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Dissecting domain-specific functions of the IGF2/M6P receptor

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by

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Thesis Abstract

The P-type lectin Mannose 6-phosphate/Insulin-like growth factor 2 receptor (M6P/IGF2R) is a type I trans-membrane protein with 15 homologous extracellular domains that bind several ligands. While domains 3, 5 and 9 bind to Mannose 6-Phosphate (M6P), domain 11 binds to IGF2 (Insulin-like growth factor 2). In mice, *igf2r* expression is monoallelic and full knock-out of the receptor maternal allele (*Igf2r^{m/+p}*) results in developmental abnormalities and neonatal lethality, marking the significance of the receptor in organogenesis and postnatal viability. What remains unknown is the contribution of each of these domains into M6P/IGF2R's roles in development. My aims are dissecting M6P and IGF2 binding domains' functions in the context of full length receptor *in vitro* and investigating Domain 11 loss-of-function *in vivo*.

In this thesis, SPR analysis of full-length M6P/IG2R mutants showed that M6P binding domains function independent of each other, and domain 3 is the dominant M6P binding domain at physiological pH. Introducing gain-of-function mutations to domain 11 binding site revealed presence of inter-loop interactions and inhibitory residues in CD and FG loop in the context of the full-length receptor. I1572A results in domain 11 loss of-function mutation with preserved binding of other ligands, suggesting that I1572A (or its homolog mutations in other mammals) could be utilized in *in vivo* studies to dissect domain 11 functions. In mice, maternal transmission of Domain 11 loss-of-binding function mutation (I1565A, mouse homolog of I1572A) resulted in increased birth weight and substantially reduced postnatal survival (~30%). The disproportional cardiomegaly and disorganised myocardium in *Igf2r^{I1565Am/+p}* neonates suggest that postnatal lethality is due to cardiac insufficiency. Furthermore, placental and embryonic oedema in *Igf2r^{I1565Am/+p}* mice is indicative of the presence of prenatal congestive cardiac failure, beginning as early as at embryonic day 14.5. In addition, surviving females developed uterine distension due to vaginal atresia. Overall, these results show that domain 11 loss-of-function, on its own, is the primary reason for the most of abnormalities that is previously reported in the full knock out mice. Thus, domain 11 is the primary domain for M6P/IGF2R's roles in development.

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Chapter 1: Introduction

Growth is indispensable for reproduction of multicellular organisms. The process of growth includes increase in size and complexity. Size increase is achieved by increase in cell number, cell volume and extracellular matrix (accretion). Growth is a dynamic, complex and tightly regulated process of cellular proliferation, apoptosis, differentiation and cellular migration accompanied with continuous synthesis and reformation of extracellular structural elements. Animals and plant's evolution to multicellularity happened separately, leading to members of each kingdom developing distinct growth patterns and mechanisms (Meyerowitz 1999). Animals, compared to plants, generally have a limited final size, grow mostly with increase in cell number rather than cell size and the cellular proliferation is not restricted to certain cell types but involves most of the tissues types.

The growth rate and pattern in the organisms varies in a temporal and spatial manner. In placental mammals, the zygote formation is followed by cleavage which is continuous cellular divisions with no significant increase in total mass of the cells until to the point of implantation to the uterine wall (Wassarman 1999). With the commencement of nutritional supply upon implantation, the loss in cellular size and lack of increase in total mass get replaced by progressive increase in total size that continues until attainment of full postnatal maturity. With the onset of organogenesis - requiring differentiation, extracellular matrix and organ-specific architecture formation - the growth rates decelerate in mouse (Snow 1986). However, generally, human foetal growth follows an "S" shaped curve that plateaus after 37th week (Peleg, Kennedy et al. 1998) While during early foetal development growth is determined by the factors that are intrinsic to the embryos, during the late gestational period uterine (maternal) conditions also influence the birth size. The maternal factors include oxygen and nutritional supply, infections, uterine anatomy and litter size. Human and mouse neonates are born with average weights of 3.5 kg and 1-2gr, respectively (Bocking 2001).

1.1 Embryogenesis

Following zygote formation as a result of fertilisation of the ovum by a sperm, several cellular rounds of cleavage divisions form a cell ball called morula. Further proliferation with concurrent structural transformation and cellular differentiation forms a round outer cell layer (trophoectoderm) with a cavity carrying a cluster of cells (embryoblast) that is attached to the cavity wall (Cockburn and Rossant 2010). This structure is called blastocyst and performs the implantation to the uterine wall. In human and mouse, morphologically, the perimplantation stages closely resemble to each other (Niakan, Han et al. 2012). Outer trophoectodermal cells of the blastocyst are specialised to initiate the implantation to the endometrium with disintegration of the zona pellucida, preparation of the endometrium for implantation through molecular mediators, responding to the cytokines released by the endometrial epithelium and finally by adhesion to and invasion of the uterine wall (Ma, Song et al. 2003, Wang and Dey 2006). The inner cell mass of embryoblasts of the blastocysts start to differentiate into two distinct cell types that will form precursors of the embryo and its membranes. These two cell populations are called epiblasts and hypoblasts. While hypoblasts form yolk sac, epiblasts differentiate into three main embryonic layers of the embryo proper: Endoderm, mesoderm and ectoderm (Sheng 2015). The endodermal layer is the origin of most of the gastrointestinal and respiratory systems, endocrine glands, and epithelium of some parts of the genitourinary and auditory systems (Gilbert 2000) The ectoderm give rise to skin and its appendices, nervous tissue and sensory organs, and the epithelia of the body openings. Finally, mesoderm differentiates to musculoskeletal, cardiovascular, genitourinary, hematopoetic and lymphopoetic systems.

1.2 Placentogenesis

Nutritional supply, gas and metabolite exchange for growth and survival of the foetus are regulated through the extensive feto-maternal exchange interface that is packed into the placenta. In rodents and human, the placenta is composed of three main zones (Rossant and

Cross 2001). Decidualised cells of the uterine endometrium and maternal vessels form the outermost layer. On the luminal side, the foetal vasculature forms a network that grows into vessels and forms the core of the placental villi. The intermediate layer contains the feto-maternal exchange interface.

Placentogenesis begins with blastocyst homing of the endometrial crypts, followed by the invasion of the luminal epithelium with the trophoectodermal cells (Cha, Bartos et al. 2014). In mouse, in contrast to human, the inner cell mass is located opposite the implantation initiation site of the blastocysts. In mouse, some of the trophoectodermal cells, through endoreduplication generate terminally differentiated polyploid primary trophoblastic giant cells (Nadra, Anghel et al. 2006, Hu and Cross 2010). Unlike the first type of trophoectoderm that differentiates immediately, the latter group of cells continues to proliferate and differentiate to form ectoplacental cone and extraembryonic ectoderm (Carson 1999). In addition to trophoectodermal components, epiblast originated allantois is also essential to form foetal placenta (Downs, Temkin et al. 2001). A derivative of epiblasts, extraembryonic mesoderm, expands caudally to form allantois (Arora and Papaioannou 2012). Allantois further elongates and attaches to the extraembryonic ectoderm (chorion) on day 8.5 (Inman and Downs 2007). Upon attachment, chorionic trophoblasts invade the endometrium to form primary villi. The primary function of the allantois is to form vasculature of the fetal placenta, filling the branching chorionic villi. The resulting layer with highly branched villi of trophoblasts and the fetal vessels inside form the feto-maternal exchange area called the labyrinth. Between endometrial decidua and the labyrinth layer lie the spongiotrophoblast and glycogen cells bordered with trophoblastic giant cells on the maternal side. On the fetal side of the labyrinth, the murine placenta is connected to the umbilical cord by the chorionic plate.

1.3 Lung organogenesis

The lungs are air-filled intrathoracic organs and vital for survival during postnatal life due to their dominant role in gas exchange. *In utero*, with the placenta being the gas exchange port for the foetus, lungs are filled with fluid and are inactive. However, during the prenatal period, lower respiratory tract epithelia contribute to the production of amniotic fluid alongside the kidneys and foetal membranes (Underwood, Gilbert et al. 2005). Lungs originate from foregut endoderm and develop in four stages: embryonic & pseudoglandular (E9.5–16.6), canalicular (E16.6–17.4), terminal saccular (E17.4 to postnatal day 5), alveolar (Postnatal days 5–28/30) (Maeda, Dave et al. 2007, Warburton, El-Hashash et al. 2010). The pseudoglandular period is characterised by formation of bronchial and bronchiolar tubules, development of mesenchymal components (smooth muscle cells, connective tissue, blood and lymphatic vessels and cartilaginous structures) supporting this tubular tree (deMello, Sawyer et al. 1997, Maeda, Dave et al. 2007). During the canalicular stage, the further branching of the respiratory tree is accompanied by luminal enlargement. Functional respiratory units are built with squamous differentiation of the terminal tubular epithelium to form sacculi and extensive capillarization of the lung parenchyma. At the end of this phase, although not completed, lung development is sufficient for postnatal survival in case of a premature birth. The terminal saccular stage is a perinatal period, characterised by differentiation of type 1 and 2 pneumocytes, and surfactant production. Further increase in vascularisation and thinning of interstitial space also occur during this phase. Finally, postnatal lung maturation is completed with the alveolar stage that is characterised by an extensive increase in gas exchange surface.

1.4 Heart organogenesis

In vertebrates, heart formation starts with the formation of the primary heart tube from the mesoderm (Wessels and Sedmera 2003). The tube consists of three concentric layers. From lumen to outside these are: Endocardial epithelium, acellular cardiac jelly and myocardial

layer. The loop bends to form two interconnected compartments that will later form the ventricles and the atria (de Boer, van den Berg et al. 2012). Afterwards, dividing septa are formed in the atrium, ventricles and the atrioventricular junction (Krishnan, Samtani et al. 2014). Separation of the truncus arteriosus (a joint vessel that originates from the ventricles) occurs with formation of a spiral wall. Atrial septation is more complicated and occurs in multiple stages. Initially, first septum (septum primum) forms. As the septum grows, a small hole called the foramen primum, between the septum and the endocardial cushions, continues to function as a passage for the blood flow. While the foramen primum becomes smaller and is destined to disappear, some septal cells go under apoptosis to form the foramen secundum to ensure continuous inter-atrial blood flow. A second septum (septum secundum) is formed later to prevent reflux of blood from left to right atria.

1.5 Genomic imprinting and parental conflict of growth regulation

Many growth factors, their receptors and carriers are identified as crucial for growth and organogenesis. Regulation of gene expression by these proteins happens in temporal and spatial, as well as in an allele specific manner. For example, some of the mammalian genes, particularly growth regulating genes are expressed in a parent-of-origin specific manner (Mochizuki, Takeda et al. 1996). Generally, the growth restrictive or promoting nature of the genes determines which parental allele is going to be chosen for expression. Evolution of such an expression pattern is explained by a conflict of the maternal and paternal interests. The mother uses her resources for the growth of the foetus. Thus, it is in the mother's interest to use these resources in an efficient, economical manner for all of her current and future offspring. On the other hand, the paternal interest is to use the maternal resources maximally without any consideration of the needs of the mother, future offspring or of the other offspring in the same litter from another father. Because of this conflict of interest between parents, maternally transmitted growth restricting genes are programmed to be expressed, while the same genes that are paternally transmitted are imprinted (not expressed). Parental conflict of

interest is a popular hypothesis that is used to explain the imprinting pattern observed in mammalian genome, however many questions still remain to be answered (Iwasa 1998). Interestingly, imprinting status of some genes is not conserved across different species. For example, some growth regulating genes, such as insulin-like growth factor 2 receptor gene (*igf2r*) is imprinted in mouse, but has biallelic expression in human (Barlow, Stoger et al. 1991).

1.6 IGF2 system and its role in embryonic growth

Multiple growth regulating molecules that are structurally and functionally closely inter-related form growth regulating systems. Probably the most well-known of these growth regulating systems is insulin-like growth factor (IGF) system (summarised at Fig 1.1). The IGF system consist of two growth factors (IGF1 and IGF2), six high affinity plasma proteins and three main cell membrane receptors (Denley, Cosgrove et al. 2005). The ligands act in an autocrine, paracrine and endocrine manner to regulate growth and metabolism (Binoux 1995). While, the ligands are produced in many organs, the main site for their synthesis is the liver. As their names suggest, these ligands structurally and functionally resemble insulin and to each other (Rinderknecht and Humbel 1978, Dupont and Holzenberger 2003). For example, IGF1, IGF2 and insulin exhibit significant sequence similarity and overlapping roles in receptor activation. The IGF system has two main signalling receptors (insulin and IGF1 receptors) and their heterodimers. Insulin and IGF1 receptors also show structural and functional similarities (Ullrich, Gray et al. 1986, Nakae, Kido et al. 2001) and both carry tyrosine kinase activity that is located in their intra-cytoplasmic tails. In mammals, the insulin receptor exists in two isoforms depending on the presence of exon 11 in the mature mRNA (Mosthaf, Grako et al. 1990). The insulin receptor isoform A (IR-A) mRNA contains exon 11 in addition to the insulin receptor isoform B (IR-B) mRNA sequence.

IR-A is primarily a growth receptor that has a high affinity for IGF2 and is expressed during intrauterine life (Belfiore, Frasca et al. 2009). IR-B, however, is essential for the metabolic

effects of insulin and predominantly expressed during adulthood. Heteromeric form of insulin receptor isoforms (IR-A/IR-B) and IR/IGF1R also exist but their functional significance has yet to be fully investigated. IGF1R is also a mitogenic tyrosine kinase receptor for IGF1 but it binds IGF2 with three fold lower affinity (Pandini, Frasca et al. 2002). The insulin receptor and IGF2R have two subunits, extracellular α , transmembrane and intracellular β subunits (Schaefer, Siddle et al. 1990, Pandini, Frasca et al. 2002). Upon ligand binding to α subunits, β subunits move close to each other. The conformational changes result in phosphorylation of cytoplasmic tyrosine and leads to recruitment and phosphorylation of many substrates leading to PI3 kinase and MAP kinase activation (Ceresa and Pessin 1998, Taniguchi, Emanuelli et al. 2006).

Plasma levels of IGF1 and IGF2 are regulated by a group of high affinity soluble proteins called insulin-like growth factor binding proteins (IGFBPs). IGFBPs 1-6 have been identified so far, each of which exhibit distinct affinities for IGF1 and IGF2 (Bach, Hsieh et al. 1993). While, IGFBP6 shows the highest affinity for IGF1 and IGF2 followed by IGFBP3; IGFBP5 and 2 has the lowest binding affinity for IGFs. IGFBPs inhibits mitogenic effects of the IGFs by reducing their concentrations in free form but at the same time store these factors and increase their half-life (Firth and Baxter 2002). In addition to their storage functions in the plasma, IGFBPs that are bound to the cell surface and the matrix also store IGFs in intercellular space and give these growth factors close access to cell surface receptors. IGFBPs' release of IGFs happens upon their interaction with extracellular matrix components or proteolytical degradation.

A unique and hybrid component of the IGF system is the IGF2/M6P receptor (IGF2/M6PR). IGF2/M6PR is similar to IR and IGF1R and located on the cell surface. Similar to the IGFBPs, IGF2/M6PR regulates extracellular levels of IGF2 with a similar affinity that of IGFBP6's. 5-10% of IGF2/M6PR is located on the cell surface and the rest circulates between intracellular vesicular compartments (Scott and Firth 2004). These vesicular compartments include, the trans-golgi network (TGN) and endosomes, and IGF2 bound on the cell surface is released to

the endo-lysosomal compartment for intracellular degradation as a result of the trafficking between these compartments.

Whether IGF2/M6PR has a signalling function or not has been a subject of debate for the last two decades. Many studies suggested that IGF2 is not only signal through IGF1R and IR-A, but also activates G protein coupled signalling pathways through its interaction with IGF2/M6PR (Minniti, Kohn et al. 1992, Nishimoto 1993, Ikezu, Okamoto et al. 1995, Groskopf, Syu et al. 1997, Zhang, Tally et al. 1997, Tsuruta, Eddy et al. 2000, McKinnon, Chakraborty et al. 2001, El-Shewy, Lee et al. 2007, Maeng, Choi et al. 2009, Harris, Crocker et al. 2011). However, these studies failed to represent conclusive evidence supporting the signalling hypothesis due to the difficulty of designing a study to dissect IGF2 stimulated signalling functions of the receptor. These difficulties include the presence of three receptors for IGF2 and multiple ligands for IGF2/M6PR; difficulty of differentiating clearance and signalling functions of the receptor, and also dissecting signalling and trafficking functions of its cytoplasmic portion. For instance, in the studies that are published before the discovery of IR-A as the second signalling receptor for IGF2, signalling related changes attributed to IGF2/M6PR could have been observed due to IR-A activation. In some of the later studies, Leu²⁷ IGF2 (an IGF2 analogue that binds to IGF2/M6PR but not IGF1R) was used to exclude IGF1R mediated signalling and measure the effects of IGF2 binding to IGF2/M6PR. However, such an experimental design could not exclude IR-A mediated signalling and reduced IGF2 clearance due to occupation of the IGF2/M6PR' IGF2 binding site by Leu²⁷ IGF2 and. In addition, the receptor's cytoplasmic tail does not carry any kinase activity and does not interact with G proteins (Korner, Nurnberg et al. 1995) which also makes it unlikely for the receptor to have any signalling functions.

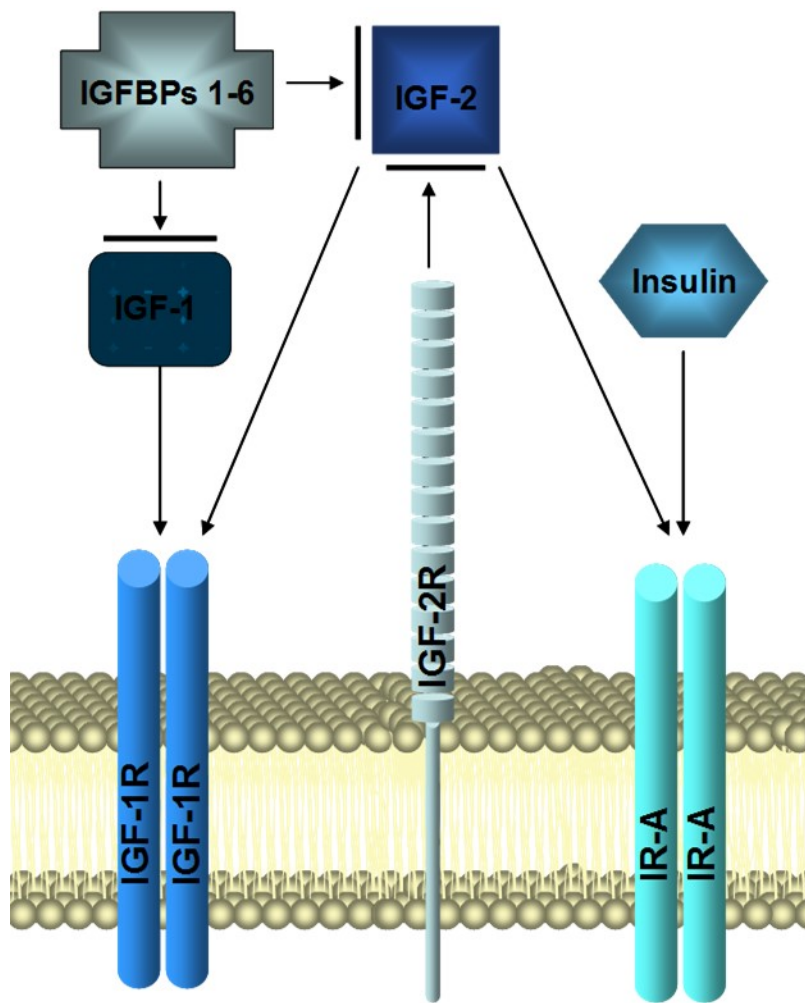


Figure 1.1 Interactions of IGF2 system: IGF1 and Insulin bind to IGF1R and Insulin receptors, respectively; and IGF2 binds to both receptors. Free IGF levels are regulated by IGFBPs and cell surface regulate IGF2 levels with cellular re-uptake.

Knock-outs of the genes that are encoding IGF system components confirmed that these genes' essential role in optimal embryonic growth. *Igf1* and *Igf2* knock-out mice exhibit similar growth restriction (60% of WT) (DeChiara, Efstratiadis et al. 1990, DeChiara, Robertson et al. 1991, Baker, Hardy et al. 1996, Efstratiadis 1998) suggesting that both ligands have similar importance in regulation of embryonic growth. *Igf1r* knock-out shows more severe phenotype compared to the *Igf1* and *Igf2* knock-outs (45% of WT). However, *Igf1/Igf1r* double knock-out showed no additional growth restriction suggesting that IGF1R is IGF1's only receptor but IGF1 is not the only ligand that the receptor binds (Ludwig, Eggenschwiler et al. 1996). When crossed with *Igf1* or *Igf1r* knock-outs, *Igf2* knock-outs weight only 30% of the WT, lower than *Igf1r* knock-outs (45% of WT) indicating presence of an additional growth regulating receptor for IGF2 in embryo. When *Igf2* and *Igf2r* knock-outs mice are crossed the resulting pups' weights are not significantly different than those of the WT. IR-A was later discovered to be the second receptor for IGF2 (Frasca, Pandini et al. 1999), and insulin receptor gene (*Insr*) and *Igf1r* double knock-out mouse has similar weight when compared to *Igf1/Igf2* or *Igf1r/Igf2* knock-outs.

In placenta, IGF system components' roles in growth change dramatically (Baker, Liu et al. 1993, Eggenschwiler, Ludwig et al. 1997, Efstratiadis 1998). While *Igf2* null mice show 25% reduction in placental size; *Igf1*, *Igf1r* and *Igf1r/Insr* knock-out mice exhibit normal placental size. Thus, an unknown receptor is responsible for IGF2's growth promoting role in placenta. Consistent with these observations, *H19*, *Igf2r* and *H19/Igf2r* null mutants have large placentas (140%, 140% and 230% of WT, respectively)

These results demonstrate key roles of the IGF system components in embryonic and placental growth; and also gave us significant information on the nature of IGF system ligand-receptor interactions.

1.7 *Igf2r* knock out mouse

To investigate the roles of IGF2/M6PR in development and growth, mouse models with *Igf2r* loss-of-function have been generated (Summarised at Table 1.1). All of these studies reported overgrowth phenotype and perinatal death. Lau et al, Ludwig et al. and Hughes et al. found the mutant heterozygous (maternal transmission) embryonic and placental weights are bigger than WT counterparts (Lau, Stewart et al. 1994, Ludwig, Eggenschwiler et al. 1996, Hughes, Frago et al. 2013). The embryonic and perinatal survival were found to be strain dependent in the paper that was published by Ludwig et al. and when the survival rates in these studies are compared (Table 1.1). When crossed with *Igf2* knock out mice, *Igf2r* knock out mice were rescued from perinatal lethality suggesting that excessive Igf2 levels was the cause of death. Except in Wang et al., other researchers identified cardiac abnormalities as a likely reason for lethality (Wang, Fung et al. 1994). The cardiac abnormalities that were described were cardiac enlargement and dilation. Although not explicitly mentioned in the text of two of the three papers, abnormal trabeculation also appears in the histological sections of the mutant hearts. Surviving mutant female mice exhibited uterine distension due to vaginal atresia (Ludwig, Eggenschwiler et al. 1996, Hughes, Frago et al. 2013). In addition, Wang et al. reported developmental immaturity in the lungs of mutant mice. Various skeletal malformations were also reported, such as polydactyly and kinked tail (Wang, Fung et al. 1994, Ludwig, Eggenschwiler et al. 1996). Other skeletal pathologies include abnormal facial features and sternal ossification defects (Wang, Fung et al. 1994). The complex phenotypes that are observed in these mice reflect the multifunctional nature of the IGF2/M6PR. As its name suggests, IGF2/M6PR, also binds to mannose 6-phosphate, a lysosomal transport tag.

Paper	Genotype	Strain(s)	Growth and survival	Organ specific effects
Wang, Zhao-Qi et al. Nature, 1994	Igf2r knock out	129/SV & 129/Sv X C57BL/6J	0-1.5% survival depending on the strain. Mutant neonates are significantly larger than the WT.	Skeletal abnormalities, such as hexadactyly, kinked tail, abnormal facial features and sternal ossification. Underdevelopment of lungs.
Lau, Michele M. H. et al. Genes & Development, 1994	Igf2r knock out	129/Sv X C57BL/6J	Faster growth of embryos and placentas . From 88 newborns, only three were carrying the mutation in their maternal allele	Cardiomegaly with hyperplastic ventricles and valvular and septal defects
Ludwig, Thomas et al. Developmental Biology, 1996	Igf2r knock out	Mixed backgrounds including C57BL/6J, 129/Sv and MF1	Embryonic and postnatal survival varies between strains. 0.7-16.2% during postnatal life. Mutant embryos and placentas are heavier	Postaxial polydactyly, kinked tail, generalised proportional organomegaly, disproportional cardiomegaly (with increased trabeculation of the ventricles) and vaginal atresia
Hughes, Jennifer et al. Plos One, 2013	Humanised receptor gene knock in resulting in hypomorphic Igf2r (less than 50 % protein expression)	C57BL/6N	Increased embryonic, placental and postnatal body weights. 25% survival at weaning	Enlarged and dilated hearts and uteri

Table 1.1: Summary of full and hypomorphic *Igf2r* knock out mice phenotype.

1.8 IGF2/M6PR as a P-type lectin

IGF2/M6PR with other glycan recognising proteins belong the family of lectins (Berg JM 2002). Different criteria are used to classify different lectins, such as the type of ion required for their functions, type of glycan they recognise or their functions. IGF2/M6PR forms the P-type lectin family alongside with cation dependent mannose-6-phosphate receptor (CD-MPR). The P-type lectin family have a unique ability to recognise phosphorylated mannose residues. CD-MPR was named as such because the initial studies suggested that divalent cations of Ca^{+2} and Mg^{+2} were required for its functions. However, it is now known that these ions increase the affinity of the receptor four-fold but are not essential for the M6P binding. Since IGF2/M6PR was not found to require any divalent cations it was called the cation-independent mannose-6-phosphate receptor (CI-MPR) in field of lectin research. As my work focuses on the multifunctional/multiligand nature of the receptor and CD-MPR is not truly a cation dependent receptor, I prefer to use "IGF2/M6PR", instead of IGF2R or CI-MPR throughout this thesis.

Phosphorylated mannose is a terminal glycan marker for lysosomal sorting that is present on approximately 60 ER and Golgi synthesized glycoprotein enzymes (Coutinho, Prata et al. 2012). These proteins are captured by P-type lectins and transferred to late endosomal compartment for its final destination, the lysosome. Most of the glycoproteins are formed with O-linked or N-linked glycosylation. "O" or "N" defines the type of the atom that the first monosaccharide forms a covalent bound with (Berg JM 2002). In O-linked glycosylation, most of the carbohydrate chain begins with N-Acetylglucosamine attached to the hydroxyl group of the serine or threonine side chains (Thanka Christlet and Veluraja 2001, Aoki-Kinoshita 2013). No consensus sequence that determines the site of modification has been identified so far for O-linked glycosylation. O-linked glycosylation is a multistep process that starts with the transfer of the first carbohydrates from phosphorylated high-energy nucleotide sugars catalysed by ER and *cis*-Golgi network (CGN) localised N-Acetylglucosamine (GalNAc)

transferase. Later, in the TGN, galactose and two *N*-acetylneuraminic (sialic) acid are added. Compared to O-linked glycosylation, N-linked glycosylation initiation is a more strictly defined step of a covalent bond formation between only N-Acetylglucosamine and an asparagine residue of a specific consensus sequence (Lodish H 2000). The N-Acetylglucosamine is bound to an oligosaccharide that is recruited to ER lumen by a membrane polyisoprenoid lipid (dolichol). The oligosaccharide (Glc₃Man₉(GlcNAc)₂) on the target peptide has a branched structure and is composed of two GlcNAcs and one mannose root chain connected to eight mannose forming three branches, one of which is capped by two glucose molecules. The oligosaccharide branches lose their terminal glucose and one mannose molecule before their departure from the ER to CGN. More mannose molecules are removed in CGN, followed by addition of GlcNAc, fucose, galactose and N-acetylneuraminic acid in the medial and trans-golgi network. In many cases, the initial mannose removal does not occur which leads to the modification chain to be broken resulting in high mannose type branched oligosaccharides as an end product. These terminal mannose branches reach to the TGN and are the substrates for the enzymes that produce the M6P tags in two-step reactions. The first step is the addition of the GlcNAc-1-phosphate to sixth carbon of the terminal mannose residues by UDP-N-acetylglucosamine 1-phosphotransferase (GlcNAc-1-phosphotransferase). As the name suggests, this enzyme uses high energy UDP-N-acetylglucosamine as a substrate. The resulting N-acetylglucosamine-1-mannose phosphodiester's GlcNAc is removed by an enzyme called uncovering enzyme or N-acetylglucosamine-1-phosphodiester α -N-acetyl-glucosaminidase. With this final reaction the lysosomal enzymes become tagged with mannose 6-phosphate and would be recognized with both P-type lectins; CD-MPR and IGF2/M6PR (CI-MPR).

In the absence of functional uncovering enzyme, the diesters could not be converted into monoesters but could still be delivered by IGF2/M6PR to its lysosomes efficiently enough that mice with knocked out uncovering enzyme gene (UCE $-/-$) exhibits increased serum lysosomal enzyme levels with no major functional or histological pathologies (Boonen, Vogel

et al. 2009). However, the first step enzyme of M6P tag generation, GlcNAc-1-phosphotransferase deficiencies could manifest with severe phenotypes. In human, Mucopolidosis (MP) 2 and 3 (also called I-cell disease and Pseudo Hurler polydystrophy respectively) is a result of dysfunctional GlcNAc-1-phosphotransferase (Bargal, Zeigler et al. 2006). The enzyme is hexameric ($\alpha_2\beta_2\gamma_2$) and its subunits are encoded by two genes. The α and β subunits are products of *GNPTAB* gene dysfunction which results in MP2 (Tiede, Storch et al. 2005). The γ subunit, however, is expressed by *GNPTAG* gene, loss-of-function which results in MP3 (Raas-Rothschild, Cormier-Daire et al. 2000). While α and β subunits are catalytic; γ subunit is for substrate recognition, expression and folding of the enzyme (Coutinho, Prata et al. 2012). Therefore, in MP2, GlcNAc-1-phosphotransferase functions are absent but in MP3, the enzyme function is partially preserved which explains the milder clinical presentation of MP3 compared to MP2.

Similar to the compensation of the absence of UCE by IGF2/M6PR, absence of one of the members of P-type lectins is compensated by the other. For example, CD-MPR loss-of function mice are normal during the development and adulthood with preserved fertility, and maternal *Igf2r* knockout mice in *Igf2* and *Igf1r* null backgrounds are viable (Ludwig, Ovitt et al. 1993, Ludwig, Eggenschwiler et al. 1996, Sohar, Sleat et al. 1998). Although both M6P receptors compensate each other, loss-of-function in *Igf2r* gene results in more severe changes in serum lysosomal enzyme levels reflecting the multiplicity of IGF2/M6PR's M6P binding sites. However, absence of functional CD-MPR, IGF2/M6PR and lack of functional IGF2 together leads to death in mice, demonstrating that the P-type lectins are essential for survival (Ludwig, Eggenschwiler et al. 1996).

In addition to its functions as a growth factor clearance receptor and a P-Type lectin that binds both diester and monoester M6P, IGF2/M6PR is also a receptor for plasminogen and urokinase-type plasminogen activator receptor (uPAR) and retinoic acid. To understand this

multifunctionality, it is crucial to understand the receptor's structure and interactions with its ligands.

1.9 IGF2/M6PR structure and its ligand interactions

IGF2/M6PR is a type 1 trans-membrane protein with 15 homologous extracellular domains. Human and bovine receptor are highly homologous to each other and bovine IGF2/M6PR constructs were used in vast majority of the work on the M6P binding domains . Amino acid sequence of the receptor reveals an N-terminal 44 (bovine)/40 (Human) amino acid signal sequence and 2269 (bovine)/2265 (human) aa ectodomain with 23 aa single trans-membrane sequence and a 163 (bovine)/164 (human) residue cytoplasmic tail. (Lobel, Dahms et al. 1988, Oshima, Nolan et al. 1988). Based on the sequence, the receptor's weight is estimated to be 275 kD. However, the receptor carries 19 potential glycosylation sites and after post-translational modification the mature receptor runs between 275 and 300 kD in western blots (Kornfeld 1992). The extracellular domains of the bovine receptor, except the domain 13, consist of approximately 147 aa repeats with 16-38% identical sequence.

Structure of domains 1-3, 5 and 11-14 have been determined so far and all of these domains showed common structural features (Olson, Yammani et al. 2004, Brown, Delaine et al. 2008, Olson, Peterson et al. 2010). The core structure of these domains is a β -barrel that is formed by nine β -strands. These β -strands form two β -sheets. This core structure is also adopted by the single extracellular domain of the CD-MPR (Roberts, Weix et al. 1998). While the β -barrel is capped with a β -hairpin at the N-terminal of IGF2/M6PR domains, an α helix is present at the same position of the CD-MPR ectodomain. On opposite side of the β -barrels, the β -strands connected to each other with four peptide loops. These loops are responsible for forming the binding sites of the IGF2 and M6P binding domains. Other ligands of the receptor are plasminogen, uPAR and retinoids.

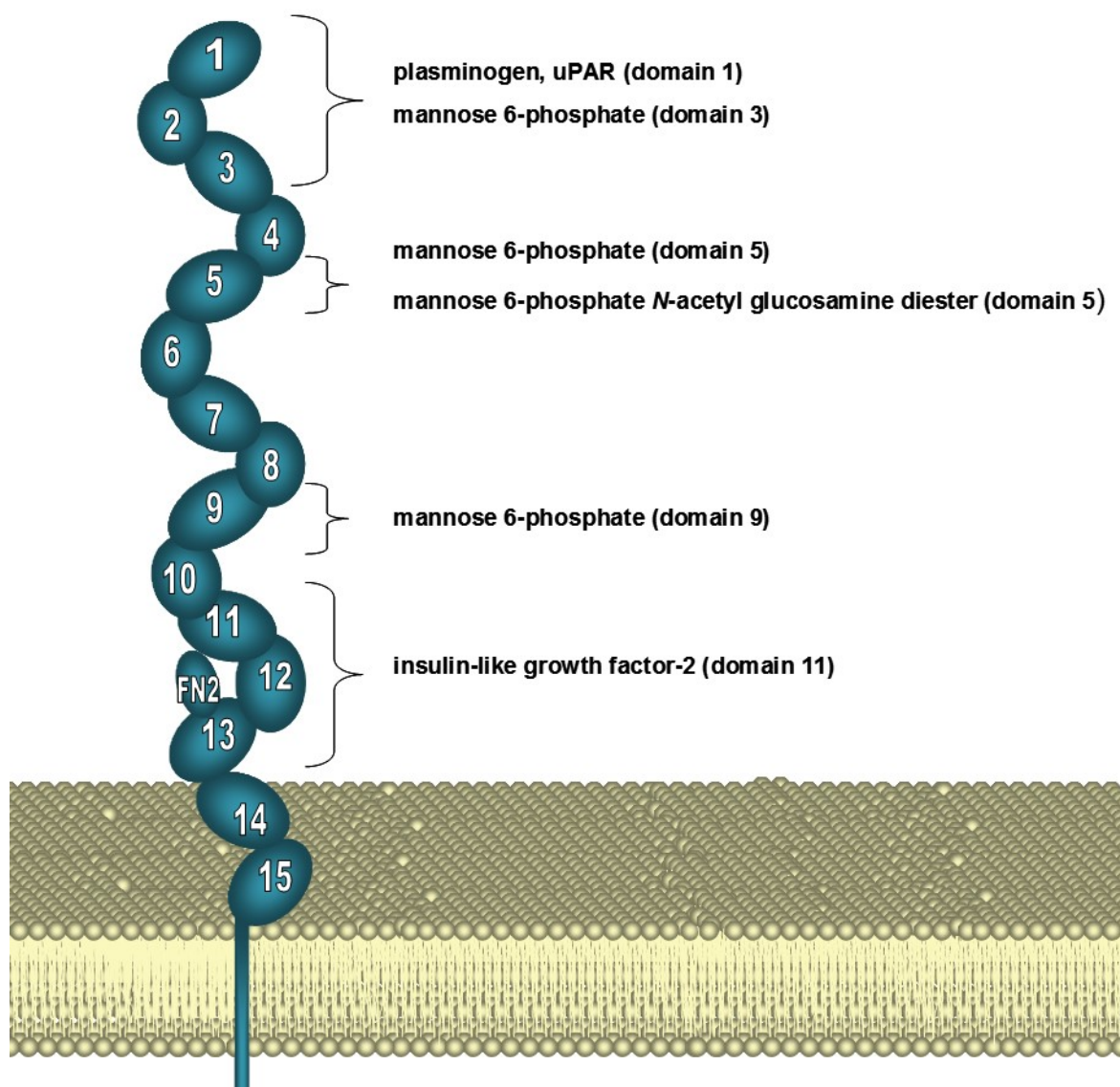


Fig 1.2. Schematic representation of IGF2/M6PR, its ligand binding sites and ligands: Domain 1 and the linker region between Domains 1 and 2 bind plasminogen and urokinase-type plasminogen receptor (uPAR). Domains 3, 5 and 9 also bind mannose 6-phosphate diesters. Domain 11 binds IGF2.

