudah A, Bouros D, Eickelberg O, Ray P, Benos PV, Kaminski N. (2010) Inhibition and role of let-7d in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* **182:** 220-229.

Pandit KV, Milosevic J, Kaminski N. (2011) MicroRNAs in idiopathic pulmonary fibrosis. *Transl Res* **157:** 191-199.

Papalouka V, Arvanitis DA, Vafiadaki E, Mavroidis M, Papadodima SA, Spiliopoulou CA, Kremastinos DT, Kranias EG, Sanoudou D. (2009) Muscle LIM protein interacts with cofilin 2 and regulates F-actin dynamics in cardiac and skeletal muscle. *Mol Cell Biol* **29:** 6046-6058.

Park WS, Lee HK, Lee JY, Yoo NJ, Kim CS, Kim SH. (1996) P53 mutations in solar keratoses. *Hum Pathol* **27:** 1180-1184.

Paul C, Lacroix M, Iankova I, Julien E, Schafer BW, Labalette C *et al*. (2006) The LIM-only protein FHL2 is a negative regulator of E4F1. *Oncogene* **25:** 5475-5484.

Pfeifer GP, You YH, Besaratinia A. (2005) Mutations induced by ultraviolet light. *Mutat Res* **571:** 19-31.

Pomies P, Louis HA, Beckerle MC. (1997) CRP1, a LIM domain protein implicated in muscle differentiation, interacts with alpha-actinin. *J Cell Biol* **139:** 157-168.

Po'uha ST, Shum MS, Goebel A, Bernard O, Kavallaris M. (2010) LIM-kinase 2, a regulator of actin dynamics, is involved in mitotic spindle integrity and sensitivity to microtubule-destabilizing drugs. *Oncogene* **29:** 597-607.

Prassler J, Murr A, Stocker S, Faix J, Murphy J, Marriott G. (1998) DdLIM is a cytoskeleton-associated protein involved in the protrusion of lamellipodia in Dictyostelium. *Mol Biol Cell*. **9:** 545-59

Pratt SJ, Epple H, Ward M, Feng Y, Braga VM, Longmore GD. (2005) The LIM protein ajuba influences p130Cas localization and Rac1 activity during cell migration. *J Cell Biol* **168**: 813-24

Qu Z, Fu J, Yan P, Hu J, Cheng SY, Xiao G. (2010a) Epigenetic repression of PDZ-LIM domain-containing protein 2: Implications for the biology and treatment of breast cancer. *J Biol Chem* **285:** 11786-11792.

Qu Z, Yan P, Fu J, Jiang J, Grusby MJ, Smithgall TE, Xiao G. (2010b) DNA methylation-dependent repression of PDZ-LIM domain-containing protein 2 in colon cancer and its role as a potential therapeutic target. *Cancer Res* **70:** 1766-1772.

Rahimi RA, Leof EB. (2007) TGF-beta signaling: A tale of two responses. *J Cell Biochem* **102:** 593-608.

Raman D, Sai J, Neel NF, Chew CS, Richmond A. (2010) LIM and SH3 protein-1 modulates CXCR2-mediated cell migration. *PLoS One* **5:** e10050.

Rasanen K, Vaheri A. (2010) Activation of fibroblasts in cancer stroma. *Exp Cell Res* **316:** 2713-2722.

Rass K, Reichrath J. (2008) UV damage and DNA repair in malignant melanoma and nonmelanoma skin cancer. *Adv Exp Med Biol* **624:** 162-178.

Rastogi RP, Richa, Kumar A, Tyagi MB, Sinha RP. (2010) Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *J Nucleic Acids* **2010:** 592980.

Rittie L, Fisher GJ. (2002) UV-light-induced signal cascades and skin aging. *Ageing Res Rev* **1:** 705-720.

Sadler I, Crawford AW, Michelsen JW, Beckerle MC. (1992) Zyxin and cCRP: Two interactive LIM domain proteins associated with the cytoskeleton. *J Cell Biol* **119:** 1573-1587.