1.9.1 IGF2/M6PR-Retinoic acid interaction

A lipophilic morphogen, retinoic acid, unlike other known ligands, binds to cytoplasmic tail of the receptor (Kang, Li et al. 1997). Functional consequences of this interaction have been investigated only in few studies. These studies suggest that retinoic acid binding to the receptor inhibits cellular proliferation (Kang, Bell et al. 1999) and modifies trafficking of the receptor between the intracellular vesicular compartments (Kang, Bell et al. 1998).

1.9.2 IGF2/M6PR-plasminogen/uPAR interaction

IGF2/M6PR domains 1-3 bind plasminogen and uPAR. The IGF2/M6PR-plasminogen interaction facilitates TGF- β activation which could be inhibited by a lysine analog called tranexamic acid (Godar, Horejsi et al. 1999). Based on this observation, alanine scanning of surface lysines of N-terminal IGF2/M6PR revealed that Lys 53 of domain 1 and Lys 125 of Domain 1 to 2 linker region are key residues for plasminogen binding (Bohnsack, Patel et al. 2010). Plasminogen binding to the receptor is mediated by the kringle domains of plasminogen and the affinity of the interaction is influenced by the conformation of the ligand.

Plasminogen is a zymogen and converted to a serine protease plasmin. Plasmin primarily functions as an anticoagulant in the plasma. Amongst many other functions of plasmin/plasminogen system, we can count regulation of angiogenesis, matrix and tissue remodelling in embryogenesis, inflammation and oncogenesis (Castellino and Ploplis 2005, Aisina and Mukhametova 2014).

Plasminogen structure is composed of an N-terminal peptide, five kringle domains and a plasmin protease domain at its C-terminal (Law, Caradoc-Davies et al. 2012). A proteolytic cleavage site present between the plasmin protease domain and kringle 5 for its conversion to plasmin. The kringle domains recognise Arginine and Lysine residues. Therefore binding of

the kringle domains to surface lysine residues of IGF2/M6PR was proposed to be the mechanism of plasminogen binding to the receptor. Supporting this model, while kringle domains 1-3 show no binding to IGF2/M6PR, domain 4 was found to be essential for the interaction, and angiostatins (cleavage products of plasminogen) with kringle domains 1-4 show only about 2.4 folds less affinity to the receptor than plasminogen (Bohnsack, Patel et al. 2010).

Plasminogen could adopt three conformations, depending on the ionic composition of its environment; open, closed and an intermediate state (Marshall, Brown et al. 1994). In plasma, plasminogen adopts the open conformation. In the absence of physiological NaCl concentrations but in the presence of acetate ions, the protein folds further to form the closed state (Warejcka and Twining 2005). Benzamide presence in the solution, however, results in plasminogen adopting an intermediate conformation.. In open conformation, plasminogen affinity to IGF2/M6PR increases probably because of increased accessibility of the receptor binding sites to the kringle domains.

IGF2/M6PR on the cell surface could be solubilised by proteolytic shedding. Soluble receptor has been shown to restrict angiogenesis and cellular invasion through inhibition of plasminogen activation. This inhibition was explained with a model of the receptor binding to uPA (urokinase-like plasminogen activator) and plasminogen concurrently, thereby inhibiting their interaction with each other in a competitive manner (Leksa, Loewe et al. 2011). Later, however, kringle domains of uPA were found to have no significant binding affinity to IGF2/M6PR. It is also possible that both plasminogen and uPAR bind IGF2/M6PR on the cell surface facilitating interaction of uPAR bound uPA and plasminogen leading to plasminogen activation. Thus, soluble IGF2/M6PR competes with the membrane IGF2/M6PR for plasminogen therefore inhibiting the plasmin production. However, further structural and functional studies are required to test this model of plasminogen-plasmin system activation.

1.9.3 M6P tagged ligands of IGF2/M6PR

Domain 3, 5 and 9 of fifteen extracellular domains of IGF2/M6PR bind to the M6P monoesters. Domain 5 can also bind M6P diesters but shows a lower affinity for the monoesters. These three M6P binding domains and CD-MPR adopt a shared M6P binding motif that consists of an Arginine, Glutamine, Glutamate and a Tyrosine. Structural studies of Domains 3 and 5 and the extracellular domain of CD-MPR show that these four preserved residues surround the mannose ring of the terminal M6P tags (Olson, Peterson et al. 2010). Comparison of the binding pockets architecture and ligand binding residues of these domains gives us insights into the overlapping and distinct binding functions of these receptors. Consistent with its very high specificity for M6P monoesters, CD-MPR's binding pocket volume is the most limited of all. The domain 5 binding groove, on the other hand, has the largest volume compared to the other two. Its extended groove-shaped architecture allows it to accommodate both M6P monoesters and GlcNAc-P-Man diesters. Domain 3 binding pocket is shallower but has a wider base compared to the CD-MPR binding pocket (Olson, Dahms et al. 2004). This open architecture is also reflected in the reduced solvent inaccessibility of the M6P phosphate upon binding to domain 3 compared to its binding to CD-MPR. Domain 3's binding pocket volume is larger than the CD-MPR's binding pocket but it forms a more restricted space compared to the domain 5 binding groove. This hybrid architecture allows the domain to accommodate M6P and mannose 6-sulfate monoesters, and methylphosphomannosyl diesters but not GlcNAc-P-Man diesters (Marron-Terada, Hancock et al. 2000). However, methylphosphomannosyl diesters and mannose 6-sulfate monoesters occur in *Dictyostelium discoideum* and Domain 3's affinity for this ligand does not have any functional implication in mammals. The difference in the volume of domain 3 and the CD-MPR is due to the presence of bulky residues in the binding pocket the latter. For example, S386 of domain 3 is replaced by a histidine residue in the same position of CD-MPR. Also, D103 and H105 of CD-MPR further restrict volume of the binding pocket. Although no structural data exist on the domain

9 binding pocket, based on sequence alignments, His-1285 is also predicted to have such a volume-restricting role.

The domain 5 binding pocket lacks the disulphide bridge that links C and D loops of the IGF2/M6PR domain 3 and CD-MPR binding site (Olson, Peterson et al. 2010). In the absence of the disulphide bridge, the pocket seems to be stabilised by the interaction of the W653 and R687 side chains. Q644, R687, E709 and Y714 form the signature motif of domain 5 that binds mannose residue of the M6P. Y679 is a conserved residue across different species and loss of its hydroxyl group results in domain 5 losing its preference for the M6P diesters over M6P monoesters (Domain 5's affinity of the diester/monoester: 14-18 folds) (Chavez, Bohnsack et al. 2007). Interestingly, when the aromatic group of the side chain of Y679 is lost, domain 5's affinity for both diesters and monoesters reduces dramatically. Y679 is also conserved in CD-MPR but the architecture of the CD-MPR binding pocket restricts its potential ability to recognize diesters. In addition to the type of the terminal M6P, branching pattern, localisation and number of the M6P on the oligosaccharide of glycoprotein ligands also influence their binding affinity to P-type lectins (Song, Lasanajak et al. 2009).

CD-MPR and CI-MPR show similar affinity to M6P (7-8 μ M) (Tong, Gregory et al. 1989, Tong and Kornfeld 1989) and both receptors were reported to show no binding below pH 5. Based on these reports, affinity reduction seems to be the primary driver of ligand-receptor dissociation in late endosomes. However, phosphatases in the late endosomal and lysosomal compartment could also complement the binding affinity loss with dephosphorylation of the penultimate M6Ps which would achieve more efficient and irreversible ligand-receptor dissociation for lysosomal delivery of the enzymes (Saftig, Hartmann et al. 1997, Suter, Everts et al. 2001). However, the pH dependency of the each M6P binding domain is different. Matching with their binding function of newly synthesized enzymes at TGN (pH 6.5-7.0), P-type lectins show optimum affinity at around pH 6.4 (Lodish H 2000). Only 10% of both lectins are localised on the cell membrane (Dell'Angelica and Payne 2001). However, at cell surface

pH, while IGF2/M6PR preserves its affinity to M6P, CD-MPR exhibits no binding. IGF2/M6PR's binding ability at such a wide range of pH is due to the fact that it has two major high affinity M6P binding sites covering different pH ranges. Domain 3 and 9's optimal binding pH is around 6.5 and 7.0, respectively (Marron-Terada, Hancock et al. 2000)

1.9.4 IGF2/M6PR Domain 11

Insulin, IGF1 and IGF2 sequences show high levels of homology (Samani, Yakar et al. 2007) and overlapping functions. For example, both insulin receptor isoform-A (IR-A) and IGF1R serve as a receptor for IGF2. Also, IGF1 and IGF2 share six serum binding proteins (IGFBPs). However, insulin receptor isoform-B (IR-B) only binds insulin and IGF2/M6PR does not bind any of the other two growth factors. IGFBPs', IGF1R's and IGF2/M6PR's binding sites on IGF2 are overlapping (Brown, Delaine et al. 2008). Such an overlap and resulting competition is important for regulation of IGF2-IGF1R interaction which is responsible for most of the growth promoting effects of IGF2. T16, L53 and F19 of IGF2 are essential for IGF2 binding to M6P/IGF2R and the last is the anchor residue for the binding interaction.

Similar to M6P binding domains of M6P/IGF2R, domain 11 binding site is located at the opening of the β -barrel that is not capped with a β -hairpin. Also, like M6P binding domains, the binding pocket is formed by four loops that connect the β -strands. According to their distance from the N-terminal, the loops are called AB, CD, HI and FG (the AB loop being the closest). The binding pocket has a shallow architecture and the walls forming the binding surface are predominantly hydrophobic with negatively charged patches matching the positive charged patches on IGF2. Upon binding, the anchor residue of the interaction, F19 of IGF2, gets oriented towards to the β -barrel centre. Between chicken and human, IGF2 structure differs little. Therefore, during evolution from chicken to placental mammals, changes in architecture and hydrophobicity of the domain 11 binding site were essential for IGF2/M6PR

to acquire high-affinity IGF2 binding. Evolution of IGF2-domain 11 occurs in two steps. The first step happens during the transition between the non-mammalian oviparous vertebrate (chicken) to monotremes (echidna and opossum). In this step, the receptor acquires a low affinity binding to IGF2 which concurs with Domain 11 CD loop shortening and the loop adopting a more rigid orientation. Simultaneously with the loss of length, chicken CD loop I1945 and L1962 are replaced by I1572 and F1567 in echidna and opossum. The second step is evolution to a high affinity IGF2 receptor which concurs with evolution of placenta in vertebrates. At this step, the IGF2 binding pocket volume and affinity increase with gain of multiple mutations in AB and FG loops.

Replacing I1572 and F1567 in domain 11 with alanine results in IGF2 loss-of-binding. Alanine scanning studies in domain 11 constructs, demonstrated that I1572 and F1567 are essential for IGF2 binding, and T1572 of CD loop increases IGF2 binding ~9 fold (Zaccheo, Prince et al. 2006). Also, Q1569, the other residue of the CD loop, has a minor role in enhancing the IGF2 binding. In placental mammals, for high affinity IGF2 binding, FG loop S1596, AB loop Y1542, S1543, K1545 residues are also required.

Three residues of IGF2 have been identified as essential for its binding to domain 11. The anchor residue of the IGF2-domain 11 interaction, F19 of IGF2 interacts with F1567, L1629 and Y1542 of domain 11. The other essential residue of the binding, IGF2 L53, interacts with K1631 of the receptor. The last essential residue, T16, is crucial for IGF2 specificity to M6P/IGF2R. In IGF1, an alanine residue is present at the equivalent position of T16 and when this residue is replaced by a threonine, IGF1 gains a weak affinity to M6P/IGF2R.

Intriguingly, suggesting the presence of inhibitory residues in the binding site, several domain 11 mutants have been identified to have higher affinity compared to human IGF2/M6PR. AB

loop E1544A and E1544K of the mutants were the first of this kind, showing three- and ten-fold higher affinity, respectively, compared to the WT construct. However, these AB loop mutants showed reduced IGF2 binding when tested in larger construct of Domains 11-13. Later, it was discovered that E1544 is essential for domain 13's FNII (fibronectin type II fold) insert's ~10-fold affinity enhancing effect on Domain 11 (Zaccheo, Prince et al. 2006, Brown, Delaine et al. 2008). These structural studies also revealed that the FN2 insert is required for fixing the AB loop in its position for high affinity binding.

With additional residue replacements, two other AB loop mutants, E4 (E1544K-K1545S-L1547V) and 3-4D (S1543A-E1544K-K1545G-G1546W-L1547G) were developed, exhibiting similar affinities, ~4.1 and ~10 folds of WT, respectively. However, when combined with a specific CD and FG mutational combination named "QPS", the IGF2 affinity of these mutants significantly increased, E4QPS and 3-4DQPS mutants showed ~41 and ~32 folds higher than WT affinity (unpublished results, Frago S et al.).

The functional consequences of these loss- and gain-of-function mutations have never been investigated in the context of the full-length receptor. So far, a single nucleotide polymorphism (G1619R) related to growth inhibition has been characterised in the full-length receptor using surface plasmon resonance and cellular models, and the mutation found to have no functional effect (Rezgui, Williams et al. 2009). However, IGF2 binding affinities of domains 1-15 (14nM), 10-13 (120nM), 11-13 (100 nM) and 11-12 (450nM) suggest the contribution of domains other than domains 10-13's to the IGF2 binding affinity of domain 11 (Linnell, Groeger et al. 2001). Furthermore, although full-length *Igf2r* loss-of-function mouse is well characterised, no domain specific loss-of-function mouse models have been generated so far. Thus, IGF2/M6PR's ligand binding domains function in the context of full-length receptor and development remains unknown. To answer these questions, I aimed to generate and characterise full-length M6P

and IGF2 binding mutants using surface plasmon resonance, characterise a domain 11 loss-of-function mouse.

Aims

- Generation of IGF2 loss and gain-of-binding function full-length IGF2/M6PR to investigate influence of these mutations on other ligands' binding affinity
- Generation of IGF2 loss and gain-of-binding function full-length IGF2/M6PR to investigate Domain 11 binding loops' functions in the context of full length receptor
- Generation and characterisation of full length M6P complete loss-of-binding function receptors
- Generation and characterisation of full length M6P loss-of-binding function receptors with single and two intact M6P binding domains
- Characterisation of heterozygous IGF2 loss-of-binding function (*Igf2^{11565Am/+p}*) in the mouse *in vivo*
- Characterisation of homozygous IGF2 loss-of-binding function (*Igf2^{11565Am/11565Ap}*) in the mouse *in vivo*

Chapter 2: Materials and Methods

2.1 Mutagenesis, Expression and Characterisation of M6P/IGF2R

Constructs

2.1.1 Vectors

2.1.1.1 Mammalian M6P/IGF2R expression vector

For expression of wild type and mutant forms of the IGF2/M6PR, a pHLSec vector construct was designed and optimized to express extracellular domains 1-15 of IGF2/M6PR fused with a GSS linker, rat CD4 domains 3&4, a Bir A biotin acceptor site and a six amino acid polyhistidine tag (Aricescu et al. 2006) (Figure 2.1)

2.1.1.2 Synthetic gene carrying plasmids

The synthetic genes produced and inserted into ampicillin resistant gene carrying plasmids by Eurofins UK (Hull, United Kingdom).

2.1.1.3 Biotin ligase expressing plasmid

A biotin ligase (Bir A) secreting plasmid was used for the in vivo biotinylation in the receptor protein. Bir A was tagged by a endoplasmic reticulum export signal, KDEL, for biotinylation of the soluble proteins prior to their excretion to the media.

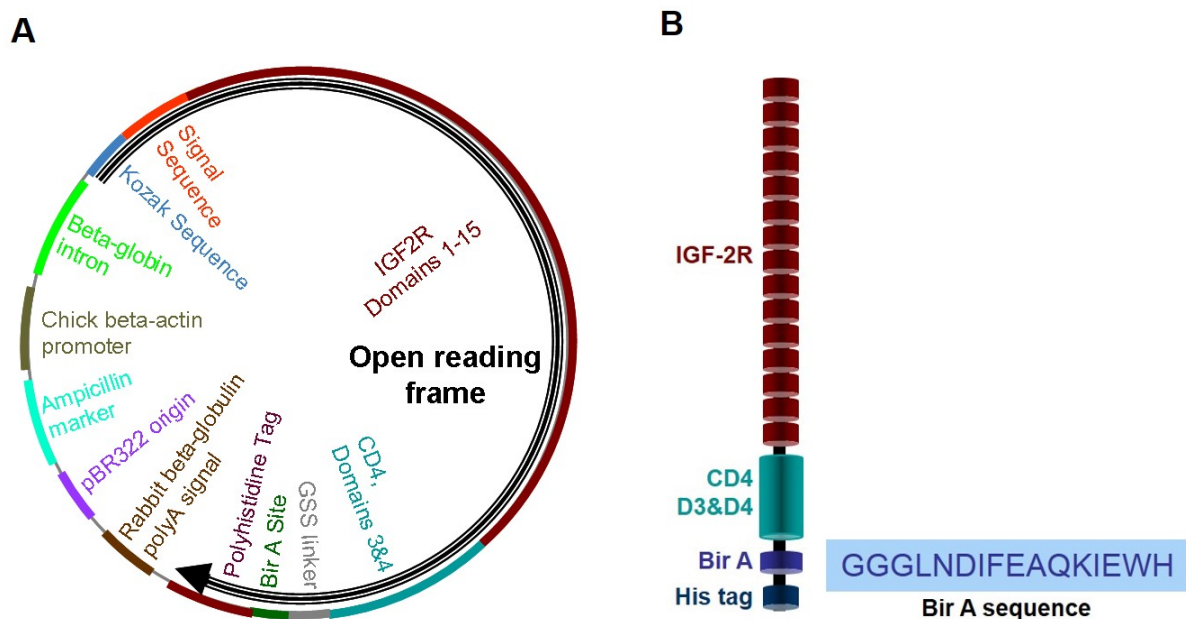


Figure 2.1: Schematic representation of mammalian expression vector and the soluble receptor to be generated by HEK293T cells. (A) The expression vector open reading frame consists of Kozak sequence for translation initiation and an endoplasmic reticulum export signal followed by the peptide sequence. **(B)** The soluble receptor consists of 15 ectodomains of IGF2/M6PR, CD4 Domains 3&4 (facilitates folding and expression), a biotin acceptor site (Bir A: GGGLNDIFEAQKIEWH) for immobilization on streptavidin coated Surface Plasmon Resonance (SPR) chips and a C-terminal polyhistidine tag for purification.

2.1.2 Cloning Strategy

2.1.2.1 DNA sequencing

Sequencing of the constructs and PCR products were performed by Source Bioscience Lifesciences® Oxford branch. Samples were prepared according to the instructions in the company's website: <http://www.lifesciences.sourcebioscience.com/>. The sequencing results were analysed using Geneious 5.1.7 software.

2.1.2.2 Restriction digests

The restriction enzymes were obtained from New England Biolabs (NEB) (Herts, UK). Restriction digests were performed according to manufacturer's instructions

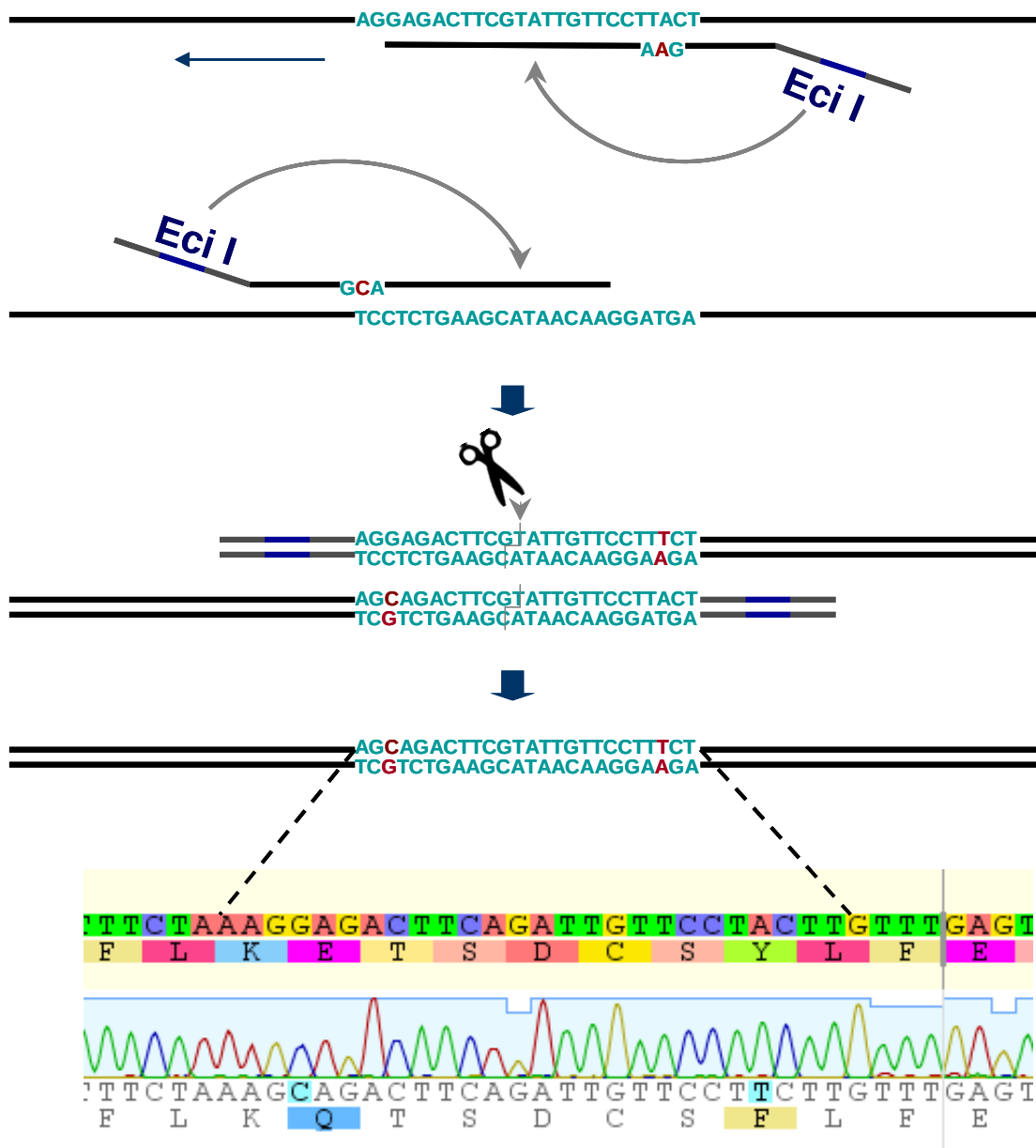


Figure 2.2: Illustration of use of Type IIS restriction enzymes for cloning: Type IIS restriction enzymes cleaves a site that is located at a certain distance from their recognition site this allows use of restriction enzyme when there is no possibility to introduce a new restriction site into the sequence of interest in the final construct

2.1.2.3 Polymerase Chain Reactions

PCR reactions carried out in MJ Mini Personal Thermal Cycler (Bio-Rad, Hemel Hempstead Hertfordshire, UK) and in MyCycler (Bio-Rad, Hemel Hempstead Hertfordshire, UK). PCR amplifications were performed using NEB Phusion® polymerase (Herst, UK) in NEB High Fidelity (HF) buffer or NEB GC buffer (For Guanosine-Cytosine rich templates where HF buffer does not work). For guanine&cytosine rich templates, 5-10% DMSO was also added to the reactions. A typical PCR reaction and programme for cloning is summarized in tables 2.1 and 2.2 respectively

Table 2.1: Examples of PCR reaction mixtures (for 100 µl) used for this thesis

Reaction Component	Volume/Amount
Template DNA	50-80 ng (0.5-2 µl)
HF buffer (x10) (Herst, UK)	10 µl
Primer 1 (10µM)	5 µl
Primer 2 (10µM)	5 µl
NEB Phusion® polymerase (Herst, UK)	1 µl (2 units/µl)
Double distilled autoclaved water	77-78.5 µl

Table 2.2: An example of PCR program used for the mutagenesis

Temperature °C	98°C	98°C	*59-76°C	72°C	72°C	4°C
Time (minutes)	2:00	0:10	**2:00-5:00	5:00	5:00	Hold
Number of Cycles	1	36			1	-

*Average of T_{m1}&T_{m2} was chosen for the first amplification attempt (See Tables 2.3 and 2.4, and the Appendix for the T_m values).

**Adjusted according to the length of the template

2.1.2.4 Oligonucleotide primers

Primers for PCR reactions were designed using Geneious 5.1.7 software. Primer melting temperatures were calculated with both an online calculator (http://www.finnzymes.fi/tm_determination.html) and manually with a formula ($T_m = 81.5 + 0.41(\%GC) - 675 / \text{Nucleotide number} - \% \text{mismatch}$). Average of these two melting temperatures ($T_{m1} \& T_{m2}$) was entered to the PCR programme. Netprimer software (<http://www.premierbiosoft.com/netprimer/>) was used to analyse the secondary structures (homodimers, heterodimers and hairpins) of oligonucleotides.

For introducing restriction enzyme recognition sites and mutagenesis of Domains 3, 5 & 9, the oligonucleotide primers listed below were used. For introducing Domain 11 mutations, synthetic genes were cloned into the full length receptor constructs (See section 2.1.1).

The primers are listed as 5'-3' in the tables 2.3, 2.4, 2.5 below (See the supplementary section for details of the primer's properties and the strategies for primer design). Name of the primers are listed on left side, "for" and "rev" refer that the primer is designed in forward or reverse direction respectively.

Table 2.3: Primers to introduce new restriction sites

Introducing MfeI site		Tm1*	Tm2**
On MfeI site (fully overlapping primers)			
MfeI4 for	GAGAAAGGAAACATTCAATTGTCTTATTCAGATGGTGATG	71.99	75
MfeI4 rev	CATCACCATCTGAATAAGACAATTGAATGTTTCCTTTCTC	71.99	75
On MfeI site (partially overlapping primers)			
MfeI over for	AACATTCAATTGTCTTATTCAG ATGGTGATGATTGTGGTCA	74.25	77.5
MfeI over rev	CTGAATAAGACAATTGAATGTT TCCTTTCTCTTTCATGGGA	73.13	77.5
On restriction site of Bsp EI			
Bsp EI for	TCATCGATCCGGACACTTCTCTATT	67.89	72.54
On restriction site of Acc 65I			
Acc 65I rev	GACTAGCGGTACCTTTGGCAGAG	67.21	75.12
Introducing SphI site			
On SphI site			
SphI_for.Scan	CTTATTCATCGTACGGGTGGTTAT	60.08	62.23
SphI_rev.Scan	CATAACCACCCGTACGATGAATAA	56.11	62.23
C-terminal to BstBI restriction site			
BstBI Rev	GGAGTGAAGTAGTCCTGTCT	49.04	64.97
N-terminal to Acc 65I restriction site			
Acc 65I for	GACTTCATCACTCTGACCTACAAA	56.11	62.3

Table 2.4: Primers to introduce loss-of-binding mutations into the M6P binding sites on domains

Introducing mutations to M6P binding site on Domain 3		Tm1*	Tm2**
1 st essential residue on Domain 3 (Glutamate 451 to Glutamine):			
Dom 3 E451Q Rev	TATATTGGCGGAATTATTTAACAGTCAACCTGCCCTGTGAATAC	69.9	69.19
C-terminal to MfeI site on domain 4			
Post-Mfe I For	TGACCACAATCATCACCATCTG	66.22	69.45
2 nd essential residue on Domain 3 (Tyrosine 456 to Phenilalanine)			
Dom 3 Y456F For	TTATATTGGCGGAATTATTGACTGCACCTTCTTCTTCACATG	68.58	68.54
Introducing mutations to M6P binding site on Domain 5			
1 st essential residue on Domain 5 (Glutamate 745 to Glutamine)			
Dom 5 E745Q Rev	TATATTGGCGGAATTATTTAACAGTCAACCTGCCCTGTGAATAC	64.05	69.04
On MfeI site on domain 4			
MfeI4 for	GAGAAAGGAAACATTCAATTGTCTTATTCAGATGGTGATG	67.88	69.05
2 nd essential residue on Domain 5 (Tyrosine 750 to Phenilalanine)			
Dom 5 Y750F For	TTATATTGGCGGATATGATAACTCCACCTTCAACTTCCGG	67.75	69.70
On restriction site of Acc 65I			
Acc 65I for	GACTAGCGGTACCTTTGGCAGAG	67.21	75.16
Introducing mutations to M6P binding site on Domain 9			
1 st essential residue on Domain 9 (Glutamate 1345 to Glutamine)			
Dom 9 E1345Q Rev	TACAGTGGCGGAGTAGGAACAATCTGAAGTCTGCTTTAGAAATA	65.51	68.92
N-terminal to Acc 65I restriction site on domain 8			
Acc 65I for	GCTTCATCACTCTGACCTACAAA	62.59	69.98
2 nd essential residue on Domain 9 (Tyrosine 1350 to Phenilalanine)			
Dom 9 Y1353F Rev	TATTATGGCGGATATTATCAGATTGTTCTTCTTGTGTTGAGTG	66.52	67.46
C-terminal to Bst EII site on domain 11			
Post- Bst EII For	AGGTGTCCTGTGCTTGGGT	65.15	69.71

2.1.2.5 Removal of plasmid templates from PCR products

Following the PCR reactions for mutagenesis, plasmid templates were removed by DpnI (NEB; Herst, UK) digestion. 1 µl of restriction enzyme (20U/µl) was added to each 100 µl of the PCR product and incubated for an hour at 37 °C.

2.1.2.6 DNA gel electrophoresis and purification of DNA from agarose gels

A sample from PCR and digestion products were run in agarose gel (0.6-2% agarose in Tris-borate-EDTA) with ethidium bromide (0.7 µg /ml) at constant voltage of 60-80 mV. See “Purification of DNA from solutions” for the purification of the PCR products that appear as a single band on the gel. In the presence of multiple bands, the bands of interest were excised under UV illumination or Dark Reader® Transilluminators from Clare Chemical Research (Dolores, CO, USA) and purified using Zymoclean™ Gel DNA Recovery Kits (Irvine, CA, USA).

2.1.2.7 Purification of DNA from solutions

DNA purified in digestion or PCR mixtures were purified using DNA Clean & Concentrator™-5 kit from Zymo Research (Irvine, CA, USA).

2.1.2.8 DNA Quantification

Concentration and purity of DNA samples were assessed using a Nanodrop 1000 (Thermo Scientific; Wilmington, DE) which measures absorbance at a continuous spectrum between 220-750 nm. Ratio of absorbance readings at 260 nm and 230 nm (260/230); and 260 nm and 280 nm (260/280) were used to determine the purity of the DNA preparations. $A_{260/280}$ values at ~1.8 and $A_{260/230}$ values between 2.0 and 2.2 were used for further experiments.

2.1.2.9 DNA Dephosphorylation

Roche dephos & ligation kit (West Sussex, UK) or Antarctic Phosphatase (AP) from NEB (Herts, UK) was used for the dephosphorylation of 5' end of vectors prior to ligation and transformation. The phosphatase was heat inactivated (2 minutes at 75 °C for Roche and 5 minutes 70 °C for AP). Dephosphorylated vector was later purified to prevent dephosphorylation of inserts.

2.1.2.10 DNA Ligation

Roche dephos & ligation kit (West Sussex, UK) and T4 ligase from Fermentas® (Thermo Fisher Scientific; Notttingham, UK) were used for DNA ligations.

2.1.2.11 E. coli Strains

JM109: endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ(lac-proAB) e14- [F' traD36 proAB⁺ lacI^q lacZΔM15] hsdR17(r_K⁻m_K⁺)

DH5α: F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d/lacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K⁻m_K⁺), λ-

2.1.2.12 Bacterial growth media

SOB (Super Optimal Broth) and SOC (Super Optimal Broth with Catabolite Repression)

For preparation of SOB medium, first 2% of (bacto-) tryptone, 0.5% yeast extract, 10mM NaCl, and 2.5 mM KCl were dissolved in distilled H₂O, the solution was then adjusted to pH 7.0 and autoclaved. Finally, filter sterilized MgCl₂ and MgSO₄ (1 ml of 2M stock) was added to the media for final concentration of 10mM for both salts. SOC medium was prepared by adding filter sterilized glucose solution to SOB medium (final glucose concentration of 20mM).

LB (Luria-Bertani) Medium

LB media were obtained from communal supply of Sir William Dunn School of Pathology, University of Oxford and composed of 1% typtone, 0.5% yeast extract with 0.5% NaCl dissolved in H₂O, the solution were then autoclaved and pH adjusted to 7.0. Ampicillin (100 µg/ml) or carbenicillin (100 µg/ml) was added to LB medium to select and grow ampicillin resistant *E.coli*. 2% agarose LB plates (with or without 100 µg/ml ampicillin) were also obtained from the communal supply of the department.

2.1.2.13 Preparation of Competent *E. coli*

Two different protocols were used to prepare competent *E. Coli* for the experiments in this thesis. Initially, a protocol based on (Mandel and Higa, 1970) was used but, to achieve a higher competency, a protocol was adapted from “DNA Cloning Techniques” (D. Hannahan et al., 1985). The following are details of the protocols.

Protocol 1 (adapted from Mandel and Higa, 1970)

1 ml of overnight inoculum was added to 100 ml of LB media and incubated in a shaker at 37 °C for approximately for two and half hours. Subsequently, the cultures were grown further until the optical density of the medium reached to 0.4-0.6 at 600 nm wavelength. When the intended optical density was achieved, the cultures were poured into precooled 50 ml centrifuge tubes and left on ice for 10 minutes. *E. coli* were collected by centrifuging the tubes at 3500 rpm, for 10 minutes at 4 °C. The cells were gently resuspended in sterile 40 ml of 100 mM CaCl₂ in ddH₂O and incubated on ice approximately for an hour. The cells were pelleted again as above and resuspended in 6 ml of 100mM CaCl₂ and 15% glycerol solution. Finally, the suspension was aliquoted to eppendorf tubes and flash frozen in methanol-dry ice mixture.

Protocol 2 (adapted from D. Hannahan et al., 1985)

1 ml of overnight culture of JM109 cells grown in SOB (Super Optimal Broth) medium were inoculated to approximately 200 ml of SOB medium and left in a shaking incubator (180 rpm) at 37 °C. After two hours, the optical density of the culture was checked at regular intervals until it reached a value between 0.35-0.6 at 550 nm wavelength and was then transferred on to ice to be chilled. The cells were then pelleted by centrifugation at 3000 rpm for 15 minutes at 4 °C and subsequently resuspended in 67 ml of RF1 (100mM KCl, 50 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 30 mM potassium acetate, 10 mM $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$ and 15% glycerol dissolved in H_2O , with 10% acetic acid its pH adjusted to 5.8 and filter sterilized using a 0.22 μm filter) solution with gentle vortexing and transferred to ice for incubation for 30 minutes. The cells were collected again as above and resuspended in 16 ml of RF2 solution (10 mM MOPS pH 6.8, 10 mM KCl, 75 mM CaCl_2 and 15% glycerol dissolved in H_2O and its pH adjusted to 6.8 using 1M NaOH and filter sterilized with 0.22 μm filter). The resulting suspension was aliquoted into small centrifuge tubes to be flash frozen in methanol-dry ice mixture.

2.1.2.14 Transformation of *E. coli*

Competent *E. coli* were thawed on ice and divided into 100 μl aliquots, then gently mixed (by flicking the tubes few times) after adding ligation mixes or purified plasmids (~50 ng). The mixtures were left on ice for 30 minutes and heat shocked for 45 seconds in heat blocks (for plasmids) or PCR thermocyclers (for ligation mixtures to enhance transformation efficiency). Subsequent to the heat shock, the tubes were returned back to the ice for two minutes, followed by addition of 900 μl of LB (for plasmids) or SOC medium (Super Optimal Broth with Catabolite Repression-for ligation mixtures for enhanced transformation efficiency) and incubation in a shaker incubator (180 rpm) for an hour at 37 °C. Later, the cells were briefly spun down for 30 seconds at 6000 rpm, approximately 900 μl of the supernatant were removed

and the cells were resuspended in the remaining medium (~100 µl). Finally, the cells were spread onto agarose petri dishes with 100 µg/ml of ampicillin for selection.

2.1.2.15 DNA minipreparations

A single colony from transformations were inoculated to 10 ml of LB with 100 µg/ml of ampicillin or carbenicillin and grown for 8-10 hours at shaker incubator (180 rpm) at 37 °C.

Qiagen HiSpeed® Spin Miniprep Kit from Qiagen Ltd (West Sussex, UK) were used to extract and purify plasmids from cultures.

2.1.2.16 DNA maxipreparations

For starter cultures, a colony of transformed bacteria was used to inoculate 10 ml of LB with 100 µg/ml of either ampicillin or carbenicillin and grown in an incubator shaker (180 rpm) at 37 °C for 8 hours. A millilitre of the starter culture was later transferred into 200 ml of LB with selective antibiotic (100 µg/ml of ampicillin or carbenicillin) for a further 16-18 hour incubation in a shaker-incubator. Finally, extraction and purification of the plasmids from the cultures were performed using Qiagen HiSpeed® Plasmid Maxiprep Kit from Qiagen Ltd (West Sussex, UK) following the manufacturer's instructions.

2.1.3 Mammalian Cell Cultures, Transfections, Protein Expression and Purification

2.1.3.1 HEK293 Cell Cultures

Human Embryonic Kidney 293 (HEK293T) cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich St Louis, MO, USA) supplemented with 10% Fetal Calf Serum (FCS; Sigma-Aldrich St Louis, MO, USA) and 1% of penicillin & streptomycin (P&S; Life

Technologies Ltd, Paisley, UK) at 37°C in 5% CO₂ incubator. Cells were passaged at 80-100% confluency using Gibco® TrypLE™ Express (Life Technologies Ltd, Paisley, UK).

2.1.3.2 Expression of Receptor Constructs in HEK293 Cells

HEK293T cells were grown in 175cm² polystyrene flasks until cells reached 60-80% confluency. Two hours prior to transfection, growth medium was replaced by serum-free DMEM media. 1.6 ml of DMEM medium was mixed with 36 µg of co-transfection expression vector mixture (34 µg of receptor expressing vector, 2 µg of biotin ligase expressing vector) followed by the addition of 144 µl of polyethylenimine (PEI) (1mg/ml) followed by vortexing of the mixture. Finally, the DNA-PEI mixture was incubated for 15 to 30 minutes and added to the tissue culture flasks.

2.1.3.3 In vivo Biotinylation of Receptor Constructs

A biotin ligase (fused with KDEL sequence) expressing pHLSec plasmid was used for *in vivo* biotinylations (kindly provided by Hans Hoppe). 2mM biotin was added to the medium between 12th and 24th hours after the co-transfection.

2.1.3.4 Protein Purification

On day 4 after the transfection, the medium from the flasks were collected in 50 ml tubes and centrifuged at 1200 RPM for 5 minutes. The supernatant collected and filtered with 0.22µm filters. The filtered supernatants later transferred to centrifugal filters with a 10-20 kDa cut off (Millipore, Carrigtwohill, Co. Cork, Ireland) and concentrated to approximately to 10-20% of its volume. Biotinylated proteins were finally purified with PrepEase his-tagged protein purification midi kits from Affimetrix UK (High Wycombe, UK). Additional number of washing steps added to the manufacturer's instructions in order to reduce biotin concentrations in the elution fractions. Finally, first and second elution fractions were transferred to Vivaspin® 500

μl (10 kDa cut off) centrifugal filter units and concentrated to 50 μl and later diluted in 500 μl of HBS-EP buffer (0.01M HEPES (pH: 7.4), 0.15 M NaCl, 3mM EDTA, 0.005% Surfactant P20) (GE Healthcare Uppsala, Sweden). The dilution-concentration step was repeated three times to clear up the imidazole in the elution buffer and to reduce biotin concentrations further.

2.1.3.5 Polyacrylamide gel preparation

6% polyacrylamide gels were prepared according to the table below (Table 2.6). Ammonium persulphate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) were added to the gel mixture last. After pouring the resolving gel into the Bio Rad cassettes, a few millilitres of water were added on the top to attain a smooth surface. Lastly, stacking gel mixture were added to the cassettes and combs were inserted to form the wells.

Table 2.5: Composition of polyacrylamide gels

Gel component	Volume (stacking gel)	Volume (resolving gel)
Deionized water	6.1 ml	16.8 ml
Buffer (stacking/resolving)	2.5 ml	8.4 ml
30% Acrylamide	1.3 ml	6.4 ml
10% Ammonium persulfate (APS)	50 μl	320 μl
TEMED (N,N,N',N'-Tetramethylethylenediamine)	10 μl	32 μl

2.1.3.6 Polyacrylamide gel electrophoresis

Prior to loading into the wells, samples were mixed with 6x or 10x reducing laemli buffer (120 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.02% bromophenol blue) and incubated at 98 °C for 5-10 minutes. The denatured proteins were run at 140-200 V in running buffer composed of 14.4 gr/L glycine, 3.02 gr/L Tris and 10gr/L SDS in double distilled water.

2.1.3.7 Probing antibodies and blocking reagents

The blocking solutions for PVDF membranes were prepared by dissolving 3% bovine serum albumin (BSA Sigma-Aldrich St Louis, MO, USA) or 5% skimmed milk in Tris-Buffered Saline with Tween 20 (TBS-T). Mouse Anti-His6 peroxidase conjugated monoclonal antibody from Roche (West Sussex, UK) diluted 1 in 10000 in the blocking reagent to probe polyhistidine tags. To detect biotinylation, high sensitivity streptavidin HRP Conjugate from pierce (Rockford, Il, USA) was diluted 1 in 20000 in the blocking reagent.

2.1.3.8 Western blots

Proteins that were separated in PAGE were transferred to polyvinylidene fluoride (PVDF) membrane by wet transfer using MiniProtean 3 system, either at 100 V, at room temperature for an hour or at 30 V at 4 °C overnight, in transfer buffer composed of 14.4 gr/L glycine, 3.02 gr/L Tris and 10% methanol in double distilled water. The PVDF membranes were blocked and probed at room temperature for one hour or at 4 °C overnight. The membranes were later washed with Tris-Buffered Saline with Tween 20 (TBS-T) (8.006 gr/L NaCl, 2.423 gr/L Tris base, 1% tween, pH 7.6) three times, around 15 minutes for each wash. Finally, blots were developed using ECL Plus Western Blotting detection system and Amersham Hyperfilm ECL (24 × 30 cm) from GE Healthcare Life Sciences (Little Chalfont, UK)

2.1.4 Surface Plasmon Resonance Analysis

All SPR studies in this thesis is performed in Biacore™ T200 for a highly sensitive real time molecular interaction evaluation

2.1.4.1 Principles and use of SPR technology for real time analysis of molecular interactions

Surface plasmon resonance (SRP) technology measures alterations in refractive index in liquid phase where two binding partners interact. One binding partner (called ligand) is always immobilised on a dextran matrix that coats a gold surface of a glass slide, the other binding partner (called analyte) is being run on the surface. Proportional to affinity of the analyte to the ligand, the analyte concentrates in the liquid phase proximal to the dextran surface which leads a change in the refractive index on the liquid phase surface.

2.1.4.1.1 Surface Plasmon Resonance Phenomenon

Under the total internal reflection conditions, when a polarised light strikes a conductive gold film layer between two medias with different refractive indexes, a reduction in the intensity of reflected light is observed. The reduction of intensity is the consequence of the absorption of electrical field (called evanescent wave) by the electrons of the gold surface. Maximum reduction in intensity is observed at a certain angle that is a function of the refractive index of the gold layer interface. Changes in the refractive indexes can be calculated by detecting the changes in the angle.

2.1.4.1.2 Generation and Evaluation of SPR Traces

Changes in the refractive index are proportional to the total mass of ligand that is covalently bound to dextran surface and the analyte that bind to the immobilised ligands temporarily. A typical sensorgram that reflects ligand-analyte interaction on the dextran surface is composed of three phases: an association, a saturation and a dissociation phase. Using a molecular binding model, association and dissociation constants, and an overall affinity value are calculated by the SPR software

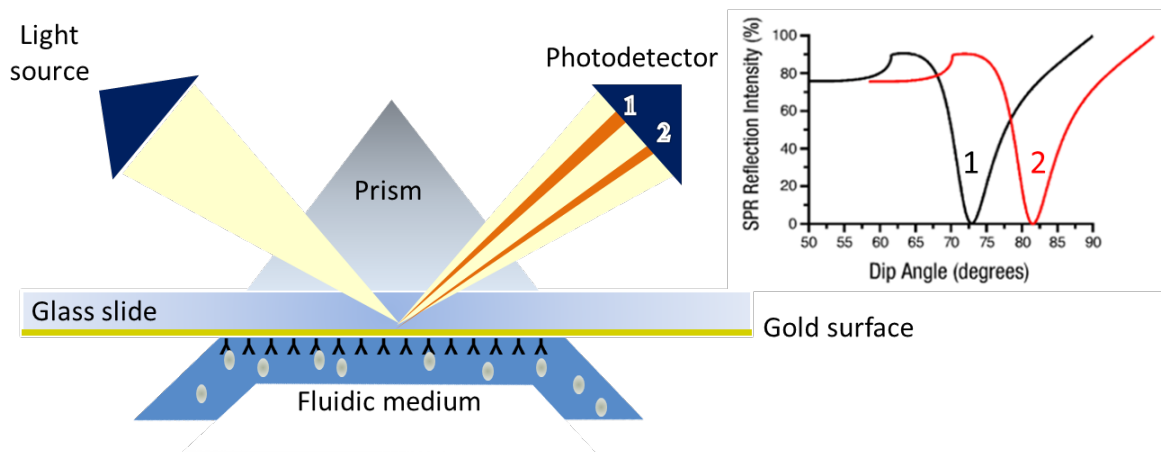


Figure 2.3: Schematic representation of the use of SPR technology in characterising biomolecular interactions: 1 and 2 represents changes in the minimum intensity measured by the photodetector.

(Drawing based on illustrations on BIAcore AB website: www.biocore.com. The sample graph is adapted from <http://biosensingusa.com>)

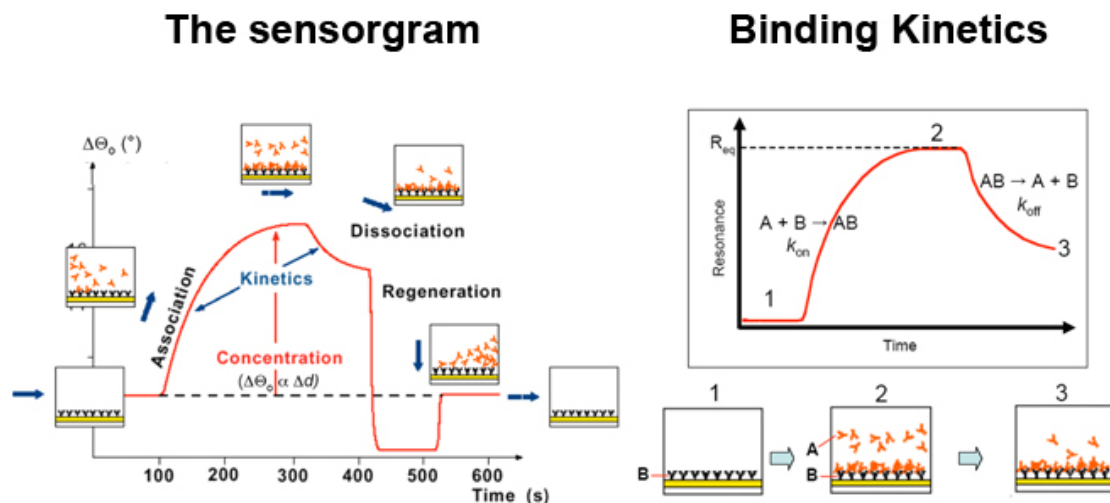


Figure 2.4: Representation of a typical sensorgram and its relationship with the analyte-ligand interactions on the chip surface

2.1.4.2 SPR buffers

HBS-EP Buffer: 0.01M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4; 0.15M NaCl, 3 mM EDTA (Ethylenediaminetetraacetic acid); 0.005% Surfactant P20 (GE Healthcare Bio-Sciences Uppsala, Sweden)

Acetate Buffer: 50mM HEPES, 150 mM Sodium Acetate, 0.005% Surfactant P20 (GE Healthcare Bio-Sciences Uppsala, Sweden)

2.1.4.3 Biacore T200 CM5 S series activation and immobilisation of receptors

CM5 S series chips were activated with mixture of N-hydroxysuccinimide and EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (10µl/min, 7-10 minutes of injection) followed by immobilization of streptavidin (0.025mg/ml in 10 mM sodium acetate buffer, pH 5.0) by amine coupling on each flow cell. Excessive reactive groups were deactivated with 1M ethanolamine, pH 8.5 (10µl/min, 7-10 minutes of injection). Finally, biotinylated and purified proteins in HBS-EP buffer were centrifuged at 13000 for 10 minutes at 4 °C and the supernatants immobilized on flow cells (Table 2.7).

Table 2.6: List of analytes used for SPR characterisation

Type of analyte and source	Binding function of the domain tested
Human glu-Plasminogen (Haematologic Technologies, Vermont, USA)	Domains 1-3
Bovine liver β -glucuronidase (Sigma-Aldrich St Louis, MO, USA)	Domains 3, 5 and 9
Recombinant Human IGF-II Protein (R&D Systems UK Abingdon, UK)	Domain 11
Mannose 6-phosphate barium salt (Sigma-Aldrich St Louis, MO, USA)	Inhibitor for M6P tagged ligand-receptor interaction
Glucose 6-phosphate barium salt (Sigma-Aldrich St Louis, MO, USA)	Negative control for M6P

2.1.4.4 SPR runs

IGF2 and β -glucuronidase (in HBS-EP Buffer), plasminogen (in Acetate buffer) serial dilutions were prepared in 10 folds range of predicted affinity of each analyte. Analytes were run at 40 μ l/min rate for 400 seconds followed by 600 seconds of buffer run for analyte-ligand dissociation. The chips were regenerated between each run by 2M MgCl₂.

2.1.4.5 SPR Analysis

Analyte run were performed using Biacore T200 Software. The resulting sensorgrams were evaluated using Biacore T200 Evaluation Software using 1:1 binding models.

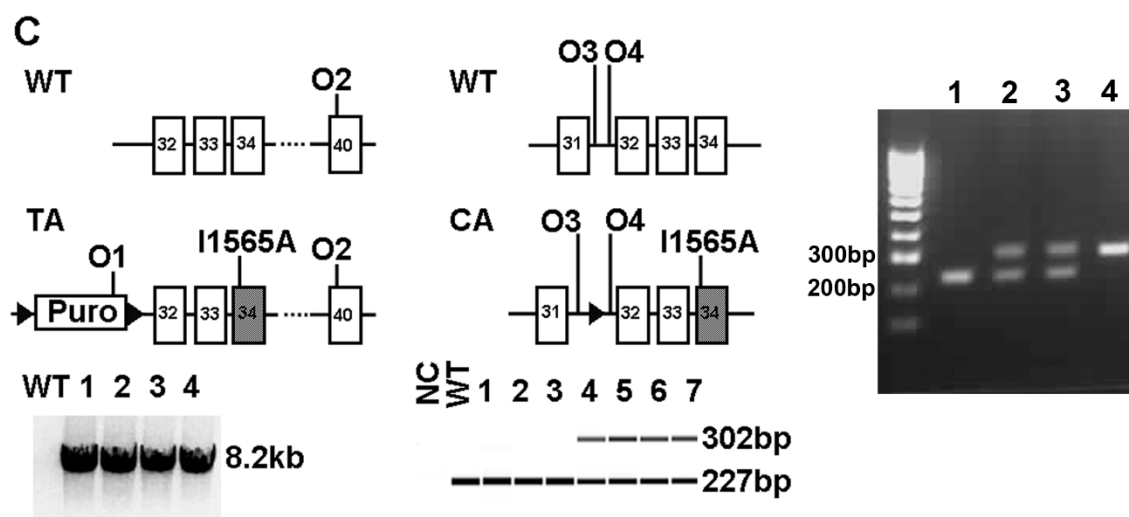
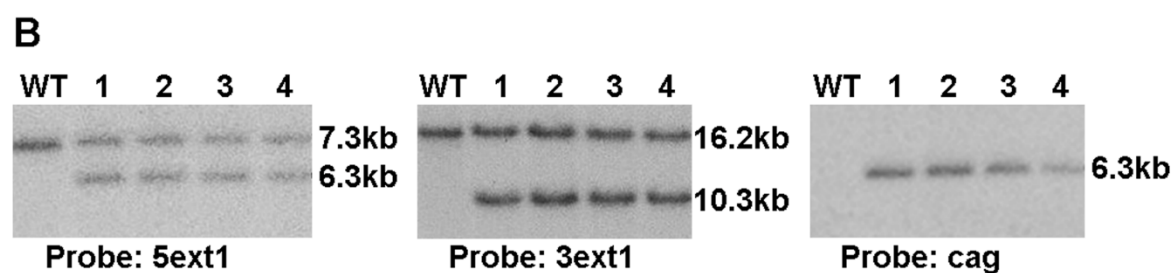
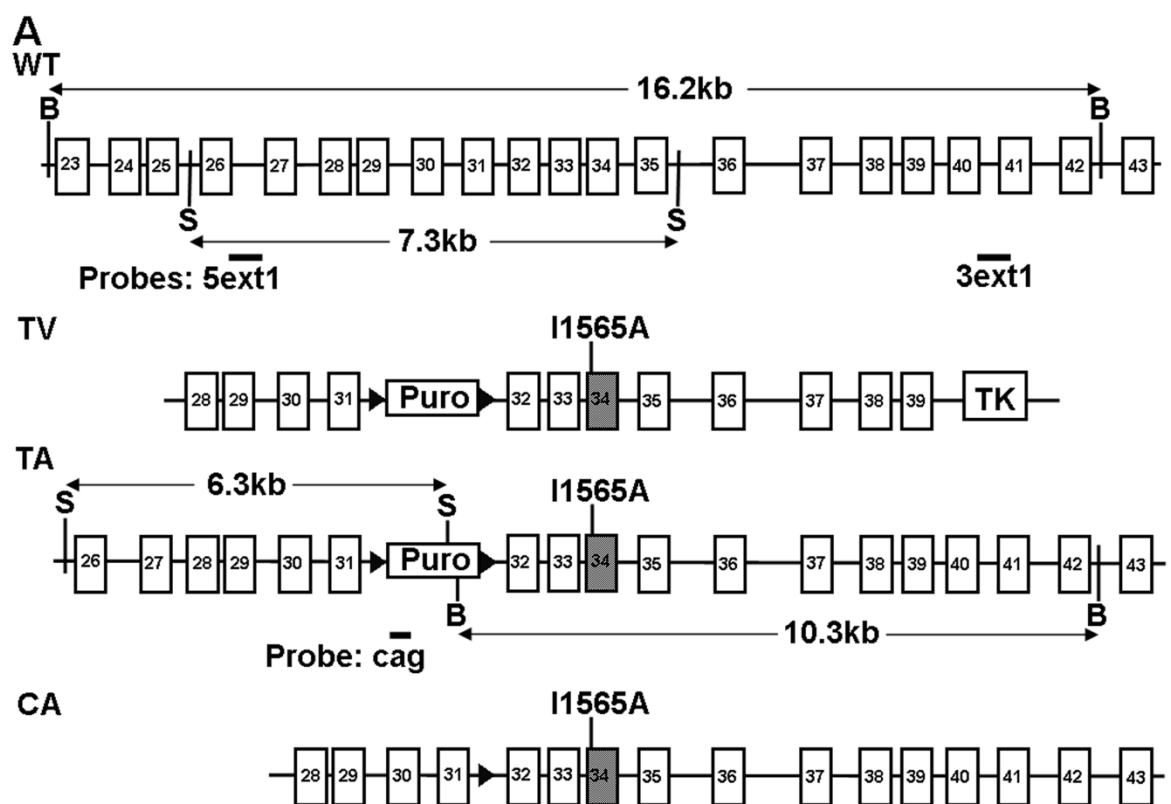
Table 2.7 Protein immobilisation levels for SPR analysis

Type of Analyte	Receptor Density	Streptavidin Density
IGF2, plasminogen	~1800 RU	12000-14000 RU
β-glucuronidase	~200 RU	9000-12000 RU

2.2 In vivo studies

2.2.1 Overview of the strategy of generating *Igf2r^{+m/I1565Ap}* mouse by TaconicArtemis®

A targeting vector containing I1565A mutation in exon 34 and the positive selection marker (Puromycin resistance - PuroR) flanked by FRT sites in intron 31 and was transfected into the TaconicArtemis C57BL/6N Tac ES cell line. The homologous recombinant clones were isolated using positive (PuroR) and negative (Thymidine kinase - Tk) selections. The selection marker was then removed with Flp recombinase. Later, the targeted ES cells were injected into blastocysts and transferred to uterine horns of pseudopregnant NMRI females. Male littermates of these females with >50% chimerism were selected for further breeding with C57BL/6 females. Finally, PCR analyses were conducted using genomic DNA extracted from mouse tail biopsies to confirm *Igf2r^{+m/I1565Ap}* genotype.



*See the next page for the legend

Figure 2.5: Strategy for generation of *Igf2r* I1565A knockin mouse. **(A)** Targeting vector (TV) carries I1565A and puromycin cassette, After homologous recombination targeted allele (TA) carries the mutation and the puromycin cassette for selection. Final constitutive knock-in allele (CA) is generated by removal of the selection cassette. **(B)** Southern Blot Analysis to detect correct homologous recombination. From left to right: Probes 5ext1 and 3ext1 detect correct 5' and 3' end homologous recombinations, respectively; probe cag detects single integration and correct 5' homologous recombination. **(C)** Left: Oligos 1&2 detect the presence of puromycin cassette. The PCR gels shows the presence of the WT allele in all clones before the homologous recombination. Middle: Oligos 3&4 detect the presence of constitutive knock-in allele (302 bp) after puromycin cassette removal; An illustration of amplification products show single 227 bp bands indicate WT and the double bands show heterozygous genotype. Right: A gel showing WT (1), heterozygous (2 and 3) and homozygous (4) genotypes.

2.2.2 Animal Breeding

Experimental animal breeding for the work in this thesis were performed under U.K. Home Office licence and approved by the local ethics committee (PPL number: 30/2695 and 30/3242). C57BL/6 background *Mus musculus* used for the breeding were housed at room temperature, under 12 hours light cycles and were fed *ad libitum*. The mice were weaned around on postnatal day 21.

2.2.3 DNA Extraction and Genotyping

2.2.3.1 DNA Extraction

As a routine practice, the mice were earclipped between 7-10 days of age for genotyping. For embryos, neonates and the post-mortem adult mice, DNA was extracted from tails. The samples were incubated at 95 °C in 250 µl DPG1 Lysis Buffer (25mM NaOH, 0.2 mM EDTA, pH 12) for an hour, followed with addition of 250µl DPG2 Neutralizing Buffer and vigorous

vortexing. Lastly, the mixture was spun for 6 minutes at 13000 RPM and 300 µl of the supernatant collected for genotyping. 1 or 2 µl of supernatants were used for genotyping that is described below.

2.2.3.2 Mouse Genotyping

Qiagen® HotStarTaq or Qiagen® HotStarTaq Plus Polymerase Kit (Hilden, Germany) used for mouse genotyping PCRs. The PCR mixtures and programmes are detailed in the table below (Table 2.8). The PCR products were run in 2.0 % agarose dissolved in 0.5% TBE buffer, at 60-80 V, in the presence of 0.7 µg/ml of ethidium bromide. DNA bands visualized under UV light.

Table 2.8 PCR conditions for genotyping

Igf2RI1565A-KI			
Reagent	Working Conc.	Volume (µl)	
Buffer	1x	5	
MgCl ₂	2 mM	4	
dNTP	0.2 mM	1	
Primers	0.1 µM	0.1	
Q solution	0		
ddH ₂ O		40	
Taq	0.08 U/µl	0.2	
	Total Volume (µl)	10	
Primer name	Sequence (5'-3')		
2RKI_37	GGTGCAGGTGTTATGACTGG	CKI	
2RKI_38	AGTACATCAGCCTGTATCCTGG		
2RKI_41	GAGTGCCATAGCTCCTGTGC	PM	
2RKI_42	GCTTGTCCAAGGAGATGAGC		
PCR Conditions			
Temp (°C)	Time	Cycles	Filename
95	5 min		2RKI
95	30 s		
60	30 s	35	
72	30 s		
72	10 min		
Products			
2% agarose	Wt - 227bp	CKI - 302bp	PM-384bp

2.2.4 Timed Matings and Embryonic Dissections

Females with paternal Domain 11 loss-of-binding mutation were mated with either males with the same genotype or WT. Females were checked twice a day for vaginal plug. The dated of the plugs were recorded and followed for the signs of pregnancy. Pregnant females were later culled on embryonic days 14, 17 and 18. Subsequently, the pregnant uteri were transferred to ice cold PBS. The embryonic membranes were dissected and embryos were culled by decapitation. After further dissection, embryos and placentas were weighted in 1.5 ml tubes, left in 37 °C incubator to dry. The dry weights were assessed after two days of drying period.

2.2.5 Newborn dissections and weightings

Mice with paternal mutation were mated to produce hetero- and homozygous progenies. Females that were found to be pregnant were checked daily for newborns. The newborns were labeled by markers for future follow up and weighted daily during the neonatal period, and some were culled on postnatal day 1 for dissections.

2.2.6 Adult Mice Dissections

Both female and male mice were left to grow to adulthood and examined. All these mice are also examined post-mortem macroscopically for any abdominal, genitourinary and thoracic abnormalities.

2.2.7 Tissue fixation and histological analysis

2.2.7.1 Tissue fixation

The tissues collected from mice were immersed in 3.6 % formaldehyde solution in PBS (Phosphate Buffered Saline; Lonza Verviers, Belgium) at room temperature for 12-24 hours. The tissue samples were later exchanged to 70% ethanol and were kept in 4 °C for until further processing.

2.2.7.2 Histological analysis

Fixated tissues were later processed for histological analysis in the University of Oxford Sir William Dunn School of Pathology facilities. Thoracic and placental sections were stained with hematoxylin and eosin (H&E) staining. Placental sections were also stained with periodic acid schiff (PAS). The sections were examined under light microscope.

2.2.8 Statistical Analysis

Chi square test was used to compare the distribution of the mice genotypes and Mendelian ratios. For comparison of weights and the weight proportions, paired T test was used.

Chapter 3: Human domain 11 binding site loops confer affinity for IGF2 independent of all the extra-cellular domains of M6P/IGF2R

3.1 Introduction

P-type lectin Insulin like-growth factor/Mannose 6-phosphate receptor (IGF2/M6PR) is a type 1 transmembrane receptor with fifteen homologous extracellular domains. (IGF2/M6PR). Domains 1-2 bind plasminogen, domains 3, 5 and 9 bind Mannose 6-phosphate (M6P), and domain 11 bind IGF2. So far, structure of domains 1-3, 5 and 11-14 have been determined (Olson, Yammani et al. 2004, Brown, Delaine et al. 2008, Olson, Peterson et al. 2010). The ecto-domains share a conserved β -barrel structure (Figure 3.1A). The β -barrels consist of nine β strands, forming two β -sheets stabilised, for most of the domains, by four disulphide bonds. Domain 13 is a unique domain and harbours an extension called FNII insert that is a 48 amino acid sequence resembling fibronectin type II tertiary structure (Figure 3.1A). The insert is formed by two β -sheets, each β -sheet is made of two β strands connected with two disulphide bridges. Core β -barrels of the domains are capped with a β hairpin in their N-terminal. On the opposite side, the strands are connected with four loops (Figure 3.1B). These loops form the binding sites of the ligand binding domains.

In domain 11, the binding loops are named as AB, CD, HI and FG, forming a predominantly hydrophobic binding pocket. Overall positive and negative charges of IGF2 and Domain 11 binding interfaces respectively also contribute to the interaction affinity (Brown, Delaine et al. 2008). IGF2 sequence and structure are highly conserved in vertebrates. Thus, evolution of M6P/IGF2R IGF2 binding pocket has played the key role for acquisition of the binding affinity. Affinity of M6P/IGF2R to IGF2 was first attained in monotremes and further evolved to a higher

affinity in placental mammals. Transformation of the CD loop seems to play a key role in this binding acquisition. The transformation of the CD loop - in addition to side chain changes - involves shortening of the loop length, resulting in reduced flexibility. Alanine scanning studies showed that, in isolated domain 11 constructs, I1572 and F1567 of CD loop are essential for IGF2 binding. These residues were also acquired during evolution from chicken to echidna. In addition, replacement of T1572 of CD loop with an alanine causes ~9 fold reduction in IGF2 binding and Q1569, the other residue of the loop, plays a minor role in IGF2 binding. Further enhancement of IGF2 binding in placental mammals concurs with increased volume and hydrophobicity of IGF2 binding pocket due to the mutations in AB and FG loops. In addition to CD loop residues, S1596 of FG loop, Y1542, S1543, K1545 of AB loop are also required for the high affinity IGF2 binding in placental mammals.

F19, L53 and T16 of IGF2 are identified as essential residues for IGF2-Domain11 binding. F19 is the anchor residue of the interaction and is buried by F1567, L1629 and Y1542 of Domain 11 upon IGF2 binding. The other one of three essential residues of the binding of IGF2, L53, interacts with K1631. The last essential residue, T16, is crucial for IGF2 specificity to M6P/IGF2R.

IGF1 and IGF2 are characterized by their high levels of sequence resemblance to each other; both act on IGF1R and bind to IGFBPs (Samani, Yakar et al. 2007). Despite structural and functional resemblance, IGF1 does not bind to IGF2/M6PR. Interestingly, with replacement of Alanine 13 of IGF1 with Threonine, IGF1 gains weak affinity to IGF2/M6PR (Brown, Delaine et al. 2008). A13 is equivalent of T16 on IGF2, an essential residue that interacts with I1572. Therefore, A13's equivalent in IGF2, T16, seems to be responsible for IGF2 specificity.

In addition to essential and affinity-enhancing residues, several Domain 11 mutants have been identified with enhanced IGF2 affinity, suggesting the presence of inhibitory residues in the binding site. First examples of such mutations that were identified in AB loop, E1544A and E1544K, increase IGF2 affinity approximately three and ten-fold respectively. When tested in Domain 11-13, E1544K, however, failed to show the same affect. Later two AB loop mutants, E4 (E1544K-K1545S-L1547V) and 3-4D (S1543A-E1544K-K1545G-G1546W-L1547G) developed, displaying ~4.1 and ~10 folds increased affinity compared to WT, respectively. Addition of a combination of CD and FG mutations named “QPS” increased the IGF2 affinity further. E4QPS and 3-4DQPS mutants showed ~41 and ~32 folds of WT affinity, respectively. These results suggest that although no single residues been identified to have an inhibitory role, IGF2 binding is inhibited collectively with the involvement of multiple residues.

Although, E1544 of AB loop is a binding inhibitor in isolated domain 11, interestingly, same mutation in Domain 10-13 results in 10 folds reduced affinity (Brown, Delaine et al. 2008). Further structural and functional studies of WT and mutant Domain 10-13 constructs revealed that E1544 is essential for interaction of domain 11 with domain 13's FNII (fibronectin type II fold) insert and this interaction fixes AB loop in its position and enhances IGF2 binding ~10 fold (Zaccheo, Prince et al. 2006, Brown, Delaine et al. 2008). In addition to domain 13's contribution IGF2 binding, IGF2 binding studies of domains 1-15 (14nM), 10-13 (120nM), 11-13 (100 nM) and 11-12 (450nM) suggest possible contribution of other domains in addition to domains 10-13 (Linnell, Groeger et al. 2001). Contributions of these domains are pH dependent, possibly reflecting the dynamic nature of the receptor functions during the trafficking between intra-cellular compartments.

While some studies have shown other domains effect on IGF2 binding, virtually no data exist on the role of domain 11 binding loops and residues' in the context of full-length receptor. The only relevant work so far is the characterisation of a growth restriction related single nucleotide

polymorphism (G1619R) in full-length receptor *in vitro* and the mutation found to have no structural and functional effect (Rezgui, Williams et al. 2009). In order to investigate, Domain 11's influence over the rest receptor's overall functions and to dissect roles of the IGF2 binding loops in the context of the full-length receptor, I aimed to generate Domain 11 IGF2 loss- and gain-of-binding function mutants and characterise them using surface plasmon resonance (SPR).

3.2 Aims

- **Generation of IGF2 loss and gain-of-binding function full-length IGF2/M6PR to investigate influence of these mutations on other ligands' binding affinity**
- **Generation of IGF2 loss and gain-of-binding function full-length IGF2/M6PR to investigate Domain 11 binding loops' functions in the context of full length receptor**

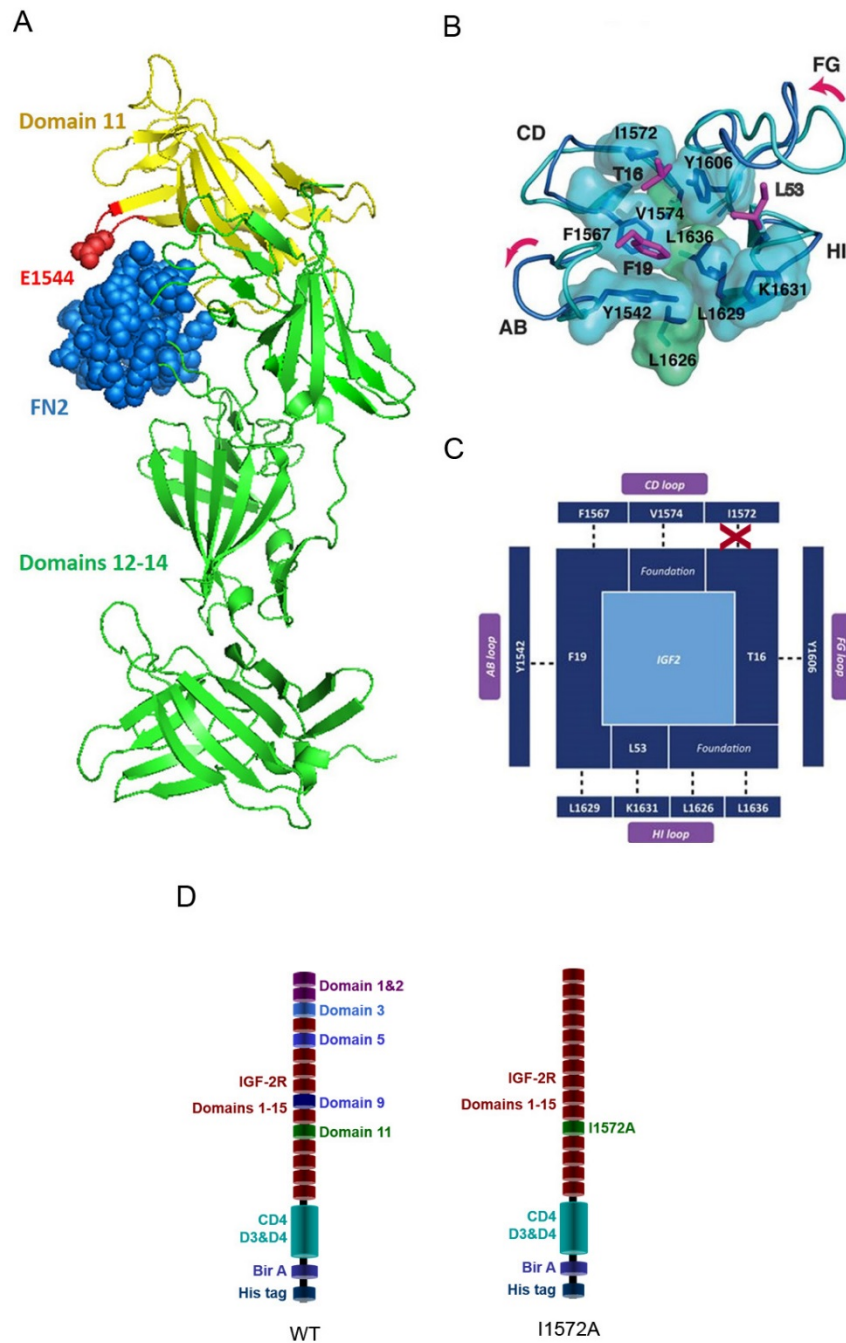


Fig 3.1 Structure and mutagenesis of IGF2/M6P receptor. **(A)** Pymol illustration of Domains 11-14 showing the interaction of AB loop E1544 (in red) and FN2 insert of Domain 13. **(B)** Interaction of the three IGF2 key residues (purple) with Domain 11 binding loops. Domain 11 AB loop is illustrated in red **(C)** An interaction map of IGF2 and IGF2/M6PR Domain 11 binding residues. **(D)** Receptor chimeras expressed by the HEK293T cells: Domains 1-15 of IGF2/M6PR fused with rat CD4 Domains 3&4, a Biotin acceptor site and polyhistidine (His6) tag. Figures 3.1B&C are adapted (Williams, Hoppe et al. 2012)

3.3 Results

3.3.1 Intact and biotinylated WT and I1572A receptor protein were secreted by HEK293T Cells

Isoleucine of the CD loop of Domain 11 (Figure 3.1C, D) was replaced by an alanine residue to generate a Domain 11 IGF2 loss-of-binding mutant. HEK293 cells were co-transfected with WT or I1572A constructs, and a biotin ligase expressing vector. In a pilot experiment using 6 well plates, media from the individual wells were collected on Days 1 to 4 (Figure 3.1C). Western blots of the collected media showed optimal protein production on day 4. After upscaling the transfections to T175 flasks, a concentration step for collected media was added to the protocol. Western Blots of the concentrated mediums collected on day 4 from HEK293T cultures transfected with WT and I1572A constructs with HRP conjugated anti-histidine antibodies and streptavidin showed bands running at the expected molecular weight (Figure 3.1D, E). Both blots showed no detectable degradation products. Streptavidin blots also confirmed that the recombinant receptors were biotinylated (Figure 3.1E). Absence of other bands in streptavidin blots also indicated absence of any other detectable biotinylated proteins.