Saeki N, Kim DH, Usui T, Aoyagi K, Tatsuta T, Aoki K, Yanagihara K, Tamura M, Mizushima H, Sakamoto H, Ogawa K, Ohki M, Shiroishi T, Yoshida T, Sasaki H. (2007) GASDERMIN, suppressed frequently in gastric cancer, is a target of LMO1 in TGF-beta-dependent apoptotic signalling. *Oncogene* **26:** 6488-6498.

Sagave JF, Moser M, Ehler E, Weiskirchen S, Stoll D, Gunther K, Bütner R, Weiskirchen R. (2008) Targeted disruption of the mouse Csrp2 gene encoding the cysteine- and glycine-rich LIM domain protein CRP2 result in subtle alteration of cardiac ultrastructure. *BMC Dev Biol* **8:** 80.

Santander C, Brandan E. (2006) Betaglycan induces TGF-beta signaling in a ligand-independent manner, through activation of the p38 pathway. *Cell Signal* **18**:1482-1491.

Sapkota G, Knockaert M, Alarcon C, Montalvo E, Brivanlou AH, Massague J. (2006) Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. *J Biol Chem* **281:** 40412-40419.

Schiller HB, Friedel CC, Boulegue C, Fassler R. (2011) Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins. *EMBO Rep* **12:** 259-266.

Scholl FA, McLoughlin P, Ehler E, de Giovanni C, Schafer BW. (2000) DRAL is a p53-responsive gene whose four and a half LIM domain protein product induces apoptosis. *J Cell Biol* **151:** 495-506.

Schmeichel KL, Beckerle MC. (1998) LIM domains of cysteine-rich protein 1 (CRP1) are essential for its zyxin-binding function. *Biochem J* **331:** 885-892.

Schmeichel KL, Beckerle MC. (1994) The LIM domain is a modular protein-binding interface. *Cell* **79:** 211-219.

Schmidt A, Hall MN. (1998) Signaling to the actin cytoskeleton. *Annu Rev Cell Dev Biol* **14:** 305-338.

Schmitt J, Seidler A, Diepgen TL, Bauer A. (2011) Occupational ultraviolet light exposure increases the risk for the development of cutaneous squamous cell carcinoma: A systematic review and meta-analysis. *Br J Dermatol* **164:** 291-307.

Scotton CJ, Chambers RC. (2007) Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Chest* **132**:1311-1321.

Selvaraj A, Prywes R. (2004) Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. BMC Mol Biol. **5**:13

Sesto A, Navarro M, Burslem F, Jorcano JL. (2002) Analysis of the ultraviolet B response in primary human keratinocytes using oligonucleotide microarrays. *Proc Natl Acad Sci U S A* **99:** 2965-2970.

Sharp TV, Munoz F, Bourboulia D, Presneau N, Darai E, Wang HW, Cannon M, Butcher DN, Nicholson AG, Klein G, Imreh S. Boshoff C. (2004) LIM domains-containing protein 1 (LIMD1), a tumor suppressor encoded at chromosome 3p21.3, binds pRB and represses E2F-driven transcription. *Proc Natl Acad Sci U S A* **101:** 16531-16536.

Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. (1997) Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* **100:** 768-776.

Smith MA, Blankman E, Gardel ML, Luettjohann L, Waterman CM, Beckerle MC. (2010) A zyxin-mediated mechanism for actin stress fiber maintenance and repair. *Dev Cell* **19:** 365-376.

Somosy Z. (2000) Radiation response of cell organelles. *Micron* **31:** 165-181.

Sousa AM, Liu T, Guevara O, Stevens J, Fanburg BL, Gaestel M, Toksoz D, Kayyali US. (2007) Smooth muscle alpha-actin expression and myofibroblast differentiation by TGFbeta are dependent upon MK2. J Cell Biochem **100**:1581-1592.

Stokes MP, Rush J, Macneill J, Ren JM, Sprott K, Nardone J, Yang V, Beausoleil SA, Gygi SP, Livingstone M, Zhang H, Polakiewicz RD, Comb MJ. (2007) Profiling of UV-induced ATM/ATR signaling pathways. *Proc Natl Acad Sci U S A* **104:** 19855-19860.