3.3.2 Nickel column purification fractions and buffer exchange of the receptor

The polyhistidine tagged proteins were purified by nickel affinity chromatography. Following extensive wash of the columns to further clear the remaining of the biotin, three elutions were collected using 300 mM imidazole. Flow through, first two washing fractions and the elution fractions were later run in western blot and probed with anti-polyhistidine antibody and streptavidin. The blots showed that the proteins were mostly concentrated in elution 1 with no detectable proteins in elution fraction 3, and the absence of any other significant bands than the bands of interest. In order to clear up imidazole and further reduce the biotin

concentrations in the elution fractions, HPS-EB buffer exchange were performed using centrifugal filters. Buffer exchanged receptors were later immobilised on streptavidin coated CM5 chips (S series) for affinity characterisation in Biacore® T200. For human IGF2 (7.4 KD) and IGF1 (7.7 KD) run, 1800 RU; for higher molecular weight analytes of bovine β -glucuronidase (290KD) and human glu-plasminogen (88 KD), 200 RU of the receptor was immobilised onto the chip surface.

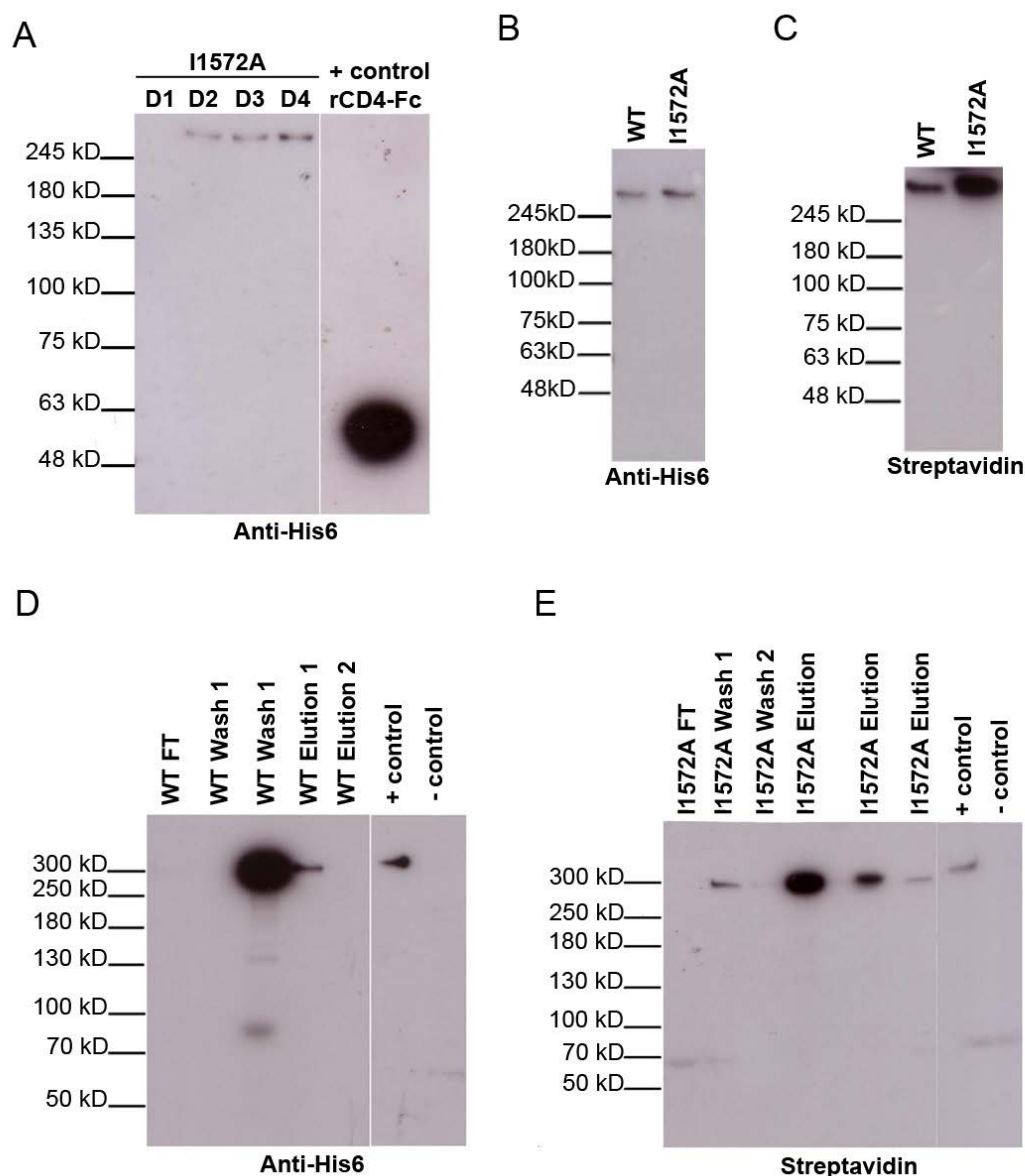


Fig 3.2 Expression and purification of full length receptor chimeras. The chimeras' molecular weights are approximately 290 kilodaltons (A) Time course of the receptor protein expression in HEK293T cells. (B, C) Detection of full length receptor proteins in western blots probed with horseradish peroxidase conjugated anti-polyhistidine antibody and streptavidin (D, E) Western blots showing the detection of full length receptor proteins in nickel column purification fractions. The blots were probed with horseradish peroxidase (HRP) conjugated anti-polyhistidine antibody and HRP conjugated streptavidin. + control: Medium collected from pilot or previous large scale transfections, - control: Medium collected from transfections without a receptor expressing plasmid.

3.3.3 Mass transport effect is observed at lower flow rates

Due to the substantial difference in size between the ligand (290 kD) and analyte (7.5 kD), high density (1800RU) chips of the ligand (IGF2/M6PR chimeras) were required for evaluation of IGF2 affinity to the receptor. Due to high binding capacity of such chips, insufficient supply of the analyte to the chip matrix could occur at lower flow rates which results in recording of lower affinity than the actual affinity. This phenomenon is called mass transport effect. In order to avoid mass transport effect, I measured dissociation constants (K_D) at a range of flow rates (5, 10, 20, 40 and 80 μ l/min) (Figure 3.3 A, B, C, D and E) and evaluated flow rate/ K_D relation (Figure 3.3 F). K_D values were doubled between flow rates of 5 and 10 μ l/min and started to plateau (~20% change) between flow rates of 10 and 20 μ l/min. Increasing flow rate 40 μ l/min to 80 μ l/min yield no significant (~6%) increase in affinity. These results indicated that 40 μ l/min flow rate is sufficient to avoid mass transport effect in the high ligand density chips that are required to evaluate IGF2 affinity.

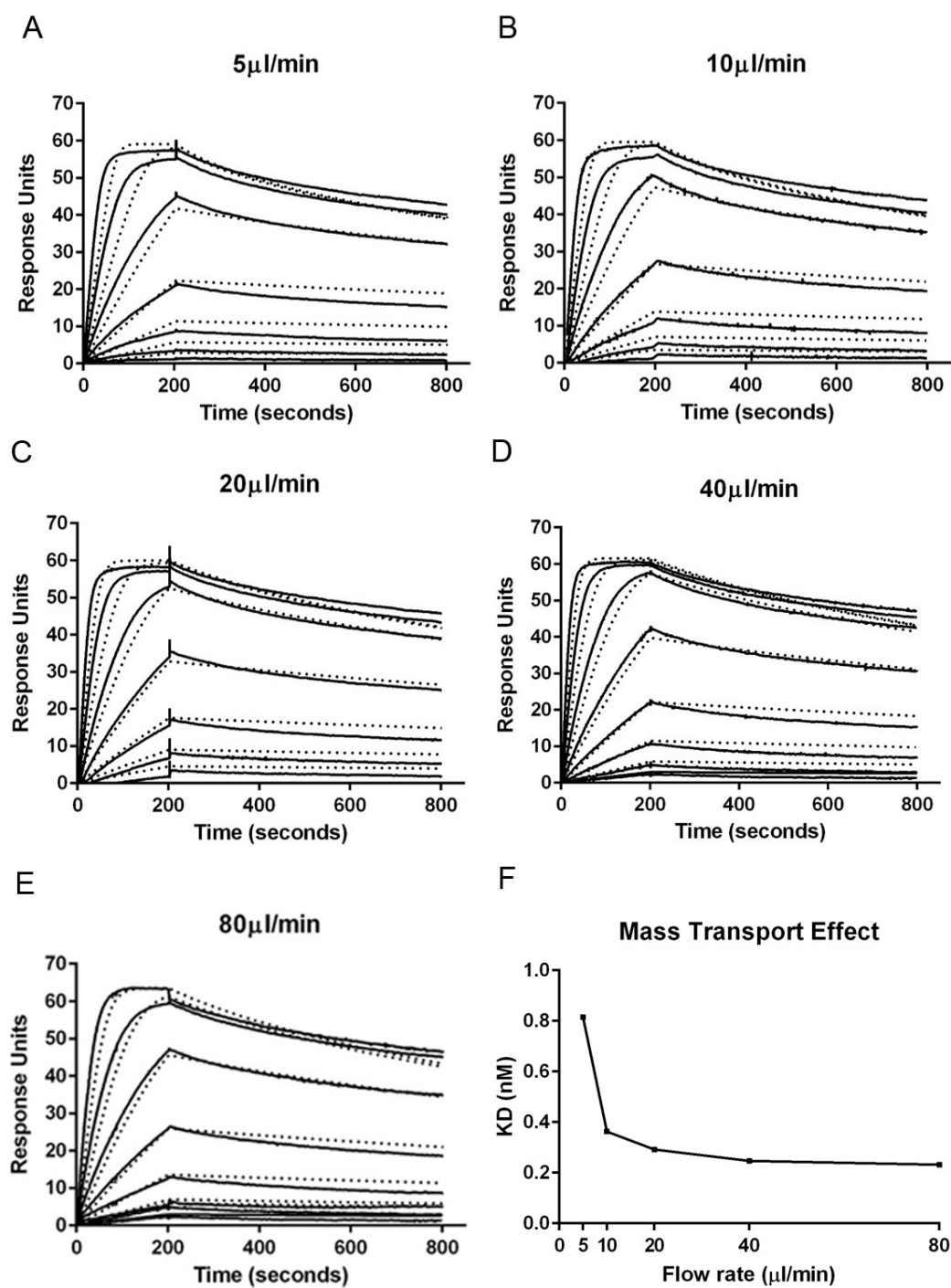


Fig 3.3 Evaluation of mass transport effect in high density receptor (1800 RU) CM5 S series chips by flowing human IGF2 at flow rates of 5-80 $\mu\text{l/min}$. (A, B, C, D, E) Sensorgrams and fittings according to 1:1 binding model at flow rates 5-80 $\mu\text{l/min}$. (F) K_D values at are plotted against flow rates showing the effect of flow rate on the affinity measurements.

3.3.4 Replacement of Isoleucine 1572 in CD loop with an Alanine results in incomplete loss-of-IGF2-binding function

WT and I1572A full length receptor were immobilised on flow cells 2 and 3 of CM5 chips flow cells. No receptor protein was immobilized on the flow cell 1, which was used as control. During the evaluation, flow cells 1 sensorgrams were subtracted from flow cells 2 and 3. WT sensorgrams showed quantifiable binding and were fitted according to the 1:1 binding model using Biacore T200 evaluation software (Figure 3.4 A). While WT exhibits 0.95 nM affinity to IGF2, I1572A sensorgrams traces showed not quantifiable minimal binding, indicating loss of IGF2 binding in the mutant (Figure 3.4 B, C).

3.3.5 I1572A mutant receptor lacks IGF1 binding.

IGF1 was also run on the WT and I1572A chips to test if replacement the Isoleucine residue with Alanine would result in IGF1 gain-of-binding in the mutant receptor due to a change in the ligand specificity of the receptor. Both WT and the mutant receptors showed flat sensorgrams after subtraction of the control flow cell traces (Figure 3.5 A, B).

3.3.6 Domain 11 loss-of-binding due to the CD loop mutagenesis do not alter other ligand binding functions of the receptor.

In order to investigate the binding function of the other domains of the receptor due to the mutation introduced, I evaluated the mutant receptor's M6P and plasminogen binding domains' functions. Bovine β -glucuronidase and human Glu-plasminogen were used to test binding function of the domains 3, 5 and 9; and domains 1-2, respectively.

Plasminogen exists in three different conformations, each of which exhibits different affinities for IGF2/M6PR. In the presence of acetate ions, plasminogen exists in an open conformation, showing the highest affinity for the receptor. To increase the sensitivity of the SPR evaluations, I performed the plasminogen binding analysis in the presence of 150 mM sodium acetate which is required for high affinity open confirmation for plasminogen. WT and I1572A receptors showed similar binding affinities to β -glucuronidase, 61.5 and 62.4 nM respectively, suggesting that the I1572A mutant has conserved M6P binding function (Figure 3.5 C, D, G). Similarly, no significant difference was found between the affinities of the mutant and the WT receptors to plasminogen (WT: 39.7 vs I1572A: 42.3 nM) suggesting preserved binding function for domains 1-2 in the mutant (Figure 3.5 E, F, G).

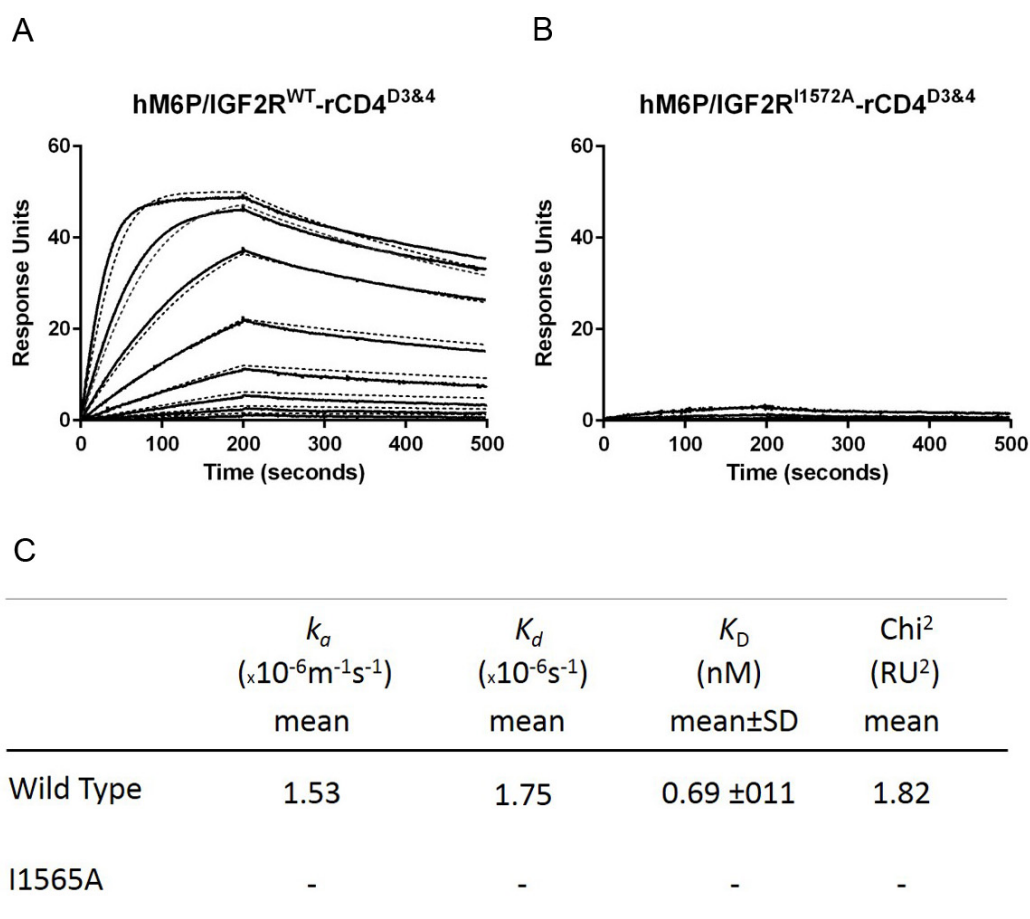
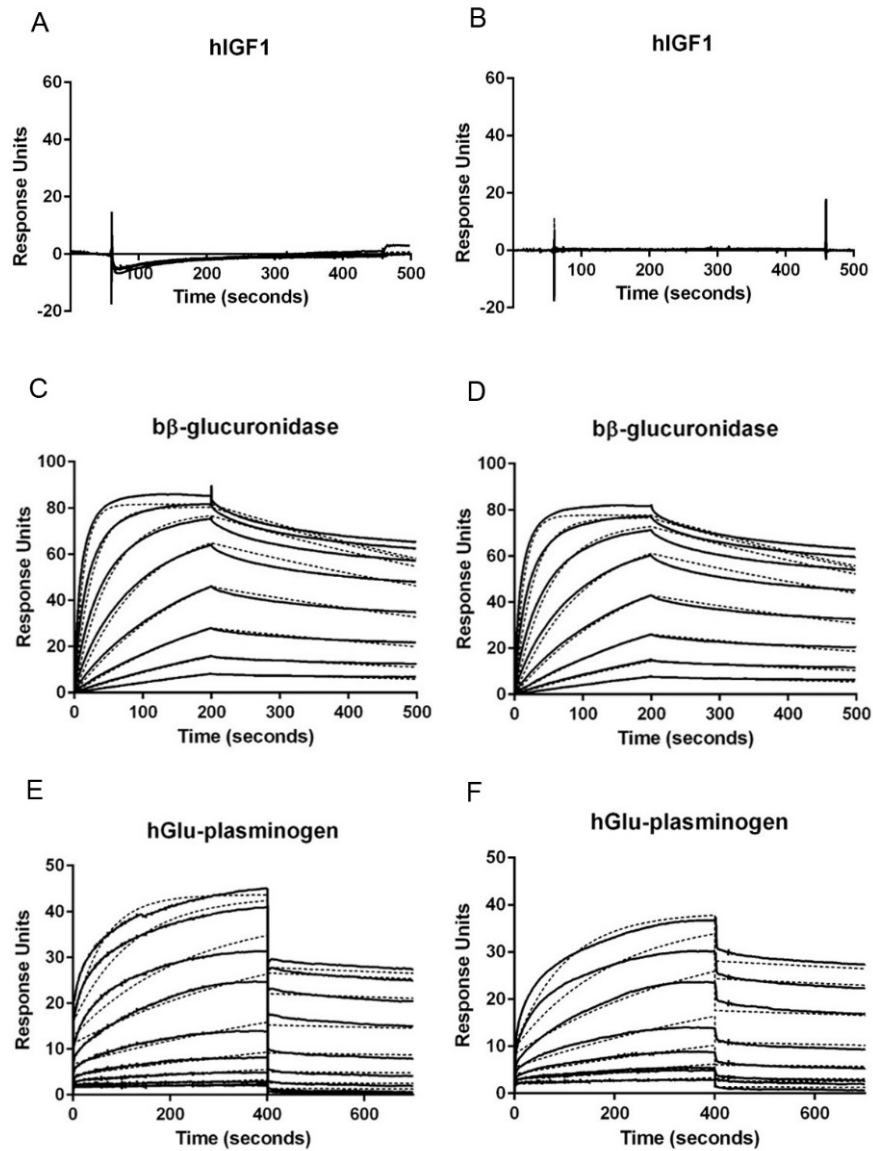


Fig 3.4 Affinity WT and I1572A full-length receptors to human IGF2. (A, B) Sensorgrams of WT and I1572A receptors' interaction with IGF2. WT receptor sensorgrams fits were performed according to 1:1 binding model. (C) Association, dissociation rates and affinity constants of the WT and I1572A interaction with IGF2 and Chi² values of their respective fits are summarised. Values shown are average of three independent experiments.



G

		k_a ($\times 10^{-6} \text{m}^{-1} \text{s}^{-1}$)	K_d ($\times 10^{-6} \text{s}^{-1}$)	K_D (nM)	Chi ² (RU ²)
		mean	mean	mean $\pm$ SD	mean
IGF1	Wild Type	-	-	-	-
	I1565A	-	-	-	-
β -glucuronidase	Wild Type	183	1130	61.5 $\pm$ 3.7	2.81
	I1565A	177	1110	62.4 $\pm$ 7.6	2.82
Plasminogen	Wild Type	3973 $\times 10^6$	157	39.7 $\pm$ 2.23	0.824
	I1565A	4744 $\times 10^6$	200	42.3 $\pm$ 3.7	1.07

*See next page for the figure legend

Fig 3.5 Affinity of human IGF1, bovine β -glucuronidase and human Glu-plasminogen binding to WT and domain 11 IGF2 loss-of-binding mutant. (A, B, C, D, E, F) Sensorgrams of human IGF1, bovine β -glucuronidase and human Glu-plasminogen binding to WT and domain 11 loss-of-binding mutant. (G) Association, dissociation rates, affinity constants of the WT and I1572A to bovine β -glucuronidase and human Glu-plasminogen, and χ^2 values of their respective fits. The values were obtained in three independent experiments.

3.3.7 Mass transport effect for gain of function mutants

In addition to the high density of the analyte binding sites on the SPR chip, high affinity of ligands to analytes could also result in mass transport effect. When both of these factors supervene, mass transport effect could be observed even at higher flow rates. Therefore, an evaluation of mass transport effect was required for higher affinity gain-of-function mutants. I measured K_D of the highest affinity mutant, E4QPS at flow rates 5, 10, 20 and 40 $\mu\text{l}/\text{min}$ (Figure 3.6 A, B, C, D). The affinities were plotted against the flow rates (Figure 3.6 E) the graph shows an abrupt plateau at 20 $\mu\text{l}/\text{min}$. This suggest that 40 $\mu\text{l}/\text{min}$ is sufficient to avoid any mass transport limitation that might arise due to high affinity nature of the immobilised gain-of-function mutants.

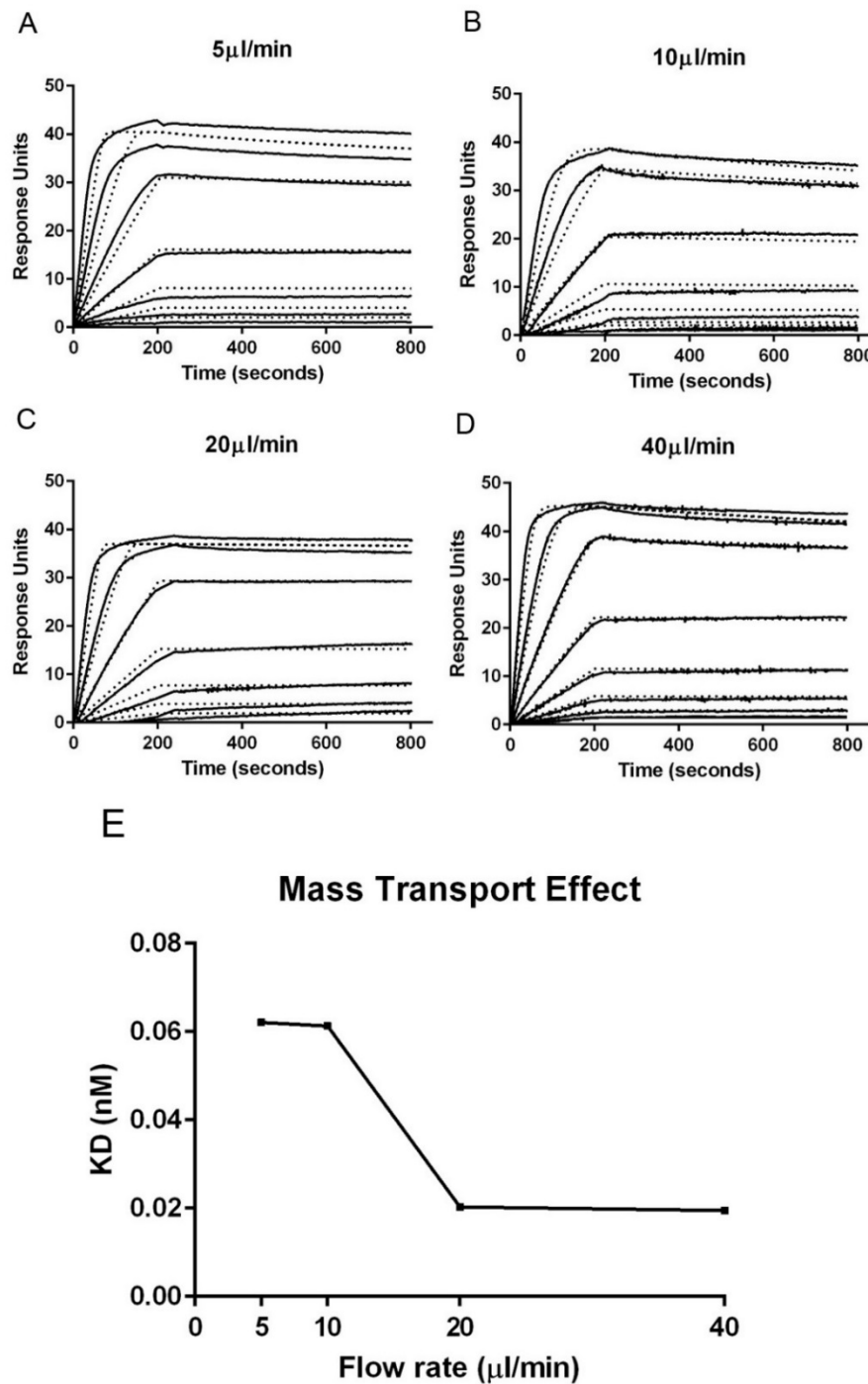


Fig 3.6 Evaluation of mass transport effect in high ligand density (1800 RU) CM5 S series chips by flowing human IGF2 at flow rate 5-40µl/min on high affinity E4QPS mutant. (A, B, C, D) Sensorgrams and fittings according to 1:1 binding model at flow rates 5-40µl/min. (E) Association constants are plotted against the flow rates

3.3.8 In the context of full-length IGF2/M6PR, the AB&CD&FG loop but not AB loop gain-of-function mutant, achieves an enhanced affinity to IGF2

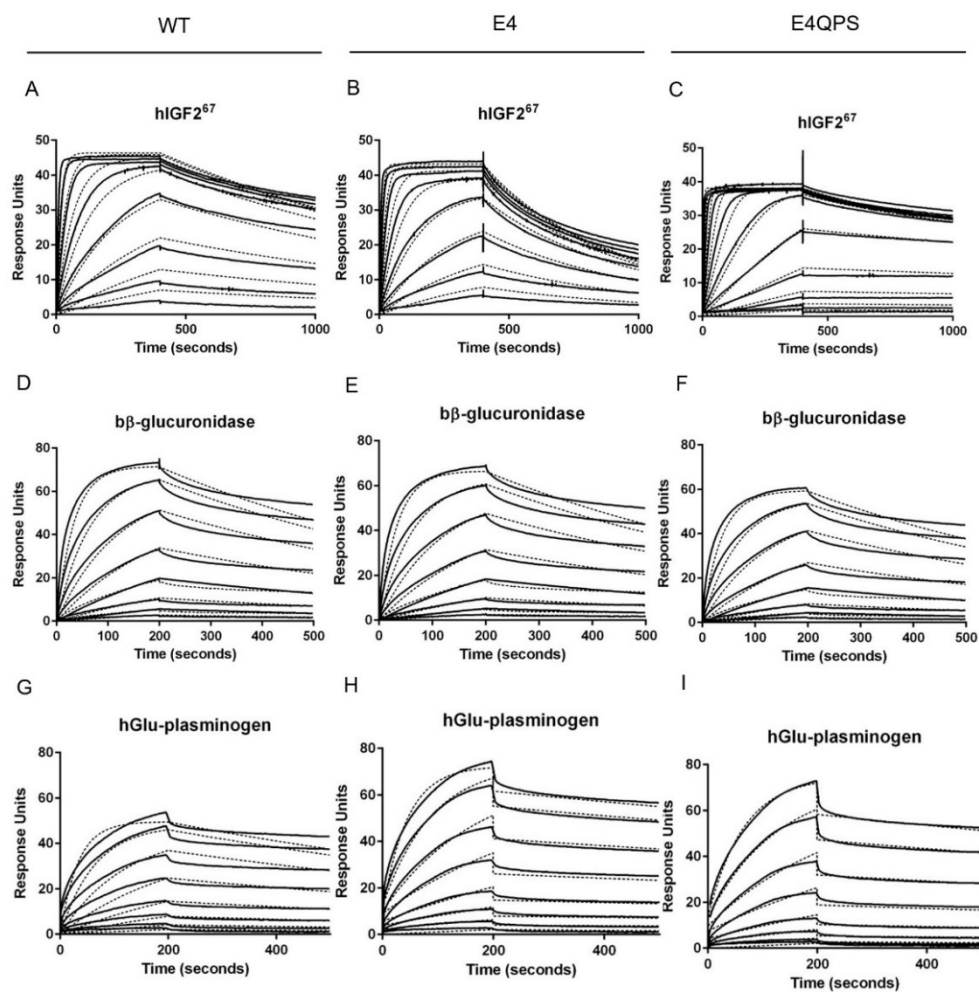
I have tested E4 (AB loop mutant) and E4QPS (AB&CD&FG loop mutant) full-length receptors affinity to IGF2. The sensorgrams were fitted to 1:1 binding model. E4 mutant showed an affinity to IGF2, 1.26 nM, that is ~60% lower affinity compared to WT receptor's affinity (0.78 nM) (Figure 3.7 A, B, J). Although AB loop gain-of-function mutations (E4) could not achieve any increase in affinity, its combination with "QPS" set of mutations increased IGF2 binding approximately three fold (E4QPS: 0.24 nM vs. WT: 0.78nM) (Figure 3.7 A, C, J).

3.3.9 Introducing multiple gain of mutations to IGF2 binding site do not disturb IGF2/M6PR's binding to its other ligands

Introducing E4 and E4QPS mutations to the full length receptor did not alter the receptor's binding to plasminogen or β -glucuronidase. Approximately same affinity of WT receptor (102.2 nM), E4 (104 nM) and E4QPS (103.1 nM) mutants; indicates presence of fully functional M6P binding domains in both of the mutants (Figure 3.7 E, F, J). N-terminal plasminogen binding function was also preserved in both mutants (WT: 23.32nM vs E4:18.8 nM & E4QPS:32.29 nM) (Figure 3.7 E, F, J).

3.3.10 P1597 and S1602 of FG loop have inhibitory role in IGF2 binding

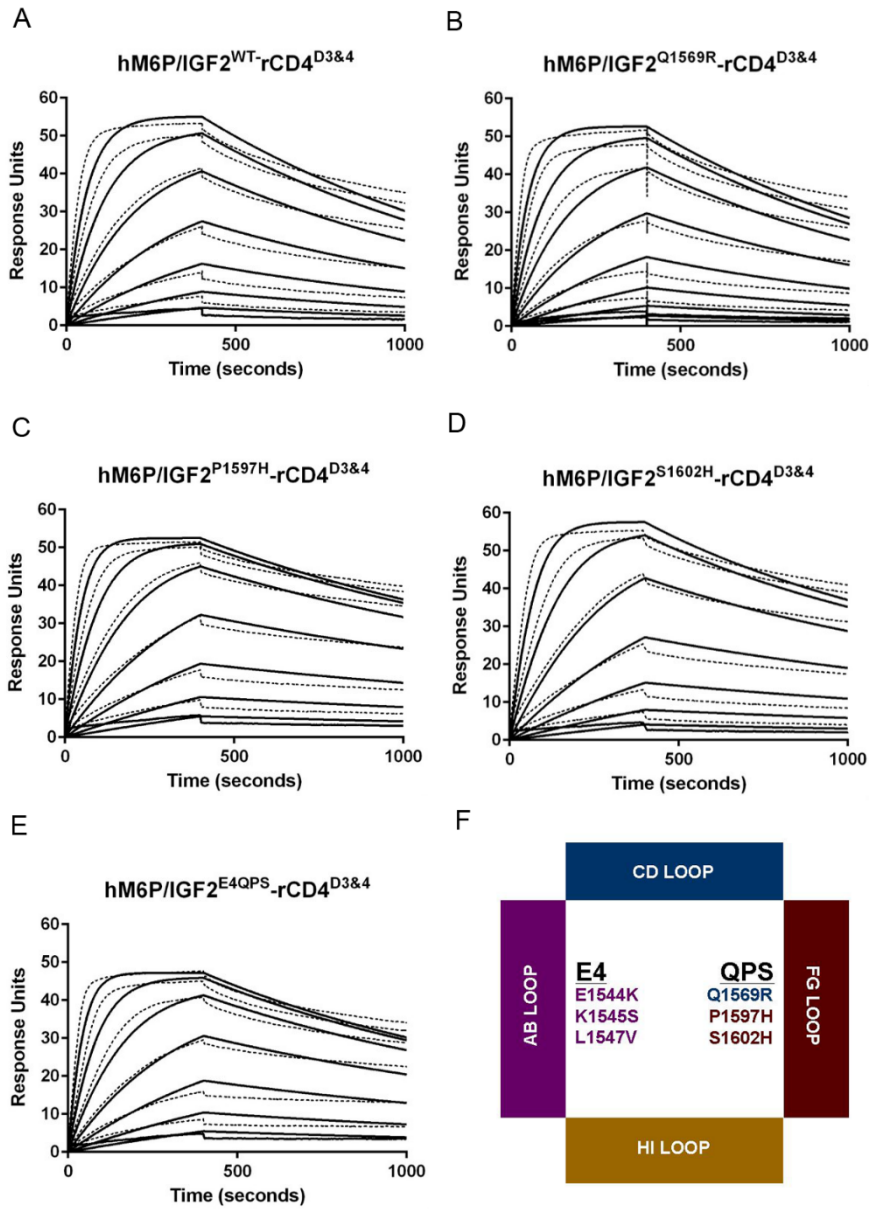
In order to investigate how addition of “QPS” increases binding affinity of the receptor in the context of full length receptor, I generated full-length receptors with single mutations that constitute the QPS combination: Q1569R, P1597H, S1602H (Figure 3.8 F). While the CD loop mutant, Q1569R showed similar affinity to IGF2 compared to WT (0.86nM vs 0.95nM); FG loop mutants showed higher affinity. P1597H mutation increases IGF2 affinity more than S1602H (0.34nM vs 0.73 nM). These results suggest inhibitory roles of P1597, S1602 residues in IGF2 binding.



		k_a ($\times 10^{-4} \text{m}^{-1} \text{s}^{-1}$)	K_d ($\times 10^{-4} \text{s}^{-1}$)	K_D (nM)	Chi ² (RU ²)
		mean	mean	mean $\pm$ SD	mean
IGF2	Wild Type	0.0838	4.4	0.78 $\pm$ 0.32	4.4
	E4	0.167	2.07	1.26 $\pm$ 0.21	2.63
	E4QPS	0.374	4.13	0.24 $\pm$ 0.16	2.68
β-glucuronidase	Wild Type	1.44	1.49	102.2 $\pm$ 0.79	3.83
	E4	1.43	1.5	104 $\pm$ 5.3	3.47
	E4QPS	1.27	4.1	103.1 $\pm$ 23	2.38
Plasminogen	Wild Type	3.1	6.86	23.32 $\pm$ 10.21	0.097
	E4	3.05	7.05	18.9 $\pm$ 4.73	0.17
	E4QPS	3.04	9.64	32.29 $\pm$ 5.74	0.162

*See next page for the figure legend

Fig 3.7 Characterisation of E4 and E4QPS mutants (A, B, C) Sensorgrams of human IGF2 binding to WT, E4 and E4QPS receptor chimeras. **(D, E, F)** Sensorgrams of bovine β -glucuronidase binding to WT, E4 and E4QPS receptor chimeras. **(G, H, I)** Sensorgrams of human recombinant glu-plasminogen binding to WT, E4 and E4QPS receptor chimeras. **(J)** Table summarising interaction parameters of the WT, E4 and E4QPS mutants with its binding partners. The values are the mean of three separate experiments.



G

	k_a ($\times 10^{-6} \text{m}^{-1} \text{s}^{-1}$)	K_d ($\times 10^{-4} \text{s}^{-1}$)	K_D (nM)	Chi ² (RU ²)
	mean	mean	mean $\pm$ SD	mean
Wild Type	1.16	10.8	0.95 $\pm$ 0.16	4.76
Q1569R	1.19	10.3	0.86 $\pm$ 0.05	6.1
P1597H	1.54	6.57	0.43 $\pm$ 0.04	4.15
S1602H	1.16	8.33	0.73 $\pm$ 0.19	5.99
E4QPS	4.29	9.99	0.28 $\pm$ 0.13	1.1

*See next page for the figure legend

Fig 3.8 Characterisation of Q1569R, P1597H and S1602H mutants. (A, B, C, D, E) Sensorgrams of human IGF2 binding to WT, Q1569R, P1597H, S1602H and E4QPS receptor chimeras. **(F)** Diagram showing the gain of function mutations. **(G)** Summary of the kinetic parameters for the gain-of-function mutants and IGF2. The values are the average of multiple independent experiments (Q1569R, P1597H, S1602H, two repeats; WT, nine repeats; E4QPS, six repeats).

3.4 Discussion

3.4.1 Co-transfection of HEK293T cells with BirA acceptor tagged full length M6P/IGF2R and biotin expressing vectors is an efficient and specific approach to produce biotinylated soluble full length M6P/IGF2R proteins.

Streptavidin-biotin interaction is an exceptionally strong non-covalent bond and is used in many applications of biotechnology (Weber, Ohlendorf et al. 1989), including protein purification and detection, and also in the study of protein-protein interactions. So far, many methods for *in vivo* biotinylation of cytoplasmic, cell surface and secreted proteins have been developed (Parrott and Barry 2001, Barat and Wu 2007, Kim, Cantor et al. 2009). I have adapted an *in vivo* biotinylation approach, involving BirA biotin protein ligase, to produce biotinylated soluble recombinant full-length M6P/IGF2R immobilised on streptavidin CM5 chips. Streptavidin probed Western Blots of the medium collected from HEK293T cells showed a single band at the expected molecular weight, indicated that BirA ligase biotinylates the full length receptor chimeras tagged with BirA acceptor site with high specificity. Based on previous protocols developed in our lab for the biotinylation of receptor fragments (such as for domains 10-14), 2mM of biotin was added to the media. The biotin in the media competes with the receptor for the immobilisation onto the streptavidin chips, which in most cases prevented sufficient immobilisation to obtain the high receptor density chips. This obstacle was overcome with the addition of extensive washes and a subsequent buffer exchange step.

Western blots of the purified protein with the modified protocol showed absence of significant degradation products. Thus, almost all of the molecules of protein that were immobilised onto the chip were intact full-length receptors. Use of the streptavidin chip also added an on-chip purification step further increasing the purity of the protein on the chip, further excluding any significant contribution of endogenous IGF2 or β -glucuronidase binding proteins' contamination from HEK293T cells.

3.4.3 CD loop I1572A mutation disrupts IGF2 binding with preserved specificity for Domain 11 and other ligand binding functions.

I1572, had previously been identified as an essential residue in isolated domain 11 and in domain 11-13 constructs (Linnell, Groeger et al. 2001, Zaccheo, Prince et al. 2006). Domain 11-IGF2 affinity is greatly enhanced with presence of other IGF2/M6PR extracellular domains. For example, IGF2 exhibits ~32 times more affinity to full length receptor compared to Domain11-12 (Linnell, Groeger et al. 2001). In our lab, we also observed that full-length receptor exhibit 100 fold higher affinity to IGF2 compared to isolated Domain 11 chimeras (unpublished data). Although IGF2 affinity is substantially higher in the full length receptor, I1572A still disrupts the IGF2 binding almost completely, demonstrating the essential role of the residue in the context of the full-length receptor. I1572 interacts with T16 of IGF2, a residue that is responsible for IGF2 binding specificity of the receptor (Samani, Yakar et al. 2007, Brown, Delaine et al. 2008). However, loss of I1572 side chain did not cause any binding to IGF1. In addition, lack of alteration in M6P and plasminogen binding suggest that the functionality of the receptor extends to its N-terminal end. Therefore, I1572A mutant could form a basis for *in vitro* and *in vivo* models to dissect domain 11 functions of IGF2/M6PR while retaining intact function of its other 14 domains.

3.4.4 Implications of the gain of function mutations for evolution

IGF2/M6PR's domain 11 gains IGF2 binding function in egg laying mammals (monotremes) and evolves further to a higher affinity receptor (Williams, Hoppe et al. 2012). In domain 11 constructs, approximately two fold of affinity increase is observed between echidna (monotreme) and opossum (marsupial). Bovine IGF2/M6PR, however exhibit similar affinity to IGF2 when compared with human receptor (Bohnsack, Richard N et al. 2010), suggesting that evolution of the receptors' affinity to IGF2 has not progressed between these two placental mammals. This could be due to the lack of possible mutations that could lead to a higher affinity receptor or the lack of a need for tighter control of IGF2 levels. So far, several domain 11 mutants with higher affinities for IGF2 than the WT have been generated. Only one of these mutations (E1544K) has been tested in the full-length receptor and failed to achieve any enhancement in IGF2 affinity. I have tested E4, EQPS, Q, P and S in the context of full-length receptor and saw no significant difference between the binding affinity of the E4 and WT full length receptor. However, P1597H and E4QPS mutants showed two and three fold increase in affinity, respectively. Nonetheless, functional significance of the increase in the affinity requires further investigation. For example, cellular uptake of IGF2 by these high affinity mutants could be investigated. Also, the effects of these mutations on protein stability also need to be determined. For an attempt to generate higher affinity mutants, E4, E4QPS, 3-4D and 3-4DQPS without E1544K or FN2 insert could be promising candidates.

Table 3.1 Summary of gain of function mutants' relative and absolute (in brackets) affinities in Domain 11 and full length receptor constructs compared to WT

	Domain 11*	Full length
WT	1 (64 nM)	1 (0.95nM)
E4	0.23 (15.3 nM)	1.26 (1.26 nM)
E4 Q1569R	0.036 (2.3 nM)	N/A
E4 P1597H	0.019 (1.23 nM)	N/A
E4 Q1569R P1597H	0.014 (0.9 nM)	N/A
E4 Q1569R P1597H S1602H	0.012 (0.74 nM)	0.29 (0.28 nM)
Q1569R	N/A	0.91 (0.86 nM)
P1597H	N/A	0.45 (0.43 nM)
S1602H	N/A	0.77 (0.73 nM)

. * Unpublished data (Frago S., et al.)

3.4.5 Interactions between Domain 11 loops is important for high affinity IGF2 binding

In the context of the full-length receptor, AB&CD&FG loop combination “E4”, showed 20.7 fold higher affinity compared to the AB loop combination “E4”. In Domain 11 constructs, however, E4QPS exhibits only 4.5 increase in affinity compared to E4 (Table 3.1) These results, apparently, suggest that the inhibitory effect of the E1544K is manifested in different proportions in the full length and the domain 11 constructs. This is likely to be a result of rearrangements in the binding pocket due to AB loop mutations. While combining the mutations of Q1569R (CD loop) and P1597H (proximal FG loop) with E4 mutant in domain 11 constructs enhances the binding by 6.7 and 12.4 fold, the same mutations increase the affinity only 1.1 and 2.2 fold in full-length WT receptor. These results also suggest that internal

rearrangements due to AB loop mutations are required for the affinity increase to happen. The impact of AB loop mutations (E4) on the affinity enhancing effect of Q1569R and P1597H is similar (6.1 vs 5.6) suggesting same level of influence of binding site rearrangements of the CD and proximal FG loops. Intriguingly, S1602H shows similar increase in affinity as a single mutation or when combined with E4-Q1569R-P1597H. This might suggest that the distal portion of FG loop, where S1602 is located, functions more independently compared to CD and proximal FG loops.

In summary, these results suggest that domain 11 functions independently and give us insights on the evolution and the roles of the domain 11 IGF2 binding loops.

Chapter 4: Human M6P/IGF2R mannose 6-phosphate binding domains function independently of each other

4.1 Introduction

Carbohydrate-protein interactions play crucial role in many processes essential to organisms, such as inter-cellular interactions, protein sorting, immunity and protein degradation. Protein partners of these interactions are called lectins. Lectins are a diverse group of proteins that are composed of many classes, some of which are grouped based on the type of carbohydrate that they recognize. For instance, galectins recognize N-Acetylgalactosamine (GalNAc) and N-Acetylglucosamine (GlcNAc); I-type lectins recognize sialic acid; and P-type lectins are unique in their abilities to recognise phosphorylated mannose residues. The P-type lectin family is composed of 46 kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and cation-independent mannose 6-phosphate receptor (CI-MPR) (Olson, Hancock et al. 1999). This nomenclature is based on the fact that divalent cations (Mg^{+2} and Ca^{+2}) enhance CD-MPR's affinity for M6P and M6P tagged ligands four-fold (Tong and Kornfeld 1989, Sun, Zhao et al. 2005). However, calcium ions are not essential for CD-MPR binding functions which makes the receptor distinct from the C-type lectins (Drickamer 1999). CI-MPR also binds IGF2 and plasminogen alongside mannose 6-phosphate (M6P) and is also known as the IGF2/MP6R.

CD-MPR and IGF2/MP6R are also the first members of a structurally related and rapidly expanding larger class of proteins called mannose 6-phosphate receptor homology (MRH) domain-containing lectins (Dodd and Drickamer 2001). Although MRH domain-containing lectins function in secretory pathways, only CD-MPR and IGF2/MP6R can recognize ~60 M6P tagged acid hydrolases (Dahms, Olson et al. 2008, Castonguay, Olson et al. 2011). Only ~10% of the P-type lectins are present on the cell surface and the rest shuttle between the trans-

golgi network (TGN), endosomes and cell surface to transport the M6P-bearing ligands (Dell'Angelica and Payne 2001). However, on the cell surface, IGF2/M6PR, but not CD-MPR, bind and take up M6P-tagged ligands (Tong and Kornfeld 1989). The acidity differs between different endosomal compartments and pH plays an important role in the receptor's affinity to M6P-tagged ligand. M6P receptors exhibit optimal binding at pH 6.4 and no binding at pH 5.0 and below (Dahms, Olson et al. 2008, Olson, Hindsgaul et al. 2008).

M6P-tagging of the lysosomal enzymes starts after the proteins are transported from endoplasmic reticulum (ER) to the Golgi. The hydrolytic enzymes that are tagged for their transport to lysosomal compartments are recognized by a group of surface lysine patches oriented in a special three dimensional position to each other (Reitman and Kornfeld 1981, Steet, Lee et al. 2005). In cis-golgi cisternae, N-acetylglucosamine (GlcNAc) 1-phosphosphate are added to penultimate mannose residues of the enzymes by UDP-N-acetylglucosamine 1-phosphotransferase (GlcNAc-1-phosphotransferase) (Coutinho, Prata et al. 2012). The GlcNAc caps of mannose 6-phosphate-GlcNAc diesters are later removed by uncovering enzyme (UCE) in the TGN (Rohrer and Kornfeld 2001) resulting in M6P monoesters. The UCE is synthesized as a propeptide and activated by furin (Rohrer and Kornfeld 2001, Do, Lee et al. 2002). In the absence of functional UCE, the hydrolytic enzymes are only tagged by M6P diesters and would be recognized by IGF2/M6PR but not CD-MPR.

Both CD-MPR's single and IGF2/M6PR's fifteen extracellular domains share a sequence homology of 14-38% (Kim, Olson et al. 2009). While the CD-MPR has a single M6P binding site, IGF2/M6PR's domains 3, 5 and 9 bind M6P. So far, the CD-MPR's extracellular domain and eight of the IGF2/M6PR's domains' structures have been determined (Domains 1-3, 5 and 11-14) all of which have been shown to have a shared β -barrel structure (Roberts, Weix et al. 1998, Olson, Yammani et al. 2004, Brown, Delaine et al. 2008). The β -barrels are composed

of nine β -strands forming two β -sheets, in most of the domains, stabilised by four disulphide bridges. N-terminals of CD-MPR and IGF2/MP6R the core β -barrels are capped by an alpha helix and a β -hairpin, respectively. Four loops that are protruding from the other side of the barrels, form the M6P binding sites. All of the MRH domains binding M6P adopt an essential conserved four residues (Glu, Gln, Arg and Tyr) binding motif surrounding the mannose ring of the M6P upon ligand binding (Sun, Zhao et al. 2005, Chavez, Bohnsack et al. 2007). The binding pocket of domain 5 possesses an extended binding groove that accommodates mannose 6-phosphate-GlcNAc diesters (Chavez, Bohnsack et al. 2007, Olson, Peterson et al. 2010). CD-MPR's binding pocket is the narrowest of all and could only accommodate M6P monoester. Domain 3, on the other hand, could be described as a hybrid binding pocket and can also accommodate mannose 6-phosphate methyl diesters (this form does not occur in mammalian cells) (Marron-Terada, Hancock et al. 2000). Another structural difference that is unique to domain 5 is the absence of a fourth disulphide bond. The stability of the binding pocket, is instead ensured by the presence of an interaction between the side chains of W653 (indole ring) and R687 (guanidinium group). Y679 is also unique to domain 5 and forms extensive interactions with the N-acetylglucosamine of the diesters which are essential for the ligand binding of the domain. In addition to the mannose ring, surrounding residues Ser431 of domain 3 and His1329 of domain 9 are also essential for the domains' recognition of M6P (Dahms, Rose et al. 1993, Hancock, Haskins et al. 2002).

Although, M6P-tagged ligands of CD-MPR and IGF2/MP6R are divided into mono- and diesters based on the presence of GlcNAc-bound penultimate phosphorylated mannoses, the length and branching patterns of the ligands' N-linked carbohydrate chains also determine each M6P binding domain's affinity (Bohnsack, Song et al. 2009, Song, Lasanajak et al. 2009). The diversity of the ligands of these domains also accounts for the fact that both receptors are required for efficient transport functions in the cell. While domains 3 and 9 of the IGF2/MP6R exhibit high affinity (K_i : $\sim 10\mu\text{M}$), domain 5 exhibits lower affinity (K_i : $\sim 5\text{mM}$) for the monoesters.

However, domain 5 exhibits 14-18 folds higher affinity to M6P diesters, compared to monoesters and is the only domain that binds to the diesters (Chavez, Bohnsack et al. 2007). M6P binding domains also show different optimal binding pH. For example, domains 3 and 9 show optimal affinity at pH 6.4-6.5 and pH 6.9-7.0 respectively (Marron-Terada, Hancock et al. 2000). In summary, M6P binding domains of IGF2/MP6R, while similar in their functions, exhibit distinct features. However, all of these data come from studies utilising fragments of the receptor and little is known of how M6P domains functions in the full-length receptor. In order to investigate the functions of each of M6P binding domains' functions in the context of the full-length receptor, I have generated full-length receptors with single functioning M6P binding domains, and later combined these constructs to test presence/absence any cooperation between the domains.

4.2 Aims

- **Generation and characterisation of full length M6P complete loss-of-binding function receptors**
- **Generation and characterisation of full length M6P loss-of-binding function receptors with single and two intact M6P binding domains**

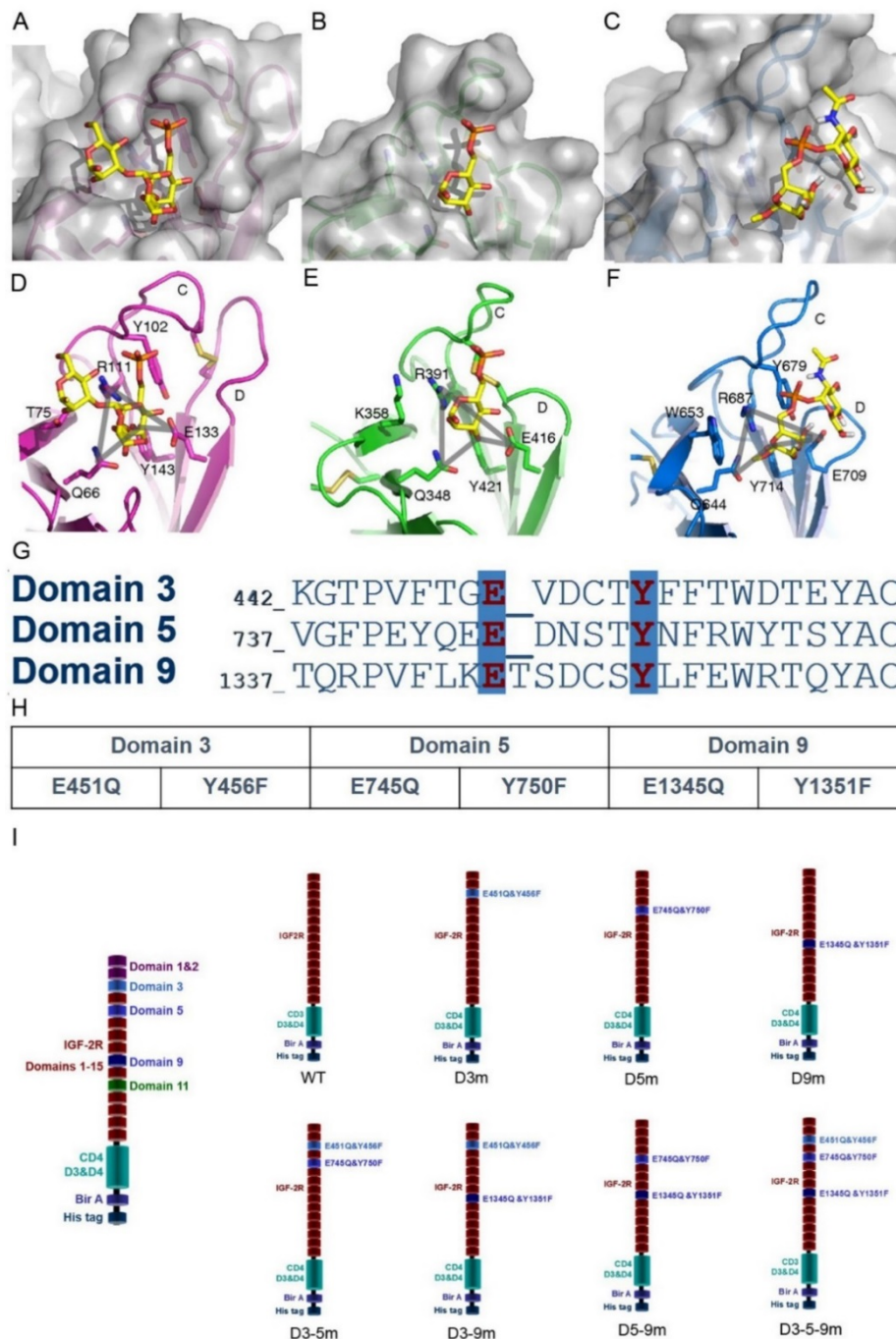


Fig 4.1 Structure and mutagenesis of M6P binding domains. (A, B, C, D, E, F) Comparison of binding sites of CD-MPR (A, D), Domains 3 (B, E) and 5 (C, F) of M6P/IGF2R. (G, H) Alignment of target residues and the mutations that are introduced to generate M6P loss-of-binding functions mutants. (I) Illustration of M6P mutants used in this chapter. The chimeras expressed by the HEK293T cells are composed of Domains 1-15 of IGF2/M6PR fused with a rat CD4 Domains 3&4, a Biotin acceptor site and polyhistidine (six Histidines) tag.

4.3 Results

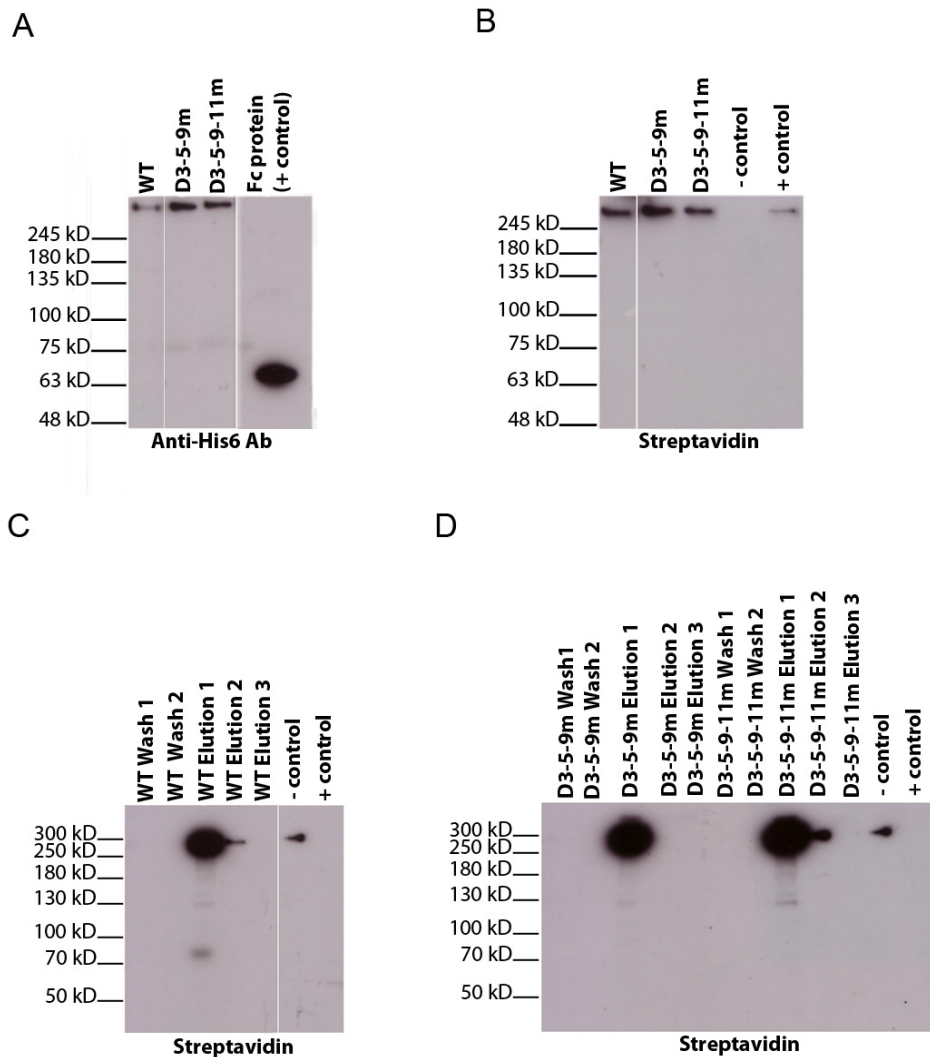


Fig 4.2 Expression and purification of full length receptor chimeras. The molecular weights of the chimeras are (A, B) An example of full length receptor proteins detected in western blots probed with horseradish peroxidase conjugated anti-polyhistidine antibody and streptavidin. The proteins appear as single bands at approximately 290 kilodaltons. + control: Medium collected from previous transfections, - control: Medium collected from transfections without the receptor expressing vector.(C, D) Examples of western blots for detection of full length receptor proteins in nickel column purification fractions using horseradish peroxidase conjugated anti-polyhistidine antibody and streptavidin for probing.

4.3.1 Generation and expression of Mannose 6-phosphate binding mutants

Glutamate and tyrosine residues of domains 3, 5 and 9 that interact with the mannose ring of the mannose 6-phosphate were replaced with glutamate and phenylalanine, respectively. The mutant domains later combined to generate D3-5m, D3-9m, D5-9m and D3-5-9 constructs. Four days after the co-transfections of the HEK293T cells with the constructs and BirA expressing plasmid, media were collected and checked for receptor expression and biotinylation (Fig 4.2A, B). To remove the biotin from the media, the chimeras were nickel column purified. The presence and the biotinylation of the receptors in the elutions were evaluated with streptavidin probed western blots (Fig 4.2C, D). The absence of significant degradation products indicates the receptors were suitable for functional evaluation in SPR.

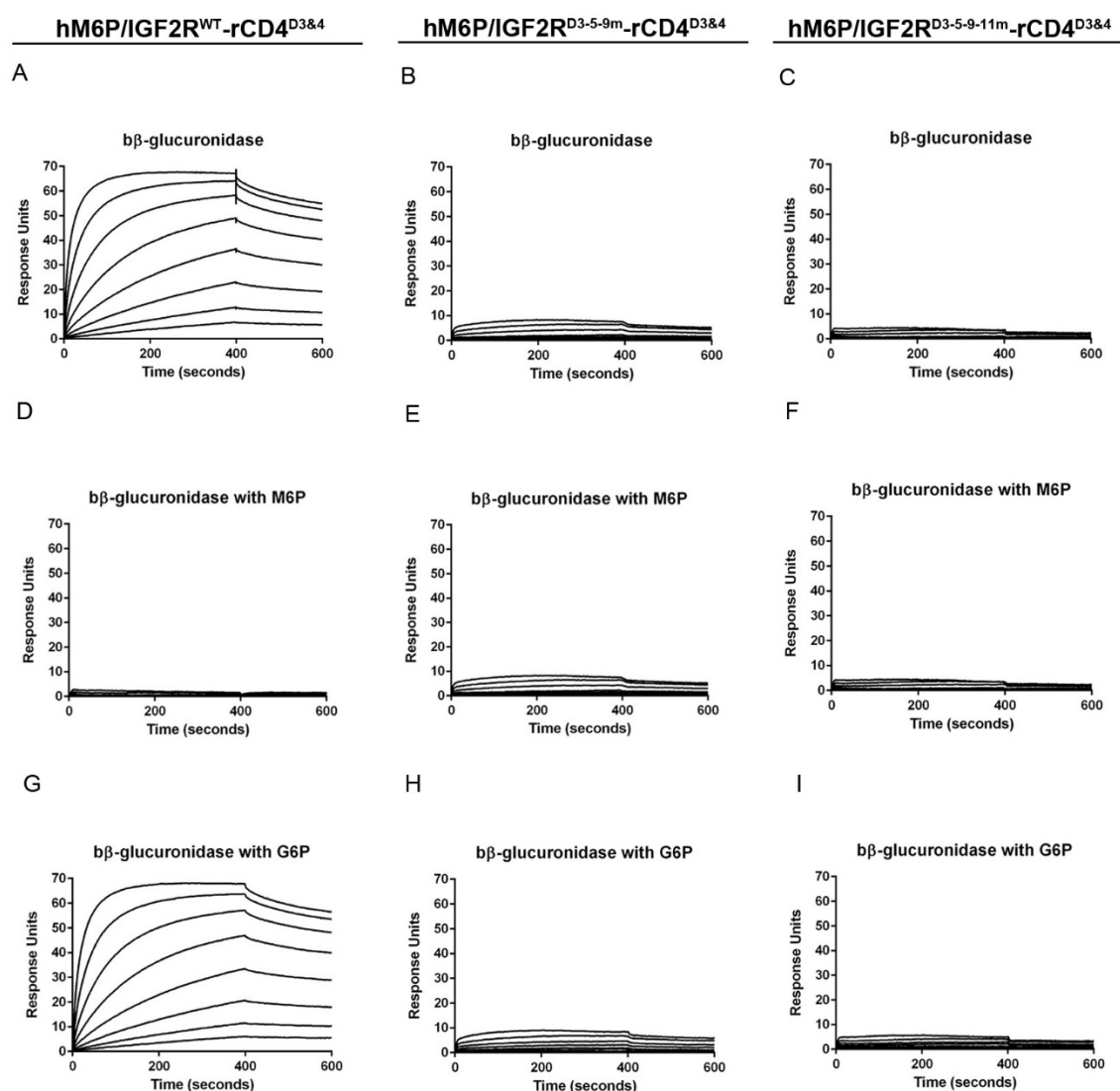


Fig 4.3 Evaluation of M6P loss-of-binding function mutants in SPR in presence and absence of glucose 6-phosphate (G6P) and mannose 6-phosphate (M6P) (A, B, C) SPR traces showing β -glucuronidase interactions with WT and M6P loss-of-binding function mutants; D3-5-9m&D3-5-9-11m (D,E,F) SPR traces showing β -glucuronidase interactions with WT and M6P loss-of-binding function mutants; (D3-5-9m&D3-5-9-11m) in the presence of the specific inhibitor of the interaction: M6P (D,E,F) SPR traces showing β -glucuronidase's interaction with WT and M6P loss-of-binding function mutants; (D3-5-9m&D3-5-9-11m) in the presence of the negative control for M6P: G6P

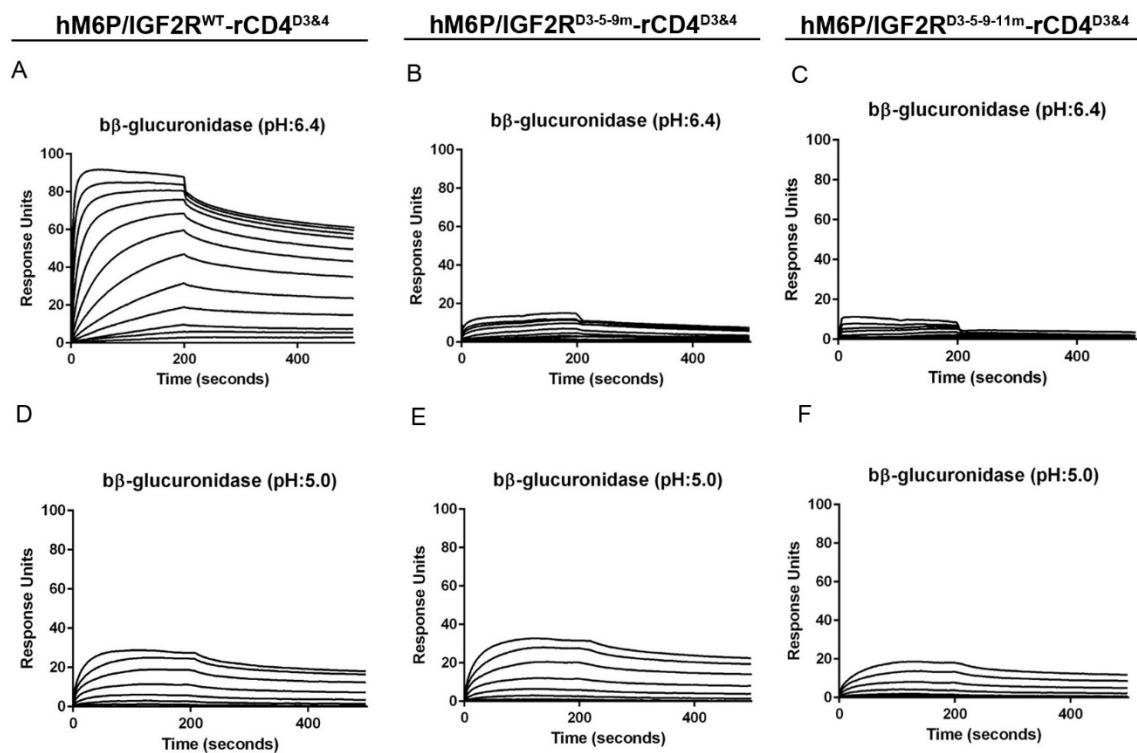


Fig 4.4 Evaluation of M6P loss-of-binding function mutants in SPR at acidic pH values of 6.4 and 5.0 (A, B, C) SPR traces showing β -glucuronidase interactions with WT and M6P loss-of-binding function mutants; D3-5-9m&D3-5-9-11m at maximum affinity pH (pH 6.4) **(C)** SPR traces showing β -glucuronidase interactions with WT and M6P loss-of-binding function mutants; D3-5-9m&D3-5-9-11m below minimum affinity pH (pH 5.0)

4.3.2 Mannose 6-phosphate binding site mutations abrogate β -glucuronidase binding at physiological pH

In order to evaluate if M6P binding site mutations cause loss-of-binding in their respected sites, I evaluated full-length receptors with the mutations in all three M6P binding sites. The mutants tested with bovine β -glucuronidase in the presence of its specific binding inhibitor; mannose 6-phosphate (M6P) and negative control for M6P: glucose 6-phosphate (G6P). M6P binding mutants (Fig 4.3B, C) showed residual binding to β -glucuronidase compared to WT (Fig 4.3A). D3-5-9m in the presence or absence of M6P and G6P showed similar levels of residual interaction suggesting that the residual binding is M6P phosphate independent non-specific binding (Fig 4.3 B, E, H). Similar but lower non-specific binding observed in D3-5-9-11m, suggesting that Domain 11 loss-of-binding reduces M6P independent residual β -glucuronidase binding (Fig 4.3 C, F, G). Intriguingly, WT receptor, compared to M6P binding mutants showed even lesser residual binding to its M6P tagged ligand, in the presence of M6P (Fig4.3 D, E, F). These results indicate that, introduction of Domain 3 (E451Q&Y456F), Domain 5 (E745Q&Y750F), Domain 9 (E1345Q&Y1351F) M6P binding site mutations abrogate M6P dependent binding of β -glucuronidase, thus D3-5-9m is a M6P loss-of-binding mutant.

Same mutants were also tested at lower pH values that mimick the intra-vesicular compartments in which M6P/IGF2R trafficking happens. At pH 6.4, the receptor exhibits maximum M6P affinity. At this pH, the M6P binding site mutants still showed β -glucuronidase binding (Fig 4.4B, C) At pH 5.0, M6P/IGF2R is expected to release all of its M6P-tagged cargo. Interestingly, however, both WT receptor and M6P binding site mutants showed still significant affinity to β -glucuronidase.

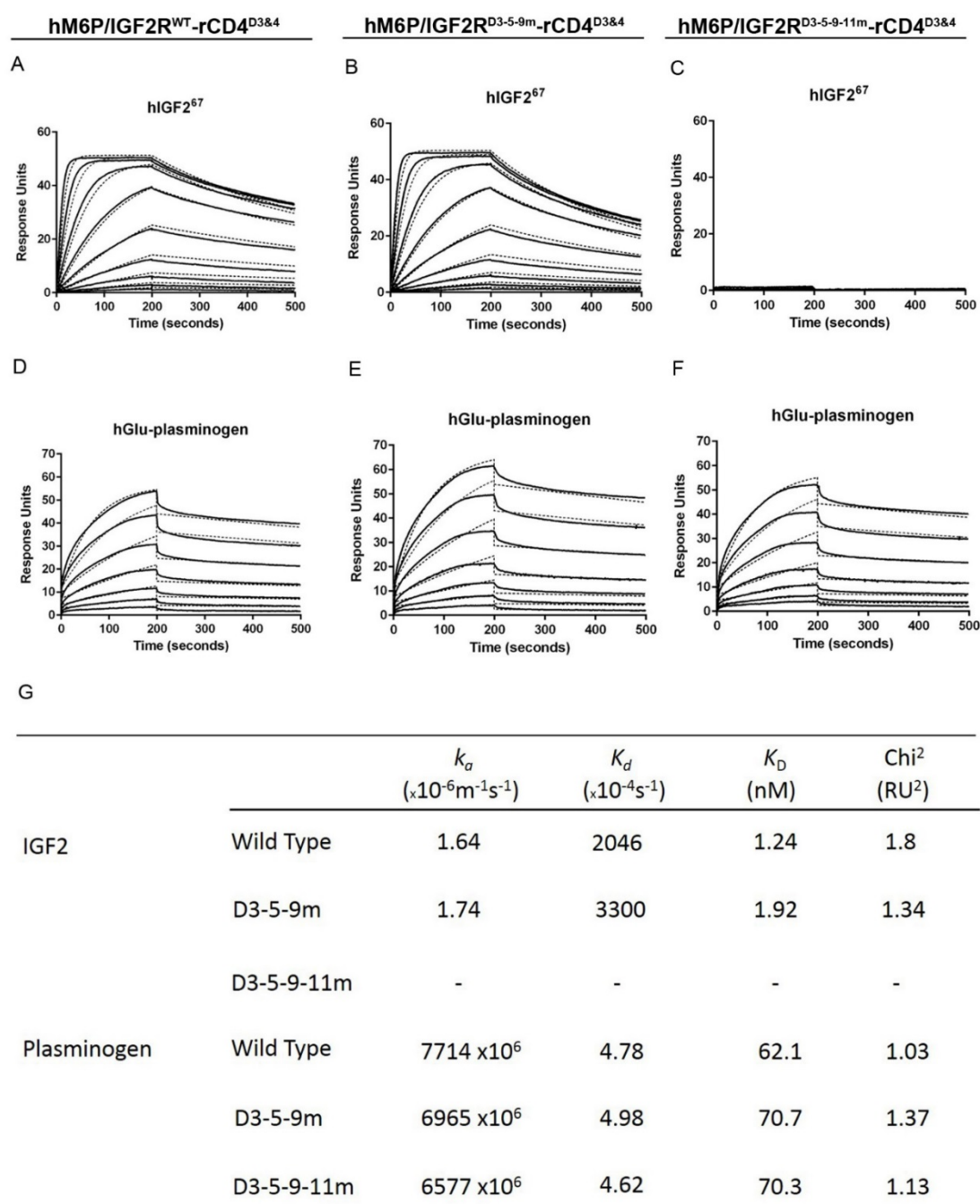
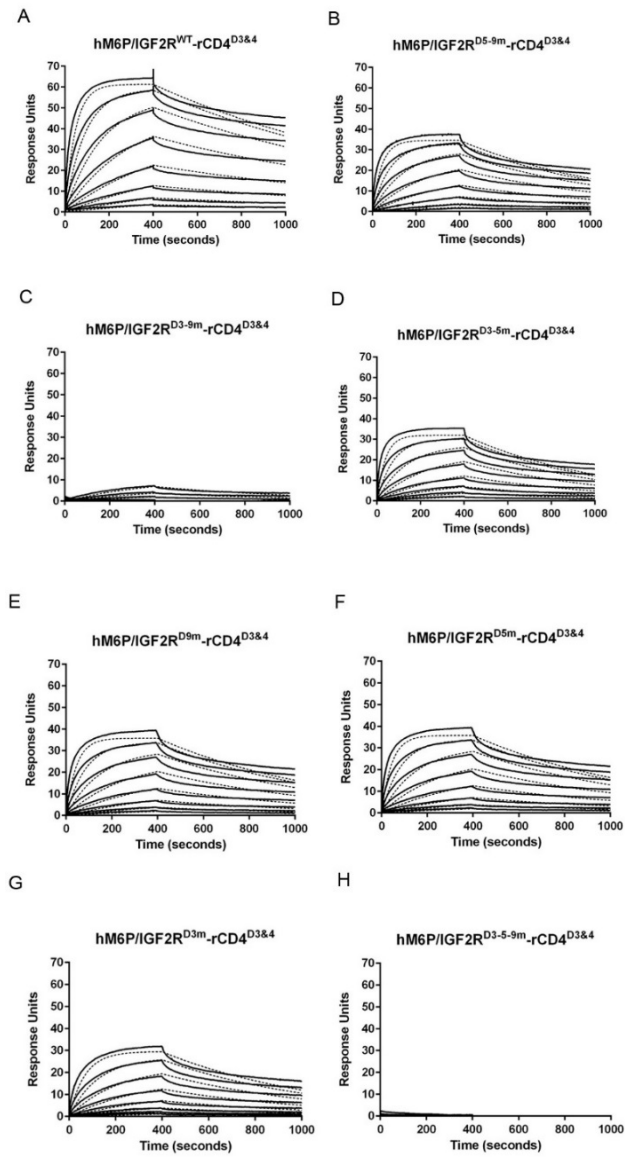


Fig 4.5 Affinity of the human IGF2 and human Glu-plasminogen binding to M6P loss-of-binding mutants. (A, B, C, D, E, F) Sensorgrams of the human IGF2 and human Glu-plasminogen binding to WT, D3-5-9m and D3-5-9-11m loss-of-binding mutants. **(G)** Table summarising association (k_a) and dissociation rates (K_d), and affinity constants (K_D) of the WT and WT, D3-5-9m and D3-5-9-11m loss-of-binding mutants to IGF2 and human Glu-plasminogen, with Chi² values of the respective fits.