Sumi T, Hashigasako A, Matsumoto K, Nakamura T. (2006) Different activity regulation and subcellular localization of LIMK1 and LIMK2 during cell cycle transition. *Exp Cell Res* **312:** 1021-1030.

Takasawa R, Nakamura H, Mori T, Tanuma S. (2005) Differential apoptotic pathways in human keratinocyte HaCaT cells exposed to UVB and UVC. *Apoptosis* **10:** 1121-1130.

Thomas C, Hoffmann C, Dieterle M, Van Troys M, Ampe C, Steinmetz A. (2006) Tobacco WLIM1 is a novel F-actin binding protein involved in actin cytoskeleton remodeling. *Plant Cell* **18:** 2194-2206.

Thomas C, Hoffmann C, Gatti S, Steinmetz A. (2007a) LIM proteins: A novel class of actin cytoskeleton organizers in plants. *Plant Signal Behav* **2:** 99-100.

Thomas C, Moreau F, Dieterle M, Hoffmann C, Gatti S, Hofmann C, Van Troys M, Ampe C, Steinmetz A. (2007b) The LIM domains of WLIM1 define a new class of actin bundling modules. *J Biol Chem* **282:** 33599-33608.

Tian Y, Wang N, Lu Z. (2010) Repression of lim only protein 4-activated transcription inhibits proliferation and induces apoptosis of normal mammary epithelial cells and breast cancer cells. *Clin Exp Metastasis* **27:** 455-463.

Tran TC, Singleton C, Fraley TS, Greenwood JA. (2005) Cysteine-rich protein 1 (CRP1) regulates actin filament bundling. *BMC Cell Biol* **6:** 45.

Trautinger F. (2001) Mechanisms of photodamage of the skin and its functional consequences for skin ageing. *Clin Exp Dermatol* **26:** 573-577.

Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK. (2007) Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci U S A.* **104**:7552-7.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95:** 779-791.

Valery C, Grob JJ, Verrando P. (2001) Identification by cDNA microarray technology of genes modulated by artificial ultraviolet radiation in normal human melanocytes: Relation to melanocarcinogenesis. *J Invest Dermatol* **117:** 1471-1482.

van Kranen HJ, de Laat A, van de Ven J, Wester PW, de Vries A, Berg RJ, van Kreijl CF, de Gruijl FR. (1997) Low incidence of p53 mutations in UVA (365-nm)-induced skin tumors in hairless mice. *Cancer Res* **57:** 1238-1240.

van Raam BJ, Salvesen GS. (2011) Proliferative versus apoptotic functions of caspase-8 hetero or homo: The caspase-8 dimer controls cell fate. *Biochim Biophys Acta* Jun 16. Epub ahead of print

Vardouli L, Vasilaki E, Papadimitriou E, Kardassis D, Stournaras C. (2008) A novel mechanism of TGFbeta-induced actin reorganization mediated by smad proteins and rho GTPases. *FEBS J* **275:** 4074-4087.

Velders M, Legerlotz K, Falconer SJ, Stott NS, McMahon CD, Smith HK. (2008) Effect of botulinum toxin A-induced paralysis and exercise training on mechanosensing and signalling gene expression in juvenile rat gastrocnemius muscle. *Exp Physiol* **93:** 1273-1283.

Visvader JE, Venter D, Hahm K, Santamaria M, Sum EY, O'Reilly L, White D, Williams R, Armes J, Lindeman GJ. (2001) The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proc Natl Acad Sci U S A* **98:** 14452-14457.

Wang H, Song K, Krebs TL, Yang J, Danielpour D. (2008) Smad7 is inactivated through a direct physical interaction with the LIM protein hic-5/ARA55. *Oncogene* **27:** 6791-6805.

Wang H, Song K, Sponseller TL, Danielpour D. (2005) Novel function of androgen receptor-associated protein 55/Hic-5 as a negative regulator of Smad3 signaling. *J Biol Chem* **280:** 5154-5162.