4.3.3 Influence of M6P binding site mutations on other ligands' binding affinity

In order to test if other domains' ligand binding function is preserved in M6P loss-of-binding receptor constructs, D3-5-9m and D3-5-9-11m affinity to IGF2 and plasminogen was evaluated. As expected, no IGF2 binding was detected in D3-5-9-11m (Fig 4.5 C). D3-5-9m showed ~35% reduction (1.92 vs. 1.24 nM) in IGF2 affinity (Fig 4.5 A, B, G), indicating a minor disturbance in IGF2 binding of domain 11, probably due to proximity of the domain 11 to domain 9. D3-5-9m and D3-5-9-11m receptors showed 70.7 and 70.3 nM affinity to plasminogen, respectively, compared to WT (62.1) (Fig 4.5 D, E, F, G), suggesting that the N-terminals of both mutant receptors show preserved binding function, therefore stayed intact.



	k_a ($\times 10^{-4} \text{m}^{-1} \text{s}^{-1}$)	K_d ($\times 10^{-6} \text{s}^{-1}$)	K_D (nM)	Chi ² (RU ²)
	mean	mean	mean $\pm$ SD	mean
Wild Type	1830.9	7.7	63.68 $\pm$ 10	5.52
D5-9m	1175.7	15.97	136.7 $\pm$ 26.4	298
D3-9m	-	-	-	-
D3-5m	5641.5	16.79	299 $\pm$ 50.1	3.9
D9m	1048.5	12.5	119.2 $\pm$ 8.27	3.25
D5m	1140	8.1	69.4 $\pm$ 8.42	4.8
D3m	5895	15	258.7 $\pm$ 31.7	2.78
D3-5-9m	-	-	-	-

*See next page for the figure legend

Fig 4.6 Evaluation single and double M6P binding domain mutants. (A, B, C, D, E, F, G, H) Sensorgrams and curve fits according to 1:1 binding model at flow rates 40 μ l/min for WT, D5-9m, D3-9m, D5-9m, D9m, D5m, and D3m **(F)** Summary of affinity values. The values are results of multiple independent experiments: WT (12 repeats); D5-9m&D3-5m&D5 (3 repeats), D3-9m&D3m&D9m (2 repeats).

4.3.4 Domain 3 of the IGF2/M6PR is the dominant M6P binding domain of the receptor

In order evaluate each M6P binding domains' M6P affinity, I inserted WT M6P binding domains back into the M6P loss-of-binding function full length receptor. These constructs are named as D5-9m, D3-9m and D3-5m with only domains 3, 5 and 9 left intact respectively. D5-9m (Domain 3 intact) showed almost half of the affinity of the WT receptor (63.7nM vs. 133.7nM) (Fig 4.6 A, B). D3-5m (Domain 9 intact) construct showed 20% off the WT receptor, 301 nM (Fig 4.6 A, D, I). D3-9m, on the other hand, showed almost unquantifiable levels of low affinity to β -glucuronidase (Fig 4.6 C). These results indicated that Domain 3 is the dominant M6P binding domain at physiological pH.

4.3.5 M6P binding domains of the receptor work independent of each other.

The full receptor construct with domain 5 and 9 left functional (D3m) showed similar affinity to β -glucuronidase compared to the receptor construct with only functional domain 9 (D3-5m) (258.7 nM vs 301 nM) (Fig 4.6 D, E, I). In addition, D9m also showed similar affinities to β -glucuronidase compared to D5-9m (119.2nM vs. 136.7 nM) (Fig 4.6 B, E, I). Both of these results indicate that Domain 5's contribution to the receptor's binding to β -glucuronidase is insignificant. Also, full-length receptor with binding site mutation only in Domain 5 showed similar affinity to β -glucuronidase compared to WT (63.7 nM vs 71.9nM) (Fig 4.6 A, F, I), further

confirming Domain 5's negligible role in β -glucuronidase binding and lack of synergism between domains 3 and 9 at physiological pH.

4.4 Discussion

4.4.1 Generation and characterisation of M6P complete loss-of binding

mutants

I introduced conservative mutations to two of four residues surrounding the mannose ring of the mannose 6-phosphate. These residues were previously identified as essential residues for M6P binding (Olson, Dahms et al. 2004, Chavez, Bohnsack et al. 2007, Kim, Olson et al. 2009). Later, I combined these binding site mutations with the domain 11 IGF2 loss-of binding mutation (D3-5-9-11m) and compared it with D3-5-9m and tested their M6P binding affinity using bovine β -glucuronidase in the presence of its specific binding inhibitor, M6P, and the negative control for the inhibitory reagent, G6P. The D3-5-9m receptor showed some residual binding that is not exceeding 10 response units (RU). When tested in the presence of M6P and G6P very similar SPR traces were also recorded for the mutants, suggesting that the residual binding is M6P independent. Thus, the M6P binding site mutations completely abrogate M6P mediated ligand binding. Similarly, D3-5-9-11m showed a minimal M6P independent β -glucuronidase binding but with lower RUs, suggesting that domain 11 loss-of-binding function further reduces M6P independent non-specific interactions of β -glucuronidase with the receptor. Of note, in chapter 3, I demonstrated that IGF2 loss of binding has no significant effect on β -glucuronidase binding. One possible explanation for this is that the reduction in non-specific component is minute, therefore negligible. Alternatively, the M6P-dependent interaction may be greatly outcompeting the non-specific component in the M6P-binding-intact receptor therefore making the influence of domain 11 loss-of-binding on M6P independent β -glucuronidase-receptor interaction unquantifiable. Interestingly, the WT

receptor showed lower R_{max} compared to D3-5-9m both in the presence and absence of M6P and G6P. It seems that most of the M6P independent component of the receptor is introduced with M6P binding site mutations. It is possible that the targeting of the M6P binding pockets of IGF2/M6PR results in gain of weak affinity to M6P free carbohydrate branches of the β -glucuronidase.

Interestingly, at pH 5.0 the M6P loss-of-binding mutants (D3-5-9m and D3-5-9-11m) showed significant binding to β -glucuronidase almost reaching to the levels of the WT receptor. IGF2/M6P receptor is expected to release its M6P tagged cargo in vesicular compartments with pH 5.0. However, these results suggest that either the cargo release is not complete and some enzyme recycles back, or the release is not only regulated by the proton concentrations and additional mechanisms might also exist to complete undocking of the hydrolytical enzymes. One such mechanism could be the presence of acid phosphatases of the acidic vesicular compartments, such as lysosomal acid phosphatase (LAP), the tartrate-resistant acid phosphatase-type 2 and 5 (Acp2 and 5) (Suter, Everts et al. 2001, Makrypidi, Damme et al. 2012).

4.4.2 IGF2/M6PR domain 5 and 15 exhibit no affinity for β -glucuronidase

For years, only domains 3, 5 and 9 of the IGF2/M6PR were known to bind M6P tagged ligands (Reddy, Chai et al. 2004). Very recently, a fourth binding site that is mapped to domain 15 has been identified and found to have similar affinity for both monoesters and diesters (Olson, Castonguay et al. 2015). In my SPR studies, there were no residual M6P dependent binding to β -glucuronidase in D3-5-9m receptor. Thus, I detected no M6P dependent binding of β -glucuronidase to any of the remaining domains, such as domain 15. This might be explained

by a steric effect due to the proximity of the domain 15 to CD4 domains 3&4 preventing interactions of β -glucuronidase with the newly identified M6P binding domain.

Domain 5 is primarily a M6P diester binding domain. WT domain 5 (D3-9m) shows residual binding to β -glucuronidase that is unquantifiable. For instance, no significant change was detected when WT domain 5 was replaced by M6P loss-of-function domain (D3m vs. D3-5m, D9m vs. D5-9m, D3-9m vs. D3-5-9m). Previous studies showed that domain 5 exhibits more than 10-fold less affinity for monoesters compared to diesters (Chavez, Bohnsack et al. 2007). Therefore, the most likely explanation for this phenomenon is that the bovine β -glucuronidase which was used in these experiments is efficiently processed by the uncovering enzyme and only carries monoesters.

4.4.3 Domain 3 is the major M6P binding domain and both of M6P binding domains function independently

The full-length receptor constructs with only domain 3 functional showed higher affinity (133.7nM), compared to the receptor with only domain 9 functional (301 nM), suggesting that domain 3 is the major M6P binding domain at the pH conditions found at the cell surface. When these two domains combined together, in the context of the full-length receptor, they reach the affinity of the WT receptor (63.7 nM vs 71.9nM). Since the contribution of domain 5 to β -glucuronidase binding is negligible, these results indicate that the M6P binding of domains 3 and 9 is additive rather than synergistic. This lack of cooperation might be specific to this ligand so a glycan array of other M6P tagged ligands would be useful for excluding the possibility of cooperation between these major M6P binding domains.

Chapter 5: Domain 11 IGF2 CD loop binding site mutation of Igf2r results in neonatal lethality

5.1 Introduction

Embryonic and postnatal growth of organisms is a finely regulated process that involves many regulators becoming active in different temporal and spatial manners. The complexity of the growth regulation also reflected in the diversity of the growth abnormalities that are observed in embryonic and early postnatal life. Such abnormalities result in a range of dysfunctions and, in some cases, lethality. Growth regulating genes also play essential role in homeostasis of the organisms during later stages of life. For example, many growth regulating genes malfunction in neoplastic disorders, resulting in continuous stimulation of signalling pathways for cellular growth and unresponsiveness to growth suppression, both of which are hallmarks of cancer (Hanahan and Weinberg 2011).

Growth, as a complex and an essential process of life, is regulated by a range of actors including soluble growth factors, membrane and intracellular receptors, signalling cascade proteins, transcriptions factors and enzymes. These elements may form growth regulating systems; such as fibroblast growth factor system and IGF system. IGF system is essential for normal *in utero* and postnatal growth; formation and functions of musculoskeletal, cardiovascular, immune and lymphopoietic systems (Denley, Cosgrove et al. 2005, Li, Cavallero et al. 2011, Huang, Harrison et al. 2013). The system is formed by two polypeptide ligands: Insulin-like growth factor 1 (IGF1) and 2 (IGF2); soluble high affinity carrier proteins: Insulin-like growth factor binding proteins 1-6 (IGFBPs 1-6); signalling receptors: Insulin receptor isoform A (IR-A), IGF1 receptor (IGF1R), IGF-1R-IR hybrids; and a clearance receptor: IGF2/M6PR.

Insulin, IGF1 and IGF2 peptide sequences and structures highly resemble to each other, reflecting their shared evolutionary origins (Rinderknecht and Humbel 1978, Brzozowski, Dodson et al. 2002). Structural resemblance also results in overlapping functions between these growth factors, such as activating the same receptors for cellular growth and sharing IGFBPs for their transport and regulation of their bioavailability (Beattie, Phillips et al. 2008). Similarly, IGF axis receptors, IR-A and IGF1R, exhibit structural and functional resemblance to each other (Nakae, Kido et al. 2001). For instance, both receptors carry tyrosine kinase activity in their cytoplasmic tails (Robinson, Wu et al. 2000). While activation of insulin receptor isoform B (IR-B) by insulin leads to metabolic changes, IR-A also binds to IGF2 and activates growth stimulating intracellular pathways upon ligand binding (Nakae, Kido et al. 2001, Pandini, Frasca et al. 2002). IGF1R, however, is activated by both IGF1 and IGF2, and responsible for MAP kinase and PI3 kinase mediated cellular proliferation (Pollak, Schernhammer et al. 2004). Bioavailability of the IGFs, but not Insulin, is regulated by IGFBPs. Six IGFBPs, with distinct binding affinities to IGF1 and IGF2 have been identified so far (Bach, Hsieh et al. 1993). IGFBPs 3 and 6 exhibit the highest affinity to IGFs and like other IGFBPs showing greater affinity to IGF2 compared to IGF1. Likewise, IGF1R also shows higher affinity to IGF2 compared to IGF1 (Pandini, Frasca et al. 2002).

IGF2/M6PR is a unique member of IGF system. It is a multi-ligand receptor with IGF2 clearance function through cellular uptake. The receptor also transports M6P tagged lysosomal enzymes, binds plasminogen and activates it to produce the end product, plasmin (Leksa, Pfisterer et al. 2012). Due to the receptor's key role in transport of hydrolytic enzymes and activation of plasmin on the cell surface, IGF2/M6P regulates proteolytic degradation of IGFBPs, a mechanism that is crucial for release of IGFs in tissues (Campbell, Novak et al. 1992, Remacle-Bonnet, Garrouste et al. 1997, Braulke, Dittmer et al. 1999, Bunn and Fowlkes 2003). Only minority (~10%) of the IGF2/M6PRs are localised on the cell surface and the rest

is distributed in intracellular membrane-bound compartments. When extracellular IGF levels increase, to enhance ligand binding and reuptake of IGF2, more IGF2/M6PRs are transported to cell surface (Braulke, Tippmer et al. 1989, Braulke, Tippmer et al. 1990, Damke, von Figura et al. 1992).

Clearance of IGF2 by IGF2/M6PR is crucial for normal development and survival of the mammals. Therefore, regulating IGF2/M6PR expression to keep the number of receptor at optimum levels on the cell surface is essential. Like other growth regulating genes, expression of IGF2/M6R depends on the parental origin of the receptor. This type of an epigenetic suppression pattern is called imprinting. Generally, growth promoting genes are maternally imprinted while growth restricting genes are maternally expressed. A reciprocal regulation is observed in the paternal alleles. The rationale behind of genomic imprinting is explained by “parent-offspring conflict” hypothesis (Moore and Haig 1991). According to this hypothesis, while the maternal genes with their imprinting pattern aim to restrict the growth of the each embryo in order to keep the mother fit for other embryos that are developing concurrently and the embryos of the future litters, the paternal genes aim to keep the size of the current offspring bigger to increase the chance of survival in expense of future litters' survival.

IGF2/M6PR knock out transgenic mice exhibit embryonic over-growth phenotype with skeletal abnormalities, such as polydactyly, kinky tail, insufficient ossification of the sternum and disproportional cranial development (Baker, Liu et al. 1993, Wang, Fung et al. 1994). In early neonatal period most mice die due to congestive heart failure. The female mice that could survive to adulthood present with complete vaginal atresia and uterine distension due to the lack of drainage openings for menstrual haemorrhage, endometrial tissues and secretions. The phenotype of complete loss of receptor's functions marks its key role in organogenesis and postnatal survival. However, the contribution of IGF2/M6PR's domains' to development

remains unknown. In order to dissect the domain specific functions of the receptor in development, I have characterised IGF2/M6PR domain 11 IGF2 loss-of-binding function mice.

5.2 Aims

- **Characterisation of heterozygous IGF2 loss-of-binding function (*Igf2r*^{I1565Am/+p}) in the mouse *in vivo***
- **Characterisation of homozygous IGF2 loss-of-binding function (*Igf2r*^{I1565Am/I1565Ap}) in the mouse *in vivo***

5.3 Results

5.3.1 I1565A is mouse equivalent of I1572A of human.

In chapter 3, I characterised I1572A in the context of full length IGF2/M6PR. I found that the mutation results in complete loss-of-binding function without any alteration of receptor's affinity to β -glucuronidase and plasminogen. Thus, I1572A mutation could form a basis for *in vivo* models to dissect domain 11's. The alignment of human, chimpanzee, bovine, rat and mouse IGF2/M6PR sequence shows that the receptor sequence is mostly preserved across the placentalia (Fig 5.1). Domain 11 of the receptor particularly falls into one of the most well preserved parts of the receptor gene. Furthermore, domain CD loop sequences of all these species are identical, indicating that the results from *in vitro* analysis human I1572A mutant can be applied to the mouse I1565A mutant.

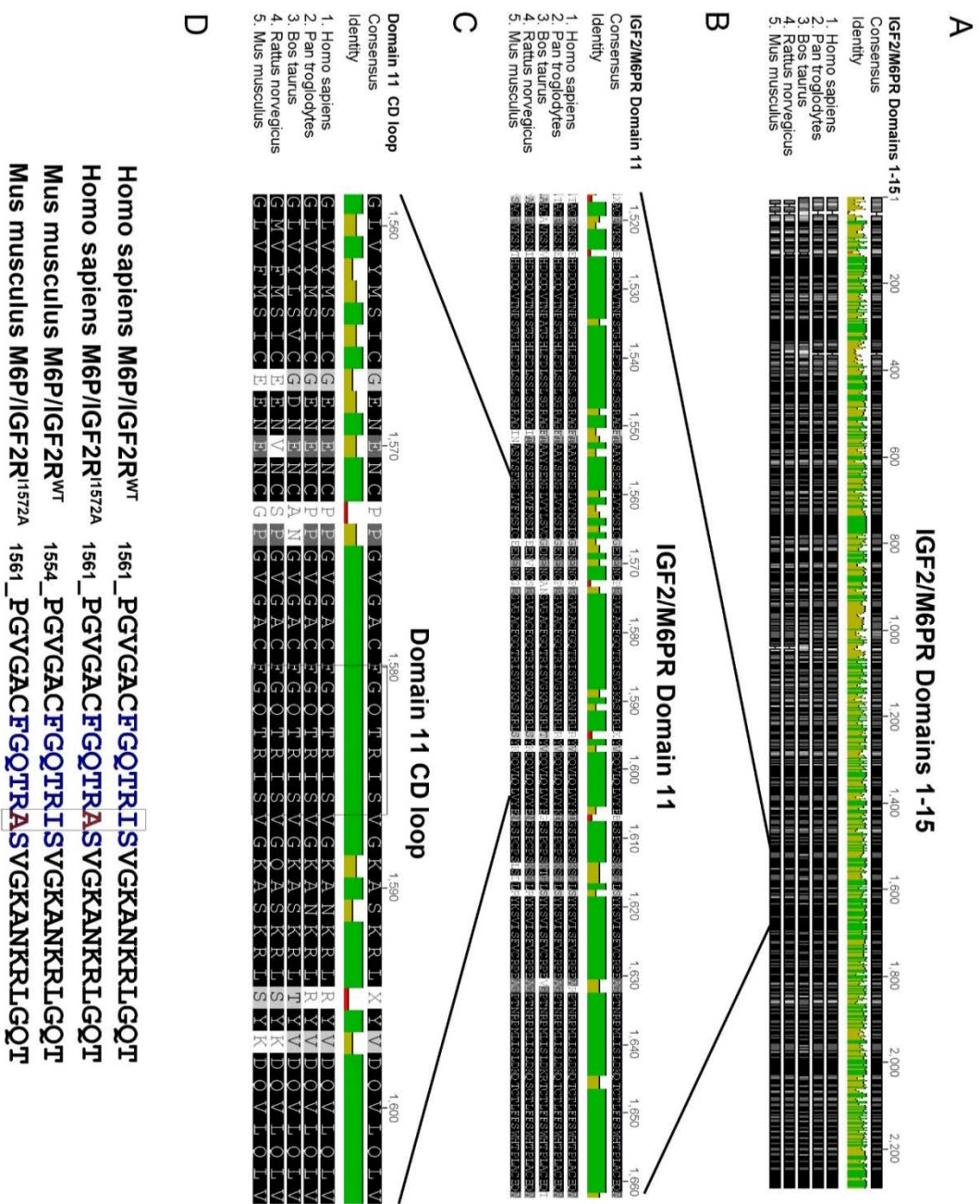


Fig 5.1: Alignment of domain 11 CD loop of WT and IGF2 loss-of-binding function mutant sequences of human and mouse IGF2/M6PR. The level of sequence similarity is represented in white to black, darker shaded sequences showing higher sequence resemblance

5.3.2. Heterozygous, but not homozygous, transmission of I1565A follows Mendelian transmission

Genotyping outcomes of the matings for paternal transmission of I1565A followed the Mendelian ratio: $Igf2^{r+m/+p}$: 25 pups and $Igf2^{I1565Am/+p}$: 29 pups (Table 5.1). The difference between the number of heterozygous pups with maternal transmission and WT pups (39 vs. 30) was not statistically significant. Similarly, heterozygous breeding did not change the transmission of the sex chromosomes (Tables 5.2, 5.3). However, when heterozygous mice were mated to produce homozygous mice, the resulting breeding outcome for the homozygous pups were significantly lower than expected (4 vs 14.75 pups) thus was not following the Mendellian laws of transmission.

Table 5.1: Summary of breeding outcomes at birth for $Igf2r$ I1565A allele transmission.

I1565A $Igf2r$ allele transmission	Summary of breeding outcomes at birth				
	Cross	Genotypes	Observed	Expected	
Maternal	$Igf2^{r+m/I1565Ap} \text{♀} \times Igf2^{r+m/+p} \text{♂}$ (11 litters, 5 pairs)	$Igf2^{r+m/+p}$	39	34.5	P=NS
		$Igf2^{I1565Am/+p}$	30	34.5	
Paternal	$Igf2^{r+m/+p} \text{♀} \times Igf2^{r+m/I1565Ap} \text{♂}$ (10 litters, 3 pairs)	$Igf2^{r+m/+p}$	25	27	P=NS
		$Igf2^{r+m/I1565Ap}$	29	27	
Homozygote	$Igf2^{r+m/I1565Ap} \text{♀} \times Igf2^{r+m/I1565Ap} \text{♂}$ (9 litters, 3 pairs)	$Igf2^{r+m/+p}$	25	14.75	P=0.0006
		$Igf2^{I1565Am/+p}$ Or $Igf2^{r+m/I1565Ap}$	30	29.5	$\chi^2=14.966$
		$Igf2^{I1565Am/I1565Ap}$	4	14.75	

Table 5.2: Summary of breeding outcomes at birth for transmission of sex chromosomes.

<i>Transmission of sex chromosomes</i> Summary of breeding outcomes at birth				
Cross	Genotypes	Observed	Expected	
<i>Igf2^{r+m/1565Ap}</i> ♀ X <i>Igf2^{r+m/+p}</i> ♂ (4 litters, 4 pairs)	XY	15	13.0	P=NS
	XX	11	13.0	

Table 5.3: Summary of breeding outcomes day 10 for transmission of sex chromosomes.

<i>Transmission of sex chromosomes</i> Summary of breeding outcomes on day 10				
Cross	Genotypes	Observed	Expected	
<i>Igf2^{r+m/1565Ap}</i> ♀ X <i>Igf2^{r+m/+p}</i> ♂ (4 litters, 4 pairs)	XY	17	16.5	P=NS
	XX	16	16.5	

5.3.3 Maternal transmission of IGF2 loss-of-binding function results in overgrowth and early neonatal lethality

Weights of the newborns with maternal transmitted of *Igf2* loss-of-binding is significantly higher than the wild type mice ($p < 0.0001$) (Fig 5.2A) and more than 60% of these mice died during the first few days of birth (Fig 5.2B). Intriguingly, when the growth of the mice was followed during first few weeks there were no statistically significant difference between the growth of *Igf2^{r1565Am/+p}* and *Igf2^{r+m/+p}* mice (Fig 5.2C). Most likely explanation for this observation was a negative correlation between neonatal weights and survival. When I looked at the survival-

weight relationship, I found that vast majority of the mutant mice that have died during the early neonatal period are significantly heavier than the population that was survived (Fig 5.2D).

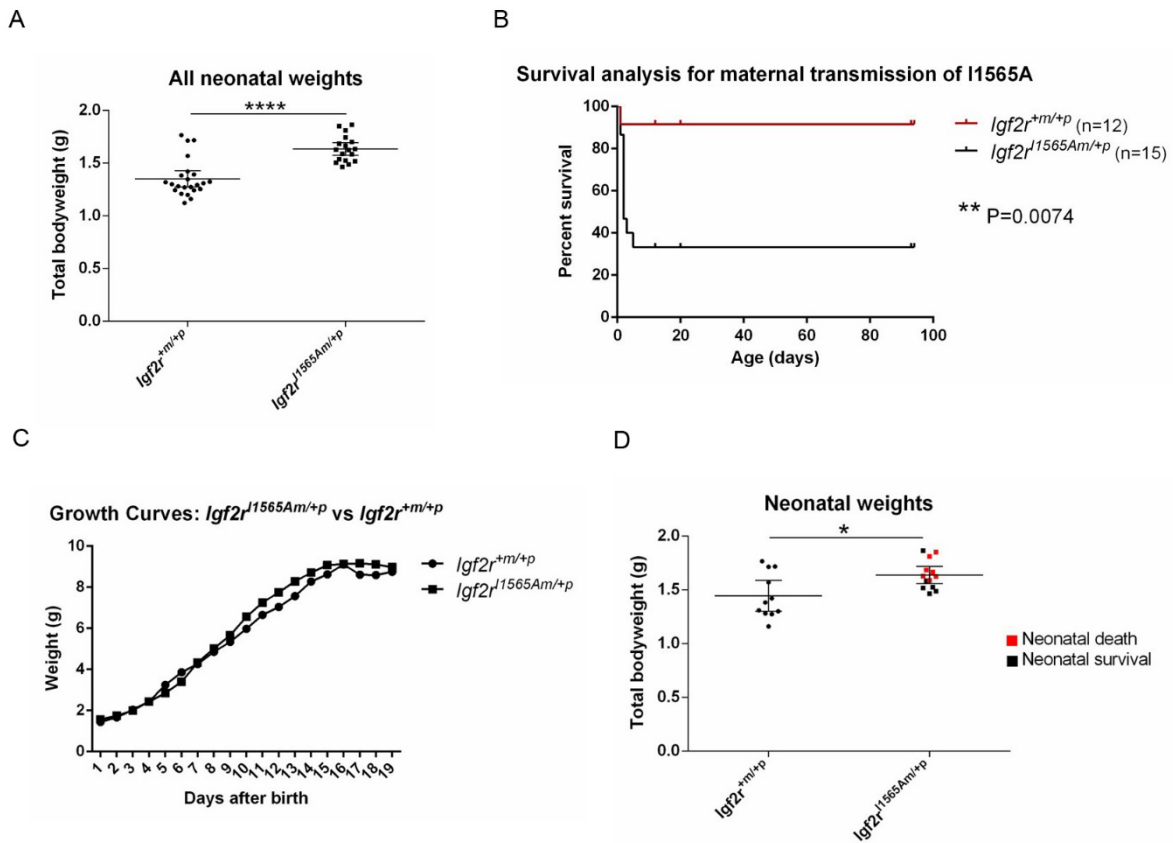


Fig 5.2: Neonatal growth, survival and size-survival relationship in IGF2 loss-binding-function mouse (A) Comparison of $Igf2r^{1565Am/+p}$ and $Igf2r^{+/m/+p}$ mice size at birth (B) Survival of $Igf2r^{1565Am/+p}$ mouse during the first three months (C) Growth of the $Igf2r^{1565Am/+p}$ and $Igf2r^{+/m/+p}$ mice during neonatal period (D) Size comparison of survived and died $Igf2r^{1565Am/+p}$ and $Igf2r^{+/m/+p}$ mice on the day of birth

Table 5.4: Relationship of survival with sex during until weaning

Sex-survival relationship at weaning				
Cross	Genotypes	Observed	Expected	
<i>Igf2^{r+m/11565Ap}</i> ♀ X <i>Igf2^{r+m/+p}</i> ♂ (5 litters, 3 pairs)	XY	13	12.0	P=NS
	XX	11	12.0	

Table 5.5: Survival of homozygous mice.

Survival of homozygous mice until weaning				
Cross	Genotypes	Observed	Expected	
<i>Igf2^{r+m/11565Ap}</i> ♀ X <i>Igf2^{r+m/11565Ap}</i> ♂ (4 litters, 1 pair)	<i>Igf2^{r+m/+p}</i>	10	6.75	P=0.313
	<i>Igf2^{r11565Am/+p}</i> Or <i>Igf2^{r+m/11565Ap}</i>	16	13.5	X ² =6.926
	<i>Igf2^{r11565Am/11565Ap}</i>	1	6.75	

5.3.4 Maternal transmission of IGF2 loss-of-binding function results in oedema in utero.

In order to investigate if the presence of the overgrowth phenotype extends back to the embryonic life, I dissected embryos on embryonic days 17.5 and 18.5. Embryos and placentas of the mice with the mutant maternal allele (*Igf2^{r11565Am/+p}*) were significantly larger than the WT (*Igf2^{r+m/+p}*) (Fig 5.3 A, B). Both for the embryos and placentas, the difference was more prominent on E17.5 when compared to the weights on E18.5. I also compared the dry weights of the same embryos and placentas (Fig 5.3 C, D). Except the placentas that are collected on E17.5, comparison of dry weights revealed that the size difference was due to water retention.

5.3.5 Homozygous transmission IGF2 loss-of-binding function results in embryonic overgrowth phenotype

The mild overgrowth of placentas with maternally transmitted IGF2 loss-of-binding function on E17.5 pointed out a possible borderline effect on whole body growth in heterozygous mice. Due to leakage from imprinted alleles, the overgrowth phenotype could be more severe following the homozygous transmission of mutant alleles. Therefore, I analysed wet and dry weights of the homozygous embryos and placentas. Similar to the heterozygous embryos, the homozygous embryos were significantly larger compared to WT embryos (Fig 5.4 A). However, except on day 18.5, there were no difference between the weights of *Igf2r*^{J1565Am/I1565Ap} and *Igf2r*^{+m/+p} placentas (Fig 5.4 B). Likewise, the dry weights of the homozygous mice placentas were also similar to the weights of placentas of WT embryos (Fig 5.4 D). Intriguingly, the presence of I1565A in the imprinted allele increased the dry mass of the mouse embryos on E17.5 and E18.5 (Fig 5.4 C).

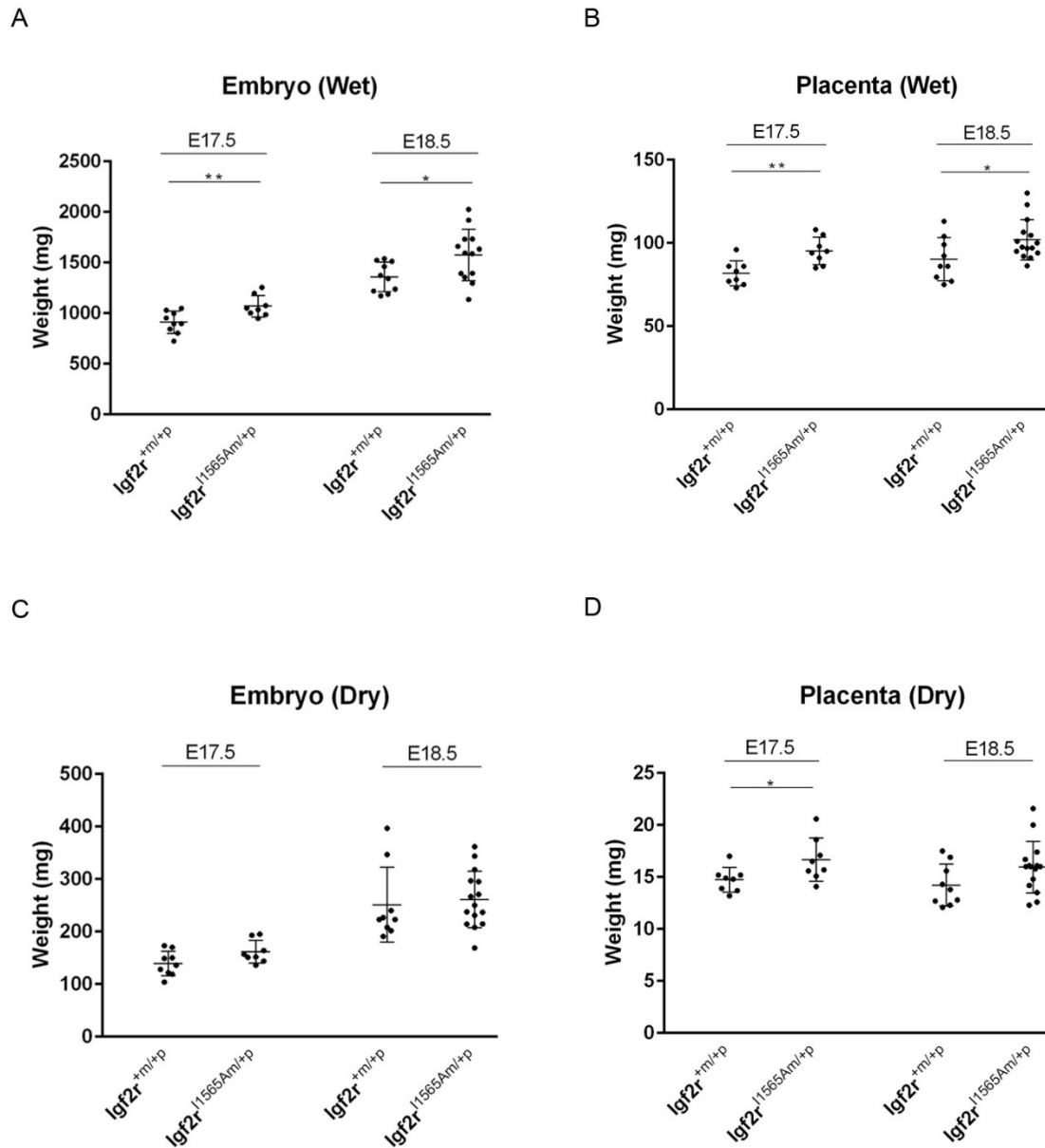
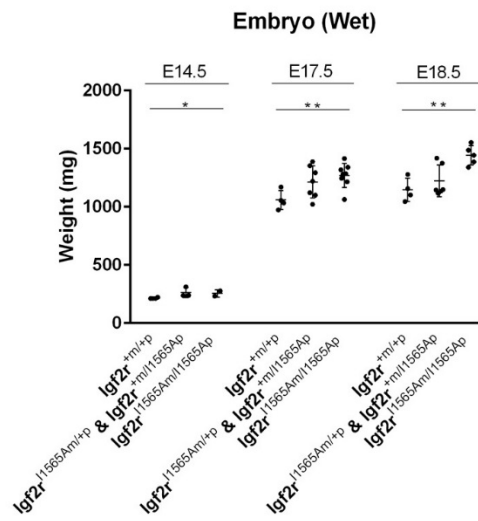
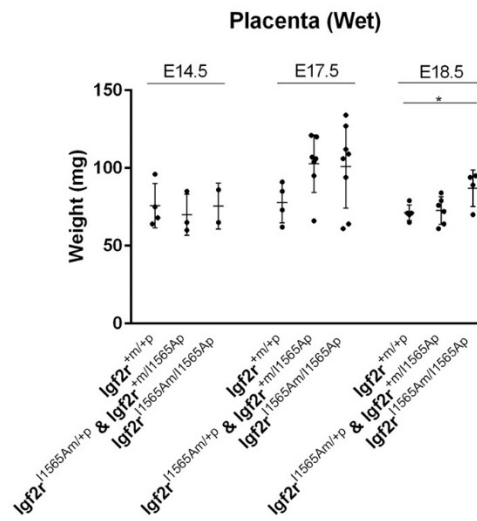


Fig 5.3: Comparison of wet and dry weights of *Igf2r*^{1565Am/+p} and *Igf2r*^{+m/+p} placenta and embryos on embryonic days 17.5 and 18.5 (A) Wet weights of *Igf2r*^{1565Am/+p} and *Igf2r*^{+m/+p} embryos on embryonic days 17.5 and 18.5, (B) Wet weights of *Igf2r*^{1565Am/+p} and *Igf2r*^{+m/+p} placentas on embryonic days 17.5 and 18.5, (C) Dry weights of *Igf2r*^{1565Am/+p} and *Igf2r*^{+m/+p} embryos on embryonic days 17.5 and 18.5, (D) Dry weights of *Igf2r*^{1565Am/+p} and *Igf2r*^{+m/+p} placentas on embryonic days 17.5 and 18.5

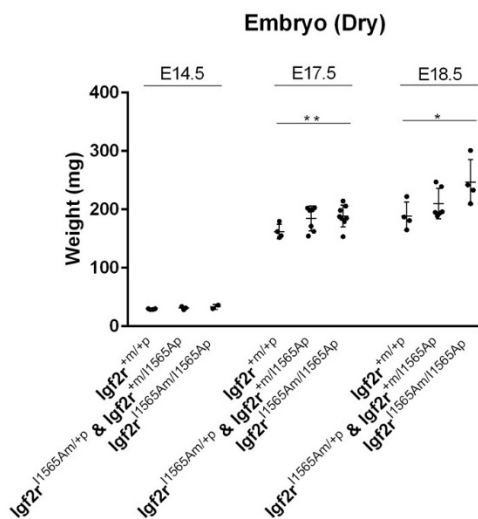
A



B



C



D

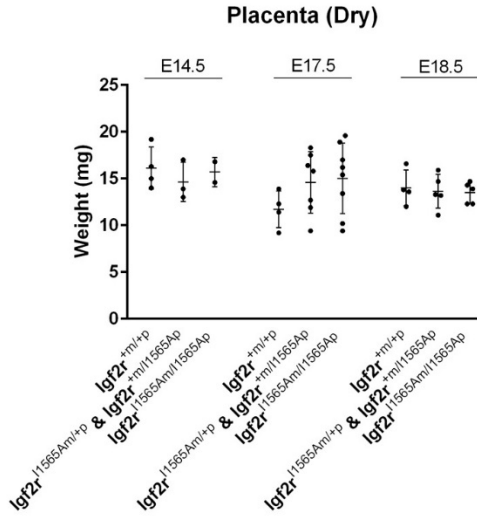


Fig 5.4: Comparison of wet and dry weights of *Igf2r*^{1565Am/1565Ap}, *Igf2r*^{1565Am/+p} *Igf2r*^{+m/1565Ap} and *Igf2r*^{+m/+p} placenta and embryos on embryonic days 17.5 and 18.5 (A) Wet weights of *Igf2r*^{1565Am/1565Ap}, *Igf2r*^{1565Am/+p} *Igf2r*^{+m/1565Ap} and *Igf2r*^{+m/+p} embryos on embryonic days 17.5 and 18.5, (B) Wet weights of *Igf2r*^{1565Am/1565Ap}, *Igf2r*^{1565Am/+p} *Igf2r*^{+m/1565Ap} and *Igf2r*^{+m/+p} placentas on embryonic days 17.5 and 18.5, (C) Dry weights of *Igf2r*^{1565Am/1565Ap}, *Igf2r*^{1565Am/+p} *Igf2r*^{+m/1565Ap} and *Igf2r*^{+m/+p} embryos on embryonic days 17.5 and 18.5, (D) Dry weights of *Igf2r*^{1565Am/1565Ap}, *Igf2r*^{1565Am/+p} *Igf2r*^{+m/1565Ap} and *Igf2r*^{+m/+p} placentas on embryonic days 17.5 and 18.5

Table 5.6: Summary of intrauterine breeding outcomes for homozygous *Igf2r* I1565A allele transmission

<i>I1565A Igf2r</i> allele transmission	Summary of breeding outcomes <i>in utero</i>			
	Genotypes	Observed	Expected	
E14.5	<i>Igf2r</i> ^{+m/+p}	4	2.25	P=0.3889
	<i>Igf2r</i> ^{I1565Am/+p} Or <i>Igf2r</i> ^{+m/I1565Ap}	3	4.5	X ² =1.889
	<i>Igf2r</i> ^{I1565Am/I1565Ap}	2	2.25	
E17.5	<i>Igf2r</i> ^{+m/+p}	4	4.75	P=0.2231
	<i>Igf2r</i> ^{I1565Am/+p} Or <i>Igf2r</i> ^{+m/I1565Ap}	7	9.5	X ² =3.0
	<i>Igf2r</i> ^{I1565Am/I1565Ap}	8	4.75	
E18.5	<i>Igf2r</i> ^{+m/+p}	4	3.75	P=0.63
	<i>Igf2r</i> ^{I1565Am/+p} Or <i>Igf2r</i> ^{+m/I1565Ap}	6	7.5	X ² =0.733
	<i>Igf2r</i> ^{I1565Am/I1565Ap}	5	3.75	
All embryos	<i>Igf2r</i> ^{+m/+p}	12	10.75	P=0.1986
	<i>Igf2r</i> ^{I1565Am/+p} Or <i>Igf2r</i> ^{+m/I1565Ap}	16	21.5	X ² =3.233
	<i>Igf2r</i> ^{I1565Am/I1565Ap}	15	10.75	

5.3.6 Maternal transmission but not homozygous transmission of I1565A results in disproportional heart overgrowth

Oedema could arise as a result of pathologies of many organs, such as because of hepatic, renal and cardiac dysfunction. Based on the reports of full IGF2/M6PR phenotype in mouse, most likely explanation for embryonic and placental oedema upon maternal transmission of *Igf2r* I1565A was a cardiac failure. Therefore, I evaluated the sizes of the hetero- and homozygous mice heart, body weights and the ratio of heart/body weights (Fig 5.5). The heart size of the *Igf2r*^{I1565Am/+p} mice was significantly larger than the *Igf2r*^{+m/+p} mice (Fig 5.5C). However, since overall body size was also bigger in the heterozygous neonates (Fig 5.5A), it

was necessary to investigate if cardiac growth was just a result of generalised overgrowth. The heart/body weight ratio indicated the presence of disproportional heart growth, pointing out possibility of structural pathologies in the heart (Fig 5.5 E). Interestingly, there were no difference of heart and body weights, and heart/body weight ratio of homozygous (*Igf2*^{J1565Am/J1565Ap}) mice when compared with WT (*Igf2*^{+m/+p}) neonates (Fig 5.5 B, D, F).

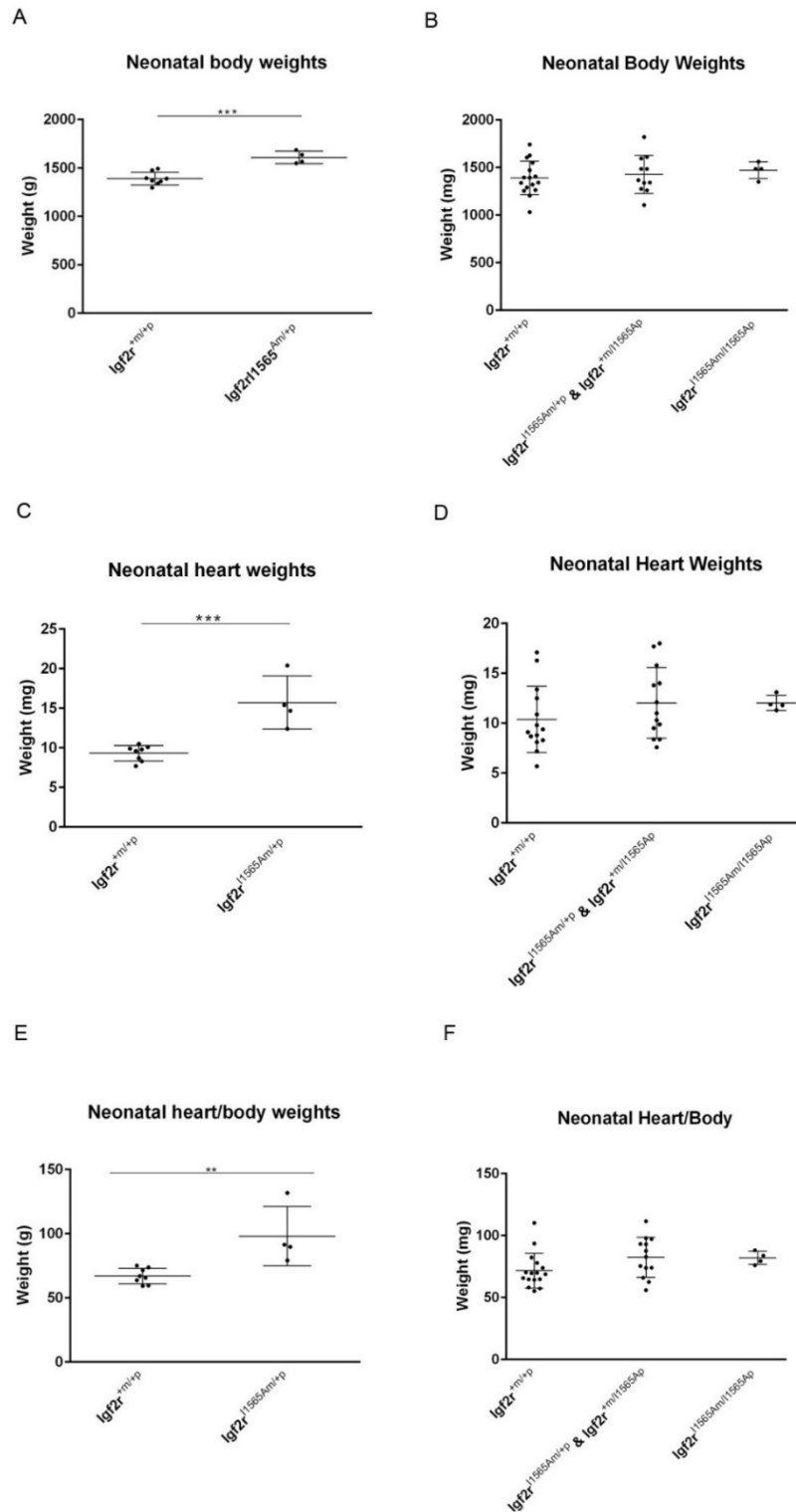


Fig 5.5: Evaluation of the consequence of IGF2 loss-of-binding function on body, heart weights, and heart/body weight ratio at birth (A, C, E) Comparison of body and heart weights, and heart/body weight ratio of $Igf2r^{1565Am/+p}$ and $Igf2r^{+/m/+p}$ mice (B, D, F) Comparison of body and heart weights, and heart/body weight ratio of $Igf2r^{1565Am/1565Ap}$, $Igf2r^{1565Am/+p}$ $Igf2r^{+/m/1565Ap}$ and $Igf2r^{+/m/+p}$ mice

5.3.7 IGF2 loss-of-binding function mice born with malformed myocardium and haemorrhagic lungs

For further evaluation of the cardiomegaly present in neonates, I have performed histopathological examination of the neonatal hearts (Fig 5.6). The H&E staining revealed structural abnormalities in the ventricular myocardium. Compared with WT (*Igf2^{+/m/+p}*) heart histology, the trabecular layer of the myocardium of the heterozygous mice (*Igf2^{1565Am/+p}*) is extended beyond the normal limits, restricting the thickness of the outer compact layer. Furthermore, lungs in the same sections showed distention of alveoli and bronchi due to haemorrhage and reduced thickness of inter-alveolar septa.

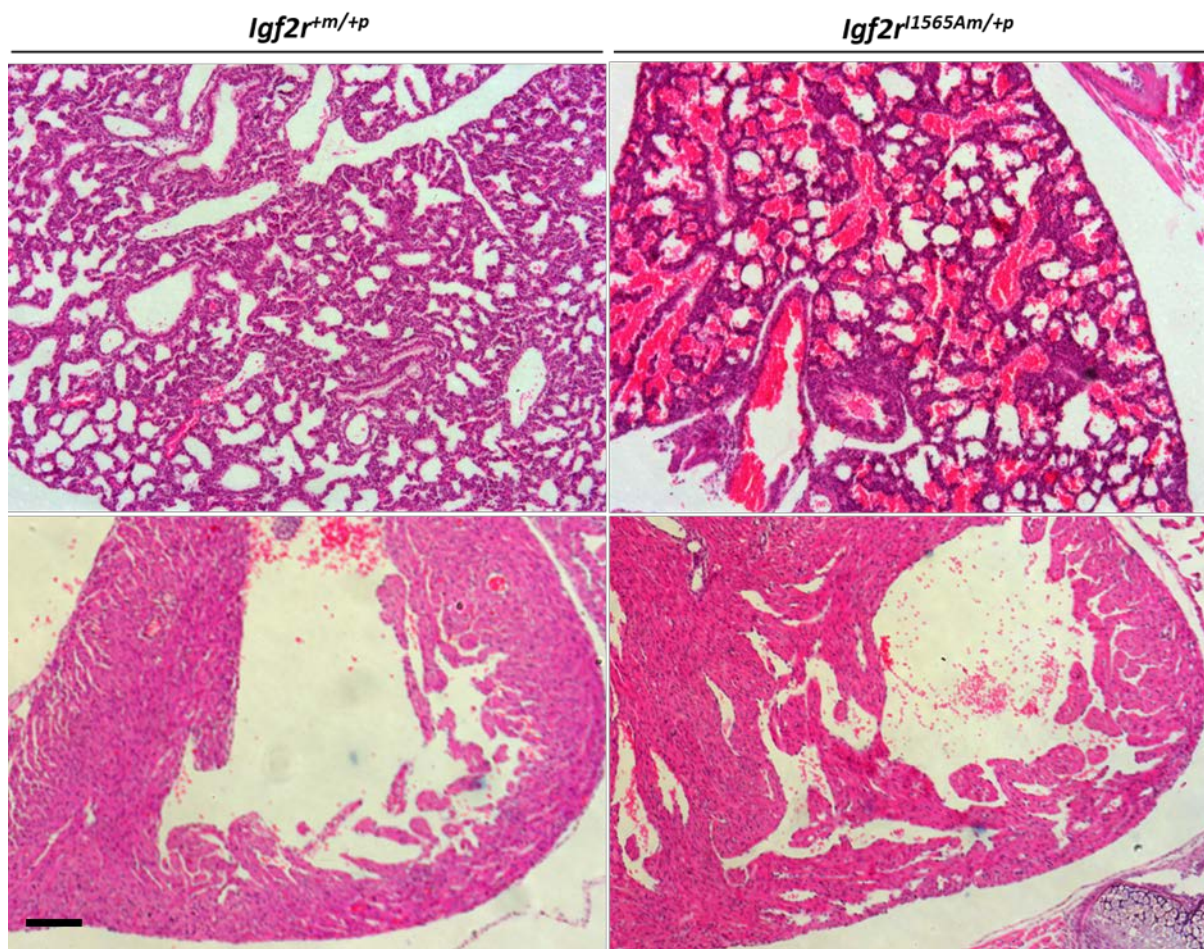


Fig 5.6: Cardiac and lung histopathology of *Igf2r*^{+m/+p} and *Igf2r*^{1565Am/+p} neonates (hematoxylin and eosin staining, n=2, bar size: 0.1 mm)

5.3.8 *Igf2r*^{1565Am/1565Ap} placentas showed mild oedema

I also examined the homozygous mice placentas to confirm the presence of oedema and examine the presence of any structural change. The histological sections on E18.5 of homozygous mice showed mild oedema without any other significant structural abnormalities (Figure 5.7).

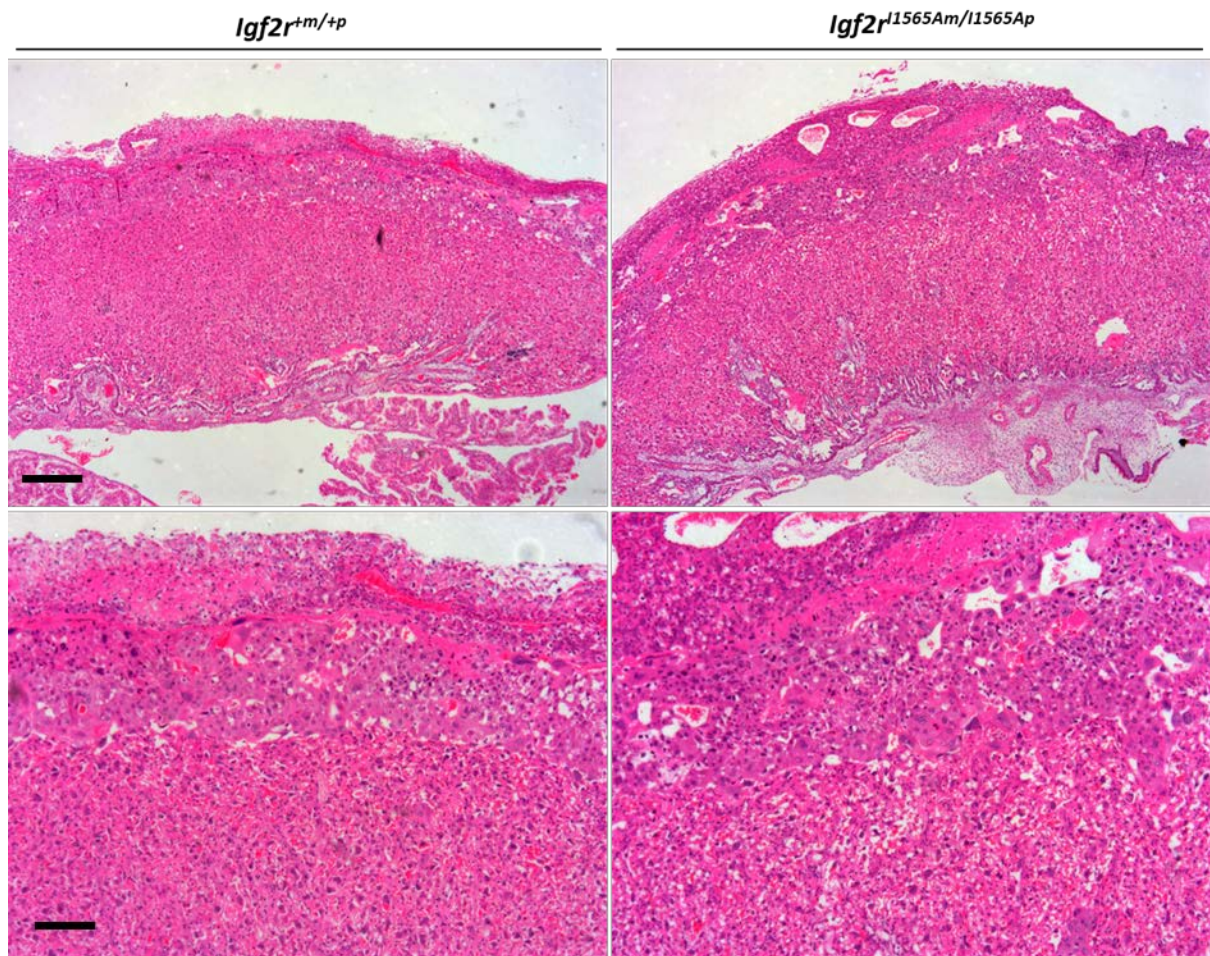


Fig 5.7: Placental histology of *Igf2r*^{+m/+p} and *Igf2r*^{I1565Am/I1565Ap} mice on E18.5 (hematoxylin and eosin staining, n=3, upper bar: 0.4 mm, lower bar: 0.1mm).

5.3.9 Female *Igf2r*^{1565Am/+p} mice that are survived to adulthood exhibit vaginal atresia

Majority of the mice with IGF2 loss-of-binding function in their maternal allele die in the early neonatal period. I have performed external examination of the survived adult mice and noted vaginal atresia accompanied by abdominal distention in the female littermate. When these mice are dissected for post-mortem examination, the abdominal and intrathoracic organs were normal except the presence of enlarged uterine horns in the mutant mice. The uterine cavity was filled with a viscous mucoid fluid with a colour ranged from yellowish to dark brown (Fig 5.8). The adult male mice, however, did not exhibit any genitourinary abnormalities upon macroscopic examination (Fig 5.8).

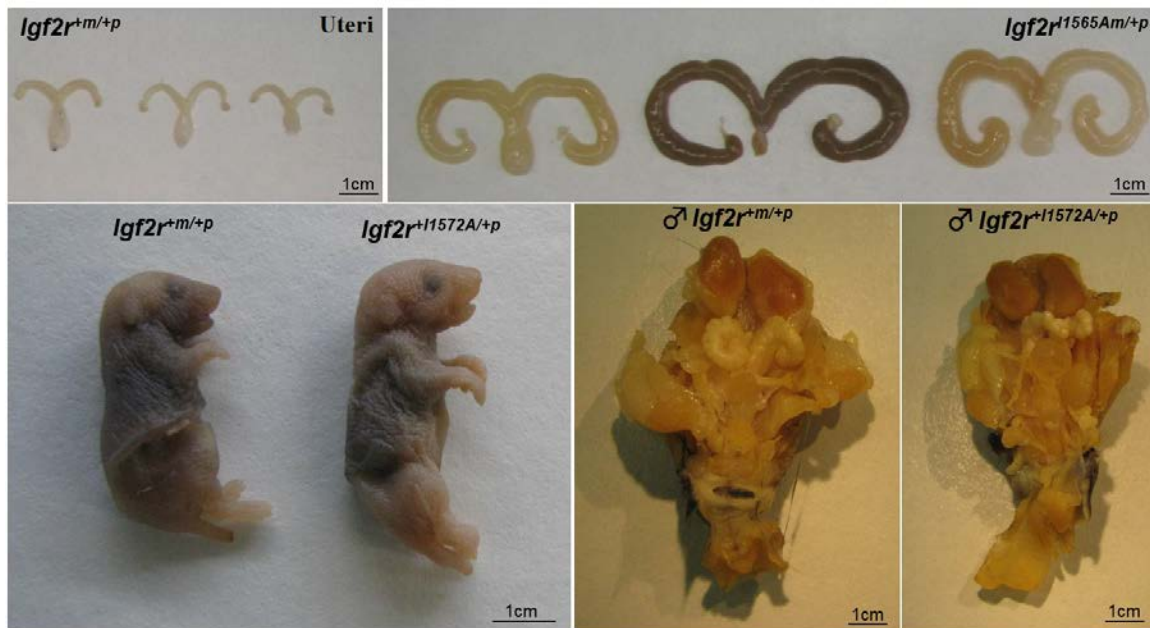


Fig 5.8: Other organ/system specific malformations in *Igf2r^{I1565Am/+p}* mice. Upper pictures are the comparison of adult mice uteri, n=4 (WT)/3(mutant). Lower left is the comparison of the WT and the mutant neonates. Lower right are the comparison of adult male abdominal genitourinary organs (n=5)

5.4 Discussion

5.4.1 I1565A is mouse homologue of human IGF2 loss-of-binding function mutation: I1572A

IGF2/M6PR gains IGF2 binding function in monotremes with increased affinity in placental mammals. IGF2, including its three essential residues for IGF2/M6PR binding (T16, F19 and L53) is conserved across vertebrata subphylum. Therefore, IGF2 binding site evolution is driver for the evolution of IGF2-IGF2/M6PR interaction. Shortened CD loop of the domain 11 binding seems to be primarily responsible for IGF2 binding in monotremes and this event coincides with acquisition of the hydrophobic residue of I1550 of CD loop in echidna (Williams, Hoppe et al. 2012). This hydrophobic residue is conserved in many mammals including opossum (I1588), mouse (I1565) and human (I1572). In human, I1572 of IGF2/M6PR domain 11 CD loop forms hydrophobic interactions with F19 of IGF2 (Brown, Delaine et al. 2008). F19 is the anchor residue of the IGF2-domain 11 interaction and I1572 residue is essential for the ligand binding in domain 11 constructs (Zaccheo, Prince et al. 2006, Delaine, Alvino et al. 2007).

The CD loop is fully conserved between mouse and human when amino acid sequences of both species are aligned (Fig 5.1), suggesting that the functions of the loop residues are well preserved. The data that I have presented in chapter 3 on the full-length IGF2/M6PR with I1572A could form a basis to understand the outcome of I1565A mutation in the ligand binding functions of the mouse full-length receptor. The binding analysis showed that the human homologue of mouse I1565A mutation results in domain 11 specific loss-of-binding function without disturbing receptor's other ligands' (M6P and plasminogen) binding functions. Thus, the mutant receptor could form a basis for *in vivo* models to dissect domain 11 functions.

5.4.2 Maternal transmission of I1565A results in size dependent neonatal lethality due to cardiorespiratory failure.

Maternal transmission of the Igf2 loss-of-binding mutation results in increased size on the day of birth. When the growth of the pups' are followed, almost one third of them die during first three days of post-natal life and remaining heterozygous pups show no significantly different growth trajectory when compared with WT pups. Retrospective analysis showed that the survival of the pups is negatively correlated with the size of the pups at birth. When heterozygous mice were followed back to the intrauterine life, comparison of the wet and dry weights showed the presence of placental and embryonic water retention. Based on the reports of the full receptor knock-out models, the oedema was most likely caused by cardiac insufficiency. The neonatal heart sizes and heart/body size ratios showed presence of a disproportional cardiomegaly. Furthermore, histological examination of the heterozygous mice revealed the maternal transmission of I1565A causes malformed myocardium with trabecular invasion to compact layer which expected to reduce ejection fractions leading to congestive heart failure which represent itself with many symptoms including generalized oedema. In addition, architecture of the lungs was disturbed, and the alveoli were haemorrhagic. Based on these observations, both cardiac and respiratory dysfunction seem to be responsible for the neonatal lethality.

5.4.3 Homozygous transmission of I1565A results in early neonatal death in newborns with pathologies related to generalised overgrowth.

Both maternal and paternal transmission of the I1565A followed Mendelian pattern but, interestingly, when the mutation was transmitted via both alleles the frequency of homozygous offsprings were lower than expected. I further investigated the transmission pattern in embryos of homozygous matings. The analysis of genotypes of embryos on E14.5, E17.5 and E18.5

shows that the homozygous allele transmission for the mice *in utero* follows Mendelian transmission rules. Such a disparity between pre- and post-natal allele transmission rates suggests that the homozygous (*Igf2r*^{J1565Am/J1565Ap}) newborns die very early, before the dissections are performed and possibly could not be recorded because of maternal cannibalism. Neonatal death also occur in *Igf2r*^{J1565Am/+p} due cardiac insufficiency. Although, there were increased in dry mass, comparison of wet and dry weights of embryos and placentas, and the placental histology on embryonic day 18.5 suggest the presence of only mild oedema which makes cardiac failure unlikely to be the sole explanation for early neonatal death. When the pups were examined on postnatal day 1, heart&body weights and the ratio of heart/body weights were found to be similar to WT pups. These results suggest that the homozygous mice with increased dry weight and oedema die probably in few hours after birth. Since the oedema is mild, congestive cardiac failure is very unlikely explanation for the cause of death. Apparently, increased dry weights of the mutant embryos suggest generalised overgrowth phenotype involving multiple organs. Independent of it nature, the condition(s) that is/are leading to death is most likely to be aggravated by stress of the birth or lack of adaptation to the postnatal life.

5.4.4 Similar to full receptor knock out mouse, Domain 11 loss-of-binding function results in hydrometrocolpos but not in musculoskeletal abnormalities.

Full knock-out of the maternal *Igf2r* allele results in skeletal malformations characterized with hexadactyly and kinked tail (Wang, Fung et al. 1994, Ludwig, Eggenschwiler et al. 1996). However, such skeletal abnormalities was not present in mice with domain 11 specific loss-of-function (*Igf2r*^{J1565Am/+p} and *Igf2r*^{J1565Am/J1565Ap}). Similarly, except the heart, male mice were also normal on external inspection; their abdominal and thoracic cavities and the organs within were macroscopically similar to their WT littermates. However, adult female mice abdomens and uteri were distended due to hydrometrocolpos as a result of vaginal atresia.

5.4.5 Igf2 loss-of-binding mutation in the imprinted allele has opposite effects on the heart and the whole body

Igf2r is a paternally imprinted gene in mice (Barlow, Stoger et al. 1991). Previous reports showed that of homozygous transmission of the full *Igf2r* knock-out results in different phenotype compared to the only maternal transmission (Baker, Liu et al. 1993, Wang, Fung et al. 1994). These studies indicate that although the paternal allele is imprinted, the imprinting is not strictly controlled, and leakage happens. In this study, I also found a difference between the hetero- and homozygous transmission of domain 11 loss-of-function receptor. While no significant change in dry weights were observed following maternal transmission, homozygous transmission of the mutant allele resulted in increased dry mass in embryos. However, intriguingly, homozygous embryos exhibit milder oedema and the homozygous pups' hearts were normal in growth on the day of birth. These results were surprising since the heart phenotype were expected to be more severe in homozygous mice. Most likely explanation for this phenomenon is that, similar to oncogenic BRAF mutations in melanomas, the MAPK pathway could have been paralysed upon hyperstimulation due to overdosage of Igf2 in mouse serum (Maddodi, Huang et al. 2010).

In summary, these results show that domain 11 regulates majority of Igf2r's role in development and organogenesis, including general growth, cardiac and genitourinary development but not skeletal formation.

Chapter 6: Discussion

Our understanding of domain specific functions of IGF2/M6PR has been very restricted up until now. Receptor fragments were used for characterisation of the receptor's ligand binding domains and virtually no data existed on how these domains function in the context of the full-length receptor. Furthermore, although several studies characterised developmental pathologies in full *Igf2r* knock out mice, IGF2/M6PR's domains' *in vivo* roles have never been examined. This thesis, utilising both *in vivo* and *in vitro* approach presents many significant insights on domain specific functions of IGF2/M6PR. In this study, I firstly showed that the receptor's ligand binding domains operate in an independent manner. For instance, plasminogen and IGF2 binding domains function independent of the M6P binding domains. Also, M6P binding domains are not operating in a synergistic manner and these domains' functions are not affected by loss- and gain-of IGF2 binding functions of domain 11. Secondly, characterisation of IGF2 loss-of-binding mice showed that, apart from the skeletal development, a functional domain 11 is essential for all known developmental regulatory functions of IGF2/M6PR. Thirdly, comparison of heterozygous and homozygous domain 11 loss-of-binding function mice suggests that the compensatory role of imprinted *Igf2r* allele -in the absence of functional maternal allele- is organ-specific. Lastly, I showed that the presence of inhibitory residues for IGF2 binding in the context of full-length receptor, and the comparison of gain-of-function mutants' affinities suggested that internal rearrangements/inter-loop interactions are important for domain 11 binding pocket functions. These results also led to new questions, in order to answer some of which I performed preliminary experiments and started mice breeding required for final experiments.

6.1 IGF2/M6PR domains function independently.

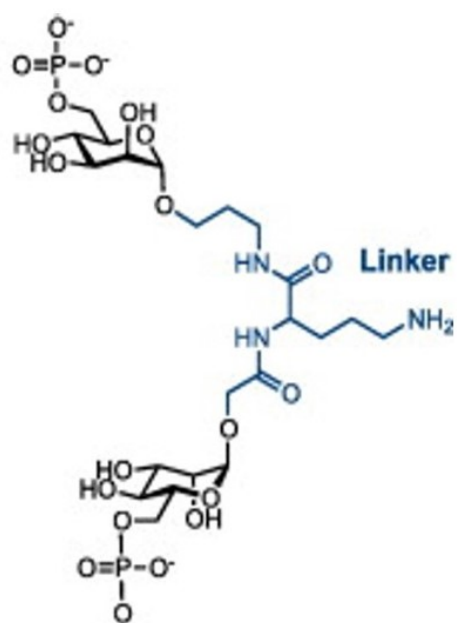
The SPR analysis in this thesis showed that loss-of-binding of M6P and IGF2 binding domains does not influence binding functions of other domains, suggesting that IGF2/M6P receptor domains function in an independent manner. Only exception was the minor affinity reduction of domain 11 to IGF2 in full loss of the M6P binding function mutant, a phenomenon that was observed probably due to the proximity of domains 9 and 11. Interestingly, however, plasminogen (which binds to domain 1 and the linker peptide between domains 1 and 2) affinity was fully preserved in the M6P binding mutants (M6P binds to domains 3, 5 and 9). Similarly, loss- and gain-of-function mutants of domain 11 also exhibited preserved M6P and plasminogen binding functions. More strikingly, evaluation of binding affinities of M6P binding site mutants showed a lack of synergism between these domains. However, it is important to bear in mind that the results also suggest that the analyte (bovine β -glucuronidase) is probably exclusively tagged with M6P monoesters but not diesters. Also, very recently discovered juxta-membrane binding site (domain 15) showed no detectable binding affinity, possibly due to steric affect. For the future work, to include domain 15 to the analysis, the linker peptide between the ectodomain and CD4 domains 3&4 could be extended or CD4 domains 3&4 could be removed or modified.

When I tested full M6P loss-of-binding full-length IGF2/M6PR domains at high proton concentrations imitating conditions that the receptor is expected to show maximum (early endosomes) and no binding (late endosomes), I found unexpected results. At the early endosomal pH, M6P loss-of-binding mutants still showed reduced but significant binding, suggesting that, under this particular pH conditions, the glutamate and tyrosine residues of the mannose surrounding motif are not essential for binding. More intriguingly, at pH 5, WT receptor showed significant β -glucuronidase binding and mutations of these two residues had no significant effect on the ligand binding. This could be due to increased M6P-independent component of the binding or shift in the share of the binding pocket residues in M6P binding.

An easy and useful experiment to differentiate the significance of these two components (M6P independent vs. other M6P binding residues) is to test these mutants in the presence of inhibitory concentrations of M6P and G6P. Later, further experiments could be directed to elucidate pH dependent roles of the residues that reside in the receptor binding pocket. It is also important to further investigate functional significance of WT M6P binding at pH 5.0 because the receptor is expected to unload all of its M6P tagged cargo at pre-lysosomal compartments. Thus, future work could be directed to elucidate dynamics of M6P tagged cargo release, including involvement of the acid phosphatases.

In order to characterise differential recognition of diester vs monoester binding functions of the M6P binding domains, exclusively diester and monoester ligands are required. These ligands can either be natural ligands or synthetic compounds. In the presence of uncovering enzyme (UCE), diesters are expected to be efficiently processed to monoesters. Thus, diester tagged glycoproteins could be obtained from UCE knock out cell lines or synthesized by *in vitro* modification. This approach carries the risk of significant level of impurities due to inefficient genetic modification or enzymatic processing. We, however, through collaboration, obtained synthetic ligands. These synthetic ligands come in three main forms and carry two M6P tags: diesters only, monoesters only, and mixed mono- and di-ester (Fig 6.1).

A



B

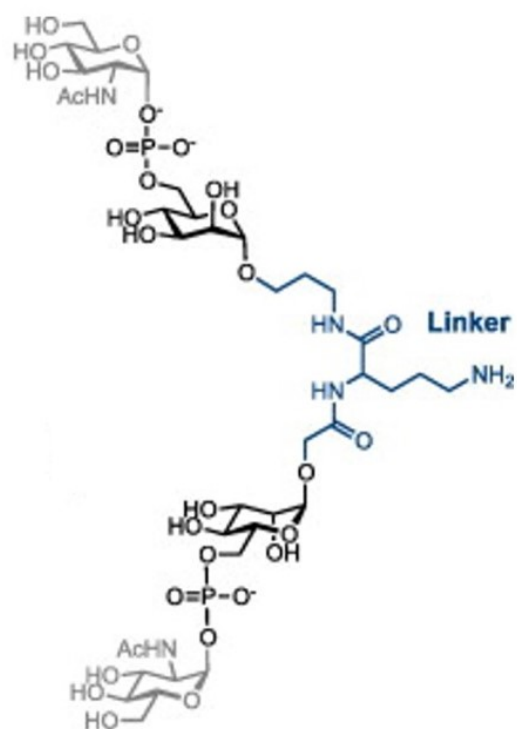


Fig 6.1: Structure of the synthetic ligands. (A) Compound with two M6P monosters, and **(B)** with two diesters.

These compounds' molecular weights were very low compared to the full-length receptor so sensitivity of SPR machines was the main limitation. I aimed to employ two strategies to overcome this technical obstacle. One was to increase the ligand density on the chip. The maximum ligand density increase that I achieved by saturating the streptavidin binding sites (three folds increase in ligand density) was not sufficient to detect binding of mannose 6-phosphate binding to the WT receptor. Increasing streptavidin density to the maximum that a CM5 chip could accommodate resulted in only minor change in ligand binding capacity of the CM5 chips. While shifting to the highest capacity chip (CM7) increased maximum streptavidin immobilisation three folds, it did not significantly change the maximum ligand density on the chips. Such a phenomenon has occurred probably because of the steric effect due to overcrowded chip. Consistent with this hypothesis, I managed increase the ligand density by reducing the streptavidin density to between 500 to 2000 RU (from between 7000-45000 RU). In this range, further future experiments for optimisation of the streptavidin density could yield higher binding capacity for the ligand immobilisation.