Wang K, Diskin SJ, Zhang H, Attiyeh EF, Winter C, Hou C, Schnepp RW, Diamond M, Bosse K, Mayes PA, Glessner J, Kim C, Frackelton E, Garris M, Wang Q, Glaberson, W, Chiavacci R, Nguyen L, Jagannathan J, Saeki N, Sasaki H, Grant SF, Iolascon A, Mosse YP, Cole KA, Li H, Devote M, McGrady PW, London WB, Capasso M, Rahman N, Hakonarson H, Maris JM. (2011) Integrative genomics identifies LMO1 as a neuroblastoma oncogene. *Nature* **469:** 216-220.

Wang W, Mouneimne G, Sidani M, Wyckoff J, Chen X, Makris A, Goswami S, Bresnick AR, Condeelis JS. (2006) The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. *J Cell Biol* **173:** 395-404.

Wang XF, Lin HY, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA. (1991) Expression cloning and characterization of the TGF-beta type III receptor. *Cell* **67:** 797-805.

Wang X, Lee G, Liebhaber SA, Cooke NE. (1992) Human cysteine-rich protein. A member of the LIM/double-finger family displaying coordinate serum induction with c-myc. *J Biol Chem* **267:** 9176-9184.

Wang X, Ray K, Szpirer J, Levan G, Liebhaber SA, Cooke NE. (1992) Analysis of the human cysteine-rich protein gene (CSRP), assignment to chromosome 1q24-1q32, and identification of an associated MspI polymorphism. *Genomics* **14:** 391-397.

Watanabe Y, Itoh S, Goto T, Ohnishi E, Inamitsu M, Itoh F, Satoh K, Wiercinska E, Yang W, Shi L, Tanaka A, Nakano N, Mommaas AM, Shibuya H, Ten Dijke P, Kato M. (2010) TMEPAI, a

transmembrane TGF-beta-inducible protein, sequesters smad proteins from active participation in TGF-beta signaling. *Mol Cell* **37:** 123-134.

Wei J, Gorman TE, Liu X, Ith B, Tseng A, Chen Z, Simon DI, Layne MD, Yet SF. (2005) Increased neointima formation in cysteine-rich protein 2-deficient mice in response to vascular injury. *Circ Res* **97:** 1323-1331.

Weinreb O, Dovrat A, Dunia I, Benedetti EL, Bloemendal H. (2001) UV-A-related alterations of young and adult lens water-insoluble alpha-crystallin, plasma membranous and cytoskeletal proteins. *Eur J Biochem* **268:** 536-543.

Weiskirchen R, Erdel M, Utermann G, Bister K. (1997) Cloning, structural analysis, and chromosomal localization of the human CSRP2 gene encoding the LIM domain protein CRP2. *Genomics* **44:** 83-93

Weiskirchen R, Gressner AM. (2000) The cysteine- and glycine-rich LIM domain protein CRP2 specifically interacts with a novel human protein (CRP2BP). *Biochem Biophys Res Commun* **274:** 655-663.

Weiskirchen R, Gunther K. (2003) The CRP/MLP/TLP family of LIM domain proteins: Acting by connecting. *Bioessays* **25:** 152-162.

Weiskirchen R, Moser M, Weiskirchen S, Erdel M, Dahmen S, Buettner R, Gressner AM. (2001) LIM-domain protein cysteine- and glycine-rich protein 2 (CRP2) is a novel marker of hepatic stellate cells and binding partner of the protein inhibitor of activated STAT1. *Biochem J* **359:** 485-496.

Weiskirchen R, Pino JD, Macalma T, Bister K, Beckerle MC. (1995) The cysteine-rich protein family of highly related LIM domain proteins. *J Biol Chem* **270:** 28946-28954.

Wikstrom P, Stattin P, Franck-Lissbrant I, Damber JE, Bergh A. (1998) Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* **37:** 19-29.

Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. (2005) Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol.* **166**:1321-32.

Wojtowicz A, Babu SS, Li L, Gretz N, Hecker M, Cattaruzza M. (2010) Zyxin mediation of stretch-induced gene expression in human endothelial cells. *Circ Res* **107:** 898-902.

Wynn TA. (2008) Cellular and molecular mechanisms of fibrosis. *J Pathol* **214:** 199-210.

Xaubet A, Marin-Arguedas A, Lario S, Ancochea J, Morell F, Ruiz-Manzano J, Rodriguez-Becerra E, Rodriguez-Arias JM, Inigo P, Sanz S, Campistol JM, Mullol J, Picado C. (2003) Transforming growth factor-beta1 gene polymorphisms are associated with disease progression in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* **168:** 431-435.

Xu J, Lamouille S, Derynck R. (2009) TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* **19:** 156-72.

Yan X, Chen YG. (2011) Smad7: Not only a regulator, but also a cross-talk mediator of TGF-beta signalling. *Biochem J* **434:** 1-10.

Yao X, Perez-Alvarado GC, Louis HA, Pomies P, Hatt C, Summers MF, Beckerle MC. (1999) Solution structure of the chicken cysteine-rich protein, CRP1, a double-LIM protein implicated in muscle differentiation. *Biochemistry* **38:** 5701-5713.

Yet SF, Folta SC, Jain MK, Hsieh CM, Maemura K, Layne MD, Zhang D, Marria PB, Yoshizumi M, Chin MT, Perrella MA, Lee ME. (1998) Molecular cloning, characterization, and promoter analysis of the mouse Crp2/SmLim gene. preferential expression of its promoter in the vascular smooth muscle cells of transgenic mice. *J Biol Chem* **273:** 10530-10537.

Yilmaz M, Christofori G. (2009) EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* **28:** 15-33.

Yoshida K, Kuwano K, Hagimoto N, Watanabe K, Matsuba T, Fujita M, Inoshima I, Hara N. (2002) MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. *J Pathol* **198**: 388-396.

Yoshigi M, Hoffman LM, Jensen CC, Yost HJ, Beckerle MC. (2005) Mechanical force mobilizes zyxin from focal adhesions to actin filaments and regulates cytoskeletal reinforcement. *J Cell Biol* **171:** 209-215.

You HJ, Bruinsma MW, How T, Ostrander JH, Blobe GC. (2007) The type III TGF-beta receptor signals through both Smad3 and the p38 MAP kinase pathways to contribute to inhibition of cell proliferation. *Carcinogenesis* **28**: 2491-2500.

Yu J, Ohuchida K, Nakata K, Mizumoto K, Cui L, Fujita H, Yamaguchi H, Egami T, Kitada H, Tanaka M. (2008) LIM only 4 is overexpressed in late stage pancreas cancer. *Mol Cancer* **7:** 93.

Yu L, Hébert MC, Zhang YE. (2002) TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *EMBO J* **21**: 3749-3759.

Zavadil J, Bottinger EP. (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24:** 5764-5774.

Zhang F, Lundin M, Ristimaki A, Heikkila P, Lundin J, Isola J, Joensuu H, Laiho M. (2003) Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas. *Cancer Res* **63:** 5005-5010.

Zhang S, Wang X, Osunkoya AO, Iqbal S, Wang Y, Chen Z, Müller S, Chen Z, Josson Z, Coleman IM, Nelson PS, Wang YA, Wang R, Shin DM, Marshall FF, Kucuk O, Chung LW, Zhau HE, Wu D. (2011) EPLIN downregulation promotes epithelial-mesenchymal transition in prostate cancer cells and correlates with clinical lymph node metastasis. *Oncogene* May 30. Epub ahead of print

Zhang Y, Chen K, Guo L, Wu C. (2002) Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading, and migration. *J Biol Chem* **277:** 38328-38338.

Zhang YE. (2009) Non-smad pathways in TGF-beta signaling. *Cell Res* **19:** 128-139.

Zheng B, Wen JK, Han M. (2008) hhLIM is a novel F-actin binding protein involved in actin cytoskeleton remodeling. *FEBS J* **275:** 1568-1578.

Zheng Q, Zhao Y. (2007) The diverse biofunctions of LIM domain proteins: Determined by subcellular localization and protein-protein interaction. *Biol Cell* **99:** 489-502.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE. (1994) Sunburn and p53 in the onset of skin cancer. *Nature* **372:** 773-776.

Zolk O, Caroni P, Bohm M. (2000) Decreased expression of the cardiac LIM domain protein MLP in chronic human heart failure. *Circulation* **101:** 2674-2677.