Another strategy, in contrast to measuring direct binding affinity, was determination of the inhibitory constants of the compounds. The compounds were synthesized in small quantities so I optimised inhibition experiments using β -glucuronidase as the M6P ligand and mannose 6-phosphate barium salts as a specific binding inhibitor as they are obtained commercially. In order to determine the minimum β -glucuronidase concentration required for the inhibition studies; 2.048 μ M, 512 nM and 128 nM of β -glucuronidase run in the presence of serial dilutions of M6P (61 nM to 10 mM) and G6P (31 μ M to 10 mM) (Fig 6.2). Only sensorgrams of the inhibition experiments with 2.048 μ M β -glucuronidase gave saturation plateaus that are suitable for steady state evaluation. These pilot experiments will determine the optimal conditions for the use of the chemical compounds for inhibition studies.

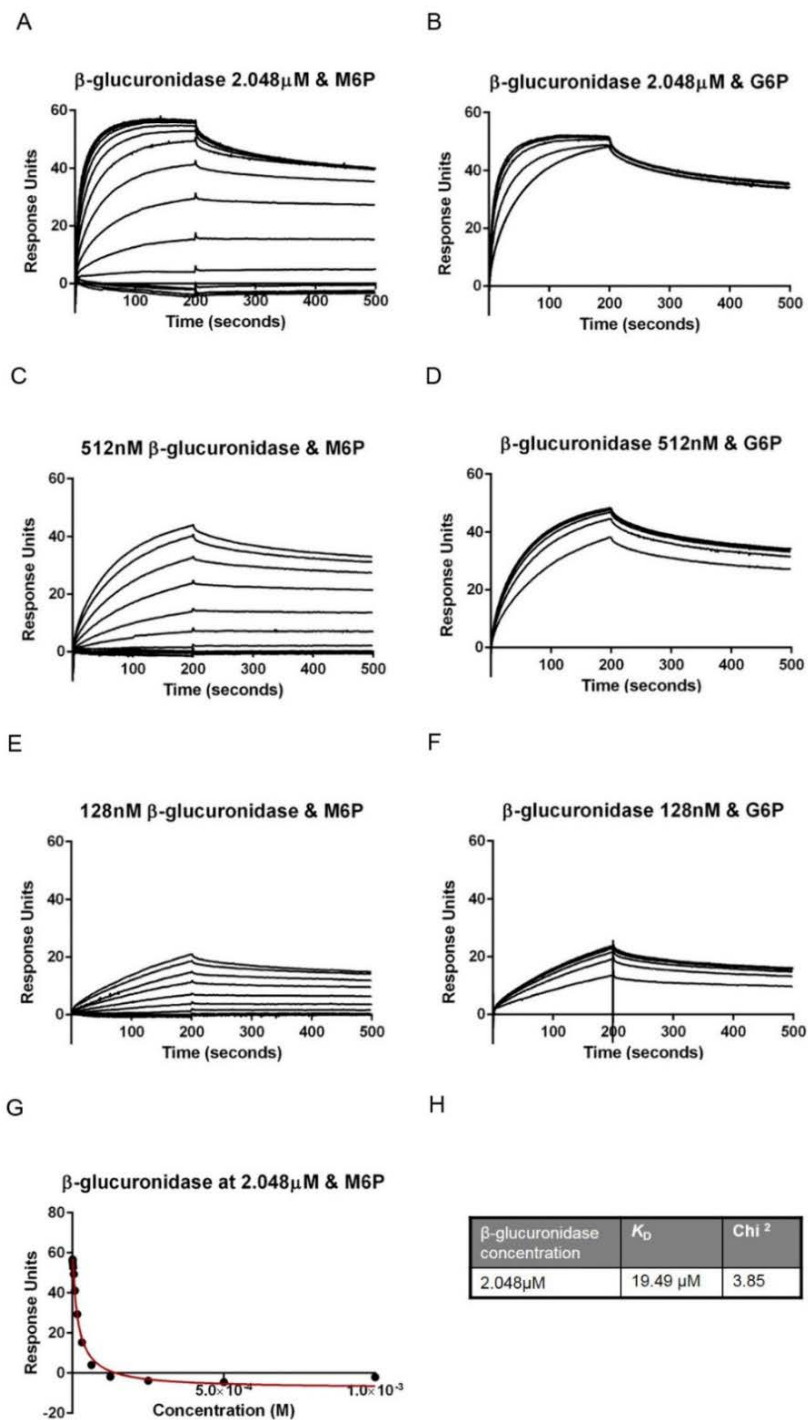


Fig 6.2: Results of preliminary experiments for inhibition studies with synthetic compounds. β -glucuronidase was used as the M6P-tagged ligand and mannose 6-phosphate barium salts as a specific binding inhibitor. G6P used as a negative control for M6P inhibition.

6.2 IGF2 binding function Domain 11 is central to IGF2/M6PR's roles in development

SPR studies of human IGF2/M6PR in this thesis suggest that ligand binding domains of the receptor function independently and introducing I1572A mutation to the full-length receptor results in a null mutant for IGF2 binding with preserved M6P and plasminogen binding. Thus, an *in vivo* model with mouse homologue of I1572A (I1565A) could serve as a model to dissect the roles of domain 11. However, in order to reach this conclusion there are two approaches that could be undertaken. One is producing recombinant full-length mouse IGF2/M6PR with I1565A and test its binding functions to mouse IGF2, M6P and plasminogen. Although this can provide us a definitive answer on the validity of utilising I1565A mutation for the mouse model to dissect the roles of domain 11, it represents substantial technical challenges because of the GC rich nature of *Igf2r*. My various attempts to employ this approach have been unsuccessful. The second approach is a mixed theoretical/experimental approach to assess inter-species conservation of the receptor and IGF2/M6PR-IGF2 interaction. While, M6P binding functions of the receptor is an ancient function; its IGF2 binding functions acquired relatively recently, co-evolving with paternal imprinting of its gene in marsupials and eutherians (Killian, Byrd et al. 2000, Killian, Nolan et al. 2001). Interestingly, the sequence, structure and domain 11 binding functions of the other partner of the interaction, IGF2, is very well preserved between birds to placental mammals emphasizing primary -or even exclusive- role of the domain 11 binding pocket transformation in achieving high affinity IGF2 binding in placentalia. The transformation is attained mainly by structural transformation and mutagenesis of the CD loop, coinciding with the evolution of placenta. However, the IGF2 binding pocket of the receptor seems to be well preserved in placentalia. Moreover, the CD loop where the I1565A/I1572A mutation is introduced is fully conserved between rodents (*Mus musculus* and *Rattus norvegicus*), bovidea (*Bos taurus*) and high primates (*Pan troglodytes* and *Homo sapiens*) (See Fig 5.1). Preservation of IGF2's binding functions to Domain 11 across species

was demonstrated by measuring affinity of recombinant human, echidna and chicken IGF2 to human, echidna and opossum domain 11 (Williams, Hoppe et al. 2012). Also, mouse and human IGF2 exhibit similar affinities to human full-length IGF2/M6PR (Hughes, Frago et al. 2013). In addition, amongst placentalia, similar affinities results were obtained when affinities of bovine and human IGF2s to their corresponding full-length IGF2/M6PR are compared. Lastly, both M6P binding pockets and M6P affinity of bovine and human IGF2/M6PR are well-preserved. In summary, structural data on IGF2/M6PR and IGF2, functional data on IGF2/M6PR's interaction with IGF2 and M6P tagged ligands and finally alignment of IGF2/M6PR sequences of placental mammals (particularly of CD loop of Domain 11) strongly suggests that the *in vitro* data obtained from full-length I1572A could be used for interpretation of phenotype that is observed in I1565A mice, and I1565A mice is a good *in vivo* model to dissect the roles of domain 11.

The most prominent feature of the maternal domain 11 IGF2 loss of binding function mice (*Igf2r*^{I1565Am/+p}) was early neonatal lethality (>60%) due to cardiac dysfunction. The heterozygous embryos exhibited embryonic and placental oedema; disproportional heart growth and cardiopulmonary pathologies upon histological examination. Perinatal lethality of cardiac origin has been universally reported in the full receptor knock out mice reports (Lau, Stewart et al. 1994, Wang, Fung et al. 1994, Ludwig, Eggenschwiler et al. 1996, Hughes, Frago et al. 2013). Moreover, the lethality phenotype was rescued in *Igf2* knock out background suggesting the central role of *Igf2* levels in perinatal death. Thus, early neonatal lethality is expected to be observed in domain 11 loss-of-function mice. Similar to full knock out mice, increased placental, embryonic and neonatal size is observed in *Igf2r*^{I1565Am/+p} mice and the difference in size is mostly attributed to increase in dry weight (Wang, Fung et al. 1994) or hyperplasia (Lau, Stewart et al. 1994). However, in several studies, embryos with hairpin-tail (Thp) mutation -a deletion mutation in chromosome 17 that is predicted to encompass a genomic region where *Igf2r* is located- reported to exhibit generalized oedema (Johnson 1974, Winking and Silver 1984, Babiarz, Donovan et al. 1988, Tsai and Silver 1991,

Filson, Louvi et al. 1993). Also, Ludwig et al. reported oedema after embryonic day 16.5 for loss-of-function of the maternal *Igf2r* allele (Ludwig, Eggenschwiler et al. 1996). Presence of oedema, in this context, is expected to be correlated with the severity of cardiac dysfunction, specifically decompensation of the heart failure. Multiple precipitating factors, both cardiac and non-cardiac, could lead to decompensation. Complexity and multiplicity of the factors makes the process more susceptible to be influenced by strain-dependent differences (Barrick, Rojas et al. 2007). In line with this, the lethality attributed to cardiorespiratory failure in *Igf2r* knock-out mice was found to be strain dependent by Ludwig et al. Thus, the disagreement on the presence of oedema between the previous reports of full *Igf2r* knock-out (*Igf2r^{m/+p}*) mice and *Igf2r^{1565Am/+p}* mice in this study could be attributed to the mouse background. Interestingly, however, *Igf2r^{1565Am/+p}* showed significantly higher post-natal survival rate compared to both oedematous and non-oedematous mice. Similarly, the oedematous mice mutants in Lau et al.'s study showed higher survival rates compared to the non-oedematous mice in other studies. When wet and dry weights of embryos were compared, it seems that the weight increase in homozygous Domain 11 IGF2 loss-of-binding mice (*Igf2r^{1565Am/1565Ap}*) is mostly due to increase in dry weight, suggesting a milder oedema phenotype in homozygous mice (*Igf2r^{1565Am/1565Ap}*) when compared to heterozygous mice (*Igf2r^{1565Am/+p}*). Moreover, in contrast to heterozygotes, homozygous neonates do not exhibit disproportional cardiomegaly. Again, interestingly, homozygous mice show significantly low post-natal survival compared heterozygous mice. All together, these observations suggest that, in addition to a possible cardiac one, a non-cardiac cause of death is also responsible for the peri-/postnatal lethality. It is very likely that these (cardiac and non-cardiac) causes of death become dominant at different level of excess IGF2 concentrations, and in a hypothetical graph that shows cause-of-death/IGF2 level relationship, the curves that represent the type of cause-of-death severity/dominance in the mice would happen to exist in an overlapping manner. Wang et al. reported different survival rates for maternal heterozygous (*Igf2r^{m/+p}*) and homozygous (*Igf2r^{m/-p}*) mice, and also detected protein expression from the paternal allele of the receptor. Similarly, Hughes et al. reported that upon homozygous transmission of the mutant allele, the

hypomorphic loss-of-function phenotype is reversed. However, in the literature (three full knock out, the hypomorphic *Igf2r* loss-of-function) and in my study, paternal loss-of-function mice do not differ from their WT siblings. These two studies (*Wang et al. and Hughes et al.*) and this current work strongly suggest that WT receptor expression from the “imprinted allele” has functional implications when the IGF2 clearance function of the receptor is disrupted due to the mutations in the maternal allele. The most likely reasons for this phenomenon are incomplete imprinting during the early stages of embryonic development or loss-of-imprinting at later stages due to high IGF2 levels in serum/extracellular fluid. Evaluation of allele-specific RNA expression in different tissues, and assessment of local and systemic protein (IGF2, IGF2/M6PR, and possibly IGF2 activated intracellular signalling proteins) levels in WT, heterozygous (paternal and maternal) and homozygous mice at different stages of development required to understand the dynamics of imprinting regulation of paternal *Igf2r* allele.

Replacement of WT paternal (imprinted) allele with domain 11 loss-of-function interestingly alleviated the heart phenotype. The homozygous embryos showed reduced oedema and the newborns did not exhibit disproportional heart weight increase. Similarly, Hughes et al. showed that homozygosity of the hypomorphic (humanized) *Igf2r* allele rescued the perinatal lethality and the heart phenotype. Interestingly, however, dry weight increase in embryos is observed in homozygous (*Igf2r*^{J1565Am/J1565Ap}) mice but not in heterozygous (*Igf2r*^{J1565Am/+p}) mice. Thus, apparently, the degree of excess IGF2 levels has different effects on heart development and on general body growth. *In vivo* and *ex vivo* studies demonstrated that IGF2 is the primary growth factor produced by the developing heart epicardium -and to a smaller degree from endocardium- for cardiomyocyte proliferation and myocardial formation (Li, Cavallero et al. 2011, Shen, Cavallero et al. 2015). For instance, in cell cultures, when IGF2 secretion from epicardial cells is inhibited, myocardial explant proliferation decreased about 70% (Li, Cavallero et al. 2011). Thus, during intrauterine development, the importance and probably the concentrations of IGF2 in the heart and the remaining of the body is different. The

MAPK/ERK pathway is essential for mouse foetal cardiomyocyte proliferation (Kang and Sucov 2005) and IGF2 stimulation constitutes the majority of the pathways activation in mice heart (Li, Cavallero et al. 2011). However, interestingly, in melanoma cell lines, hyperstimulation of the MAPK pathway due to mutant BRAF^{V600E} overexpression results in reduced proliferation. (Maddodi, Huang et al. 2010). This paradoxical effect on proliferation due to overstimulation of the MAPK pathway could explain the reversal of cardiac phenotype due to introduction of loss of IGF2 binding function the paternal *Igf2r* allele. Systemic and organ specific evaluation of MAPK pathway activation could help to elucidate the differential effect of the IGF2 levels on the cardiac and the whole body development.

Interestingly, in disagreement with the reports of full knock-out mice, maternal transmission of IGF2 loss of binding function allele did not change dry weights of the embryos (dry weights increased only upon homozygous transmission) suggesting the increased weights in mutant embryos is due to oedema. This disparity between my work and full receptor knock out mice might be due to the physiological variations in different mouse strains or more probably due to higher levels of IGFs in the full receptor knock down mice compared to the domain 11 loss-of-function mice. Since, the SPR data suggest that I1565A mutant is a complete IGF2 loss-of-binding mutant, probably loss in plasminogen and M6P binding functions of the receptor in full knock out mice is the source of the difference. The fact that *Igf2/Igf2r* null mice show milder growth deficiency (65-75% of WT) compared to *Igf2* null mice (60% of WT) suggests that IGF2/M6PR could also regulate growth through IGF2 independent mechanisms. *Igf2/Igf1r* null and *Igf2/Igf1r/Igf2r* null mice shows similar growth restriction (30% of WT) suggesting IGF1, the only other ligand of IGF1R, is responsible for the milder phenotype. In addition to IGF1R, IGFBPs are also shared by IGF1 and IGF2. IGFBP3 knock out mice exhibit an overgrowth phenotype and IGFBP3s overexpression *in vivo* results in growth restriction suggesting that storage function of IGFBPs is important in negative regulation of growth (Modric, Silha et al. 2001, Yamada, Mehta et al. 2010). Thus, one possible mechanism for the IGF2 independent growth increase in *Igf2r* knock out mice is proteolytic degradation of IGFBPs due to missorting

of proteolytic enzymes by IGF2/M6PR. This, alongside strain-dependent differences, could provide an explanation for the milder phenotype that I observed in *Igf2r*^{1565Am/+p} mice compared to *Igf2r*^{m/+p} mice. Moreover, IGFBP3 levels were shown to increase four to six fold in *Igf2r* knock out mice (Lau, Stewart et al. 1994). The changes in growth phenotype due to possible degradation of IGFBPs are expected to be even more prominent in mice with increased IGF2 levels due to the lack of clearance function of IGF2/M6PR. Another possible mechanism of interplay between M6P and IGF2 transport pathways is steric hindrance. SPR studies in this thesis suggest that M6P and IGF2 binding domains function independently and small molecular size of IGF2 makes presence of receptor-bound IGF2 insignificant thus such a binding interference is unlikely. However, further functional studies, such as cellular re-uptake assays, could be helpful in excluding this possibility.

Unlike heterozygous (*Igf2r*^{1565Am/+p}) mice, homozygous (*Igf2r*^{1565Am/1565Ap}) mice dry weights showed not only increased water content but also increase in solid components of the body suggesting a real increase in organic and mineral content. This increase could be due to hyperplasia (increased cell number), hypertrophy (increased cellular contents) and/or extracellular matrix. Hyperplasia and hypertrophy could be assessed by nuclear count and assessment of nuclear-cytoplasmic ratio, respectively. DNA content analysis is also very useful to assess the rate of cellular proliferation by mitotic stage determination and calculating cell numbers, could give us both cross-sectional and cumulative data on growth rate. However, unlike dry weight measurements, evaluation of whole body growth using these approaches represents a major technical obstacle so their use is restricted to organs or tissues.

The most prominent feature lacking in domain 11 IGF2 loss of binding mice is the absence of bone malformations that are widely reported in full receptor knock out mice. These abnormalities include polydactyly, kinked tail, and sternal fusion defects. From the onset of ossification to death, bones continuously undergo osteoclastic resorption and osteoblastic new bone formation. Regulation of these two antagonistic processes is crucial for both optimal

skeletal morphology and mechanical qualities. Osteoclastic digestion of bone matrix involves extracellular release of lysosomal enzymes (Goto, Yamaza et al. 2003, Blair and Athanasou 2004, Everts, Korper et al. 2006, Henriksen, Sorensen et al. 2006), the transport of which is particularly IGF2/M6PR because IGF2/M6PR with its four M6P binding sites recognizes a wide range of glycan tags with penultimate M6Ps and playing crucial role in lysosomal enzyme transport (Song, Lasanajak et al. 2009) Thus, intact M6P binding function is probably the reason for lack of bone deformations in domain 11 loss-of-function mice. However, since plasminogen is also involved in bone remodelling (Daci, Udagawa et al. 1999, Daci, Everts et al. 2003, Kawao, Tamura et al. 2013), it is possible that preserved functions of the mutant receptor's N-terminal could also be responsible for the difference observed between two mutant mice. For instance, lack plasminogen activation in full-knock out receptor might lead to insufficient bone resorption and postaxial hexadactyly.

6.3 Mechanism of development of cardiac phenotype and lethality

Maternal transmission of *Igf2r* IGF2 loss-of-binding function results in cardiac and lung malformations both of which could lead to the death observed in the heterozygous neonates. IGF2 levels are implicated in development of both organs. Loss of IGF2 expression in paternal allele results in underdeveloped lung with thick interstium and haemorrhage (Haley, Barnes et al. 2012) Interestingly, *Igf2r*^{1565Am/+p} neonates also exhibited lung haemorrhage, suggesting that increased levels of IGF2 could also result in similar lung pathologies. I have extensively discussed the significance of IGF2 in cardiac development, previous reports of full *Igf2r* loss-of-function and current Domain 11 IGF2 loss-of-binding mice in the previous section. Although my results, in the light of the literature, suggest that the neonatal death occurs due to excess IGF2 related cardiac dysfunction, direct evidence is lacking. In order to test if lethality associated with maternal *Igf2r* disruption is due to cardiac associated IGF2 supply, I aimed to generate a cross with cardiac specific *Igf2* null mice. Multiple steps were required for

generation of the cross from *Igf2^{fl/fl} Apc^{fl/fl} Rosa 26 YFP*, *Igf2^{J1565Am/+p}* and *Nkx2-5Cre* (Fig 6.3). First, I crossed *Igf2^{fl/fl} Apc^{fl/fl} Rosa 26 YFP* with a WT mouse to segregate *APC* and floxed *Igf2* genes to produce off spring with no floxed *APC* allele. In parallel to this breeding, I also started breeding of *Igf2^{J1565Am/+p}* with *Nkx2-5Cre* mice. As a result, I managed to generate mice with the genotypes needed for the final breeding (showed with a red arrow in Fig 6.3). Survival and phenotype of the embryos resulting from the final breeding are going to be assessed to evaluate possible cardiac origin of the lethality and its IGF2 dependency and if the cross could survive to the adulthood, a viable adult mice with non-cardiac IGF2 loss-of-binding function would be a useful model to study the roles of excessive IGF2 in the rest of the body in post-natal development and adulthood such as its roles in genitourinary and cognitive development and functions, and even in carcinogenesis.

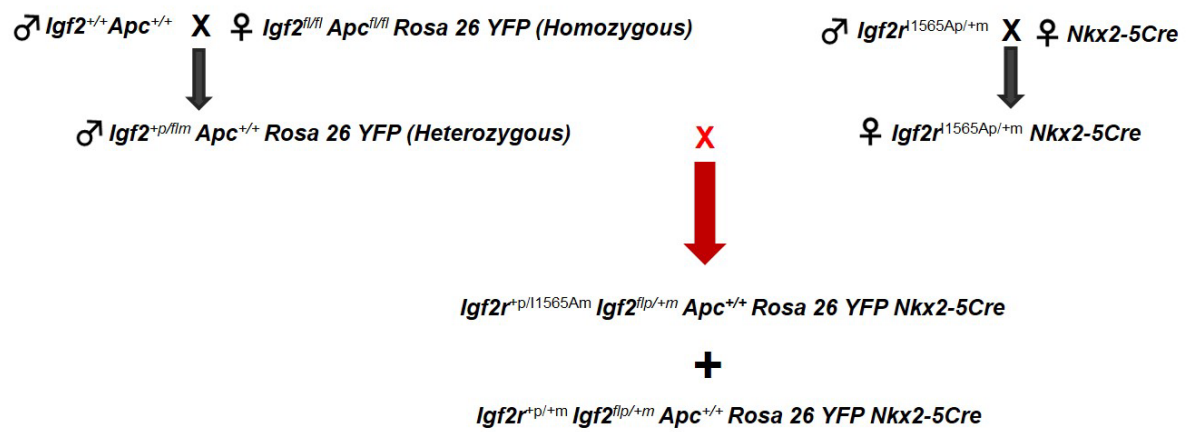


Fig 6.3: Generation of IGF2 loss-of-binding function/cardiac-specific IGF2 null mice

6.4 Conclusions

In summary, at physiological pH, domains 3, 5 and 9 function independent of each other and domain 3 is the dominant M6P binding domain. Loss-of-binding in M6P binding domains have a slight effect on IGF2 binding and no effect on plasminogen binding. Similarly, domain 11 loops do not appear to have any influence on other ligand binding domains functions (M6P and plasminogen). However, intra-domain inter-loop interactions occur in the context of the full-length receptor. These results, overall, suggest that IGF2/M6PR ligand binding domains function independent of each other.

In the context of the full length receptor, I1572A results in IGF2 loss of binding function with preserved binding for other ligands. In addition, the receptor's ectodomain sequence, particularly the ligand binding sites, is well preserved in placentalia suggesting that I1572A homologues could be used in *in vivo* studies to dissect domain 11 functions in many mammals, including mice. In my study, maternal transmission of domain 11 loss-of-binding in mice results in increased neonatal weight with early neonatal death (~70% of all neonates). The disproportional cardiomegaly and disorganised myocardium, and lung malformations in *Igf2r^{I1565Am/+p}* neonates suggest that the postnatal lethality is due to cardiorespiratory dysfunction, however, further studies are needed to prove the aetiology and the mechanism. Furthermore, the congestive cardiac failure begins as early as embryonic day 14.5. In addition, domain 11 loss-of-binding function results in vaginal atresia in adult female mice. Lastly and very intriguingly, on heterozygous maternal transmission, IGF2 loss of binding mutation only resulted in increased water content, however, homozygous transmission increased dry mass in embryos. In contrast and paradoxically, the cardiac phenotype upon maternal transmission of the mutation is reversed in homozygous mice possibly due to paralyzed MAPK pathway as result of an additional increase in IGF2 concentrations.

To conclude, these results suggest that, in the context of full-length receptor, IGF2/M6PR's ligand binding domains function independently and domain 11 bears inhibitory residues which

also seem to be involved in inter-loop interactions. Also, when IGF2 loss-of-binding mutation is introduced to the maternal *Igf2r* allele, the receptor expression from the paternal allele becomes physiologically significant with opposite cardiac and whole body effects. Lastly and most prominently, domain 11 loss-of-function is the primary reason for the most of abnormalities that is previously reported in the full knock out mice. Thus, domain 11 is the primary domain for M6P/IGF2R's regulatory functions in prenatal development.

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Supplementary: Design of the primers used in this thesis

SITE 1 (Before restriction site of BspEI)

CCTCAACTCTTCATCAAACACATAGC (in non-coding sequence)

GCTATGTGTTTGATGAAGAGTTGAGG (reverse complementary: Primer sequence)

Tm:

66.0 °C (calculator)*

72.4°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

	Hairpin	Dimer	Cross Dimer	
Primer	NO	NO		
Overlapping			-6.96	
Partially overlapping			-6.95	

* http://www.finnzymes.fi/tm_determination.html

** Tm:81.5+0.41(%GC)-675/Nucleotide number-%mismatch

SITE 2 (After restriction site of NsiI)

ATCCATCTCGTCTGCTCCAG (in coding sequence)

CTGGAGCAGACGAGATGGAT (reverse complementary: Primer sequence)

Tm:

64.9 °C (calculator)*

70.3°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

	Hairpin	Dimer	Cross Dimer	
Primer	NO	NO		
Overlapping			-4.75	
Partially overlapping			-4.52	

* http://www.finnzymes.fi/tm_determination.html

** Tm:81.5+0.41(%GC)-675/Nucleotide number-%mismatch

SITE 3 (After restriction site of Acc651)

GGATCTTTGAGCATCATGTATGTCA (in coding sequence)

TGACATACATGATGCTCAAAGATCC (reverse complementary: Primer sequence)

Tm:

66.2 °C (calculator)*

70.9°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

	Hairpin	Dimer	Cross Dimer	
Primer	NO	-5.64		
Overlapping			-4.53	
Partially overlapping			-5.87	

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \% \text{mismatch}$

Previous Primers

Overlapping primers

MfeI forward

GAGAAAGGAAACATTCAATTGTCTTATTCAGATGGTGATG

MfeI reverse

CATCACCATCTGAATAAGACAATTGAATGTTTCCTTTCTC

Tm:

75 °C (calculator)

75 °C (formula)

Non-overlapping (partially overlapping) primers

MfeI forward

AACATTCAATTGTCTTATTCAG ATGGTGATGATTGTGGTCA

MfeI reverse

CTGAATAAGACAATTGAATGTT TCCTTTCTCTTTCATGGGA

Tm:

74 °C (calculator)

77.5°C (formula)

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \% \text{mismatch}$

On restriction site of Bsp EI

TCATCGATCCGGACACTTCTCTATT (coding sequence: Primer sequence)
(Grey nucleotides does not match, but will be cleaved after amplification)

Tm:

67.89 °C (calculator)*

72.54 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
NO	-12.9	
		-5.77

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

On restriction site of Acc 65I

GACTAGCGGTACCTTTGGCAGAG (reverse complementary: Primer sequence)
CTCTGCCAAAGGTACCGCTAGTC (in coding sequence)
(Grey nucleotides does not match, but will be cleaved after amplification)

Tm:

67.21 °C (calculator)*

75.16°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
NO	-9.78	
		-5.77

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

Single site directed mutagenesis on Domain 9
(Tyrosine 1352 to Phenylalanine); Selected Type 2S restriction enzyme: Eci I

TATTATGGCGGA TATTATCAGATTGTTCCCTTCTTGTTTGAGTG
coding sequence: Primer sequence) (Forward)

Random nucleotides before and after recognition site for restriction enzyme: TACAGT and AGTGA

Recognition site: GGCGGA

Full matching nucleotides: TCAGATTGTTCCCTTCTTGTTTGAGTGG

Restriction enzyme will cut between these two nucleotides: TT

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: TTC



T_m (first cycles: non-italic part):

66.52 °C (calculator: mismatches ignored)*

67.46 °C (formula) **

T_m (whole primer):

75.84 °C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-4.53	
		No

* http://www.finnzymes.fi/tm_determination.html

** T_m: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Primer after Bst EII site on domain 11

ACCCAAGCACAGGACACCT (in coding sequence)

AGGTGTCCTGTGCTTGGGT (reverse complementary: Primer sequence)

T_m:

65.15 °C (calculator:)*

69.71 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	No	
		No

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Single site directed mutagenesis on Domain 9

(Glutamic acid 1345 to Glutamine); Selected Type 2S restriction enzyme: Eci I

TACAGTGGCGGAGTAGGAACAATCTGAAGTCTGCTTTAGAAATA

coding sequence: Primer sequence) (Forward)

Random nucleotides before and after recognition site for restriction enzyme: TACAGT and GTA

Recognition site: GCGGA

Full matching nucleotides: ACAATCTGAAGTCTGCTTTAGAAATA

Restriction enzyme will cut between these two nucleotides: TC

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: CTG



Tm (first cycles: non-italic part):

65.51 °C (calculator: mismatches ignored)*

68.92 °C (formula) **

Tm (whole primer):

75.84 °C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-4.89	
		5.47

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Primer before Acc 65I site on domain 8

GCTTCATCACTCTGACCTACAAA (in coding sequence: Primer sequence)

Tm:

62.59 °C (calculator:)*

69.98 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	No	
		-7.97

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Target Residues on Domain 3

CCTGTATTCACAGGGGAGGTTGACTG|CACCTTCTTCTTCACATGGGACACG
GGACATAAGTGTCCCCTCCAAGT|ACGTGGATGAAGAAGTGTACCCTGTGC

"|" is where Eci I (Type 2S restriction enzyme) cuts
GAG and **TAC** are the target codons on coding sequence

GAG to CAG (3.42 % codon usage), TAC to TTC (2.03 % codon usage)

Single site directed mutagenesis on Domain 3 (Forward)

(Tyrosine 456 to Phenylalanine); Selected Type 2S restriction enzyme: Eci I

TTATATTGGCGGAATTATTGACTGCACCTTCTTCTTCACATG

(coding sequence: Primer sequence) (Forward)

Random nucleotides before and after recognition site for restriction enzyme: TTATATT and ATTA

Recognition site: GGCGGA

Full matching nucleotides: TTGACTGCACCTTCTTCTTCACATG

Restriction enzyme will cut between these two nucleotides: GC

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: TTC



Tm (first cycles: non-italic part):

68.58 °C (calculator: mismatches ignored)*

68.54 °C (formula) **

Tm (whole primer):

78.31 °C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-7.05	
		6.37

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Primer after MfeI site on domain 4

TGACCACAATCATCACCATCTG (Primer sequence)

Tm:

66.22°C (calculator:)*

69.45 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	No	
		-5.37

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Target Residues on Domain 5

CCTGTATTCACAGGGGAGGTTGACTG|CACCTTACTTCTTCACATGGGACACG
GGACATAAGTGTCCCCTCCAACTG|ACGTGGATGAAGAAGTGTACCCTGTGC

CGTGTCCCATGTGAAGAAGTAGGTGCA|GTCAACCTCCCCTGTGAATACAGG
GCACAGGGTACACTTCTTCATCCAC|GTCAGTTGGAGGGGACACTTATGTCC

GTGTCCCATGTGAAGAAGTAGGTGCA|GTCAACCTCCCCTGTGAATACAGG
GCACAGGGTACACTTCTTCATCCAC|GTCAGTTGGACGGGACACTTATGTCC

Single site directed mutagenesis on Domain 3 (Reverse)

(Glutamate 451 to Glutamine); Selected Type 2S restriction enzyme: Eci I

TATATTGGCGGAATTATTTAACAGTCAACCTGCCCTGTGAATAC

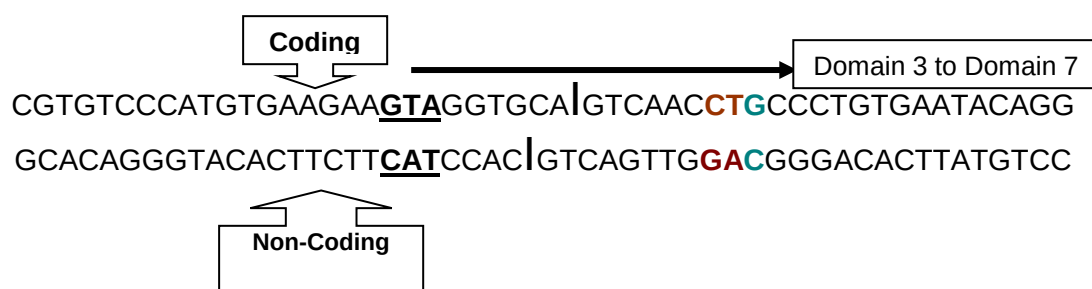
Random nucleotides before and after recognition site for restriction enzyme: TATATT and ATTATTTAA

Recognition site: GGCGGA

Full matching nucleotides: CAGTCAACCTGCCCTGTGAATAC

Restriction enzyme will cut between these two nucleotides: AG

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: CTG



Tm (first cycles: non-italic part):

69.96°C (calculator: mismatches ignored)*

69.19 °C (formula) **

Tm (whole primer):

77.75 °C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-0.59	-5.38	
		-7.36

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

Primer before Age I site

CCGTGCTGCTGATGGGTT (in coding sequence: Primer sequence)

Tm:

67.88 °C (calculator:)*

69.05 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	No	
		-7.36

* http://www.finnzymes.fi/tm_determination.html

** Tm: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

TTCCCTGAATATCAGGAAGAGGATAACTC|CACCTACAACTTCCGGTGGTACACC
AAGGGACTTATAGTCCTTCTCCTATTG|AGGTGGATGTTGAAGGCCACCATGTGG

TTCCCTGAATATCAGGAAGAGGATAACTC|CACCTTCAACTTCCGGTGGTACACC
AAGGGACTTATAGTCCTTCTCCTATTG|AGGTGGATGTTGAAGGCCACCATGTGG

GGTGTACCACCGGAAGTTGTAGGTGGALGTTATCTGTTTCCTGATATTCAGGGAA
CCACATGGTGGCCTTCAACATCCAC|CTCAATAGACAAGGACTATAAGTCCCTT

Single site directed mutagenesis on Domain 5 (Forward)
(Tyrosine 750 to Phenylalanine); Selected Type 2S restriction enzyme: Eci I

TTATATTGGCGGATATGATAACTCCACCTTCAACTTCCGG

(coding sequence: Primer sequence) (Forward)

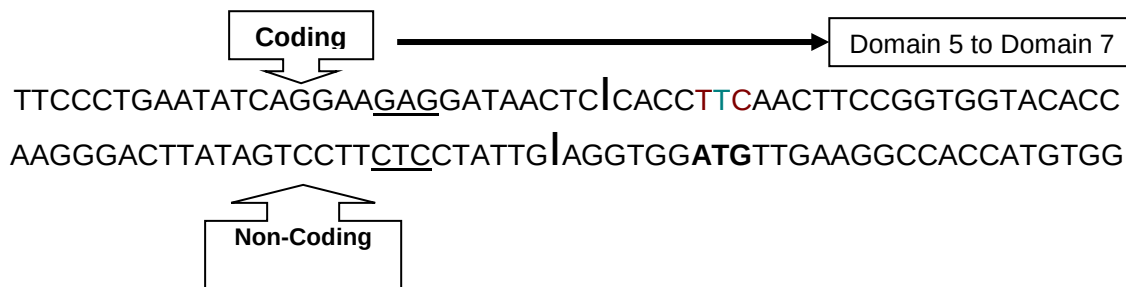
Random nucleotides before and after recognition site for restriction enzyme: TTATATT and ATTA

Recognition site: GGCGGA

Full matching nucleotides: GATAACTCCACCTTCAACTTCCGG

Restriction enzyme will cut between these two nucleotides: CC

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: TTC



Tm (first cycles: non-italic part):

67.75°C (calculator: mismatches ignored)*

69.70°C (formula) **

T_m (whole primer):
78.3 °C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-1.76	-10.75	
		-7.68

* http://www.finnzymes.fi/tm_determination.html

** T_m: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

On restriction site of Acc 65I

GACTAGCGGTACCTTTGGCAGAG (reverse complementary: Primer sequence)
CTCTGCCAAAGGTACCGCT**AG**TC (in coding sequence)
(Grey nucleotides does not match, but will be cleaved after amplification)

T_m:
67.21 °C (calculator)*
75.16°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
NO	-9.78	
		-7.68

Single site directed mutagenesis on Domain 5 (Reverse) (Glutamate 745 to Glutamine); Selected Type 2S restriction enzyme: Eci I

TATATT**GGCGGA**GTTATTACGGAGTTATC**CTG**TTCCTGATATTTCAG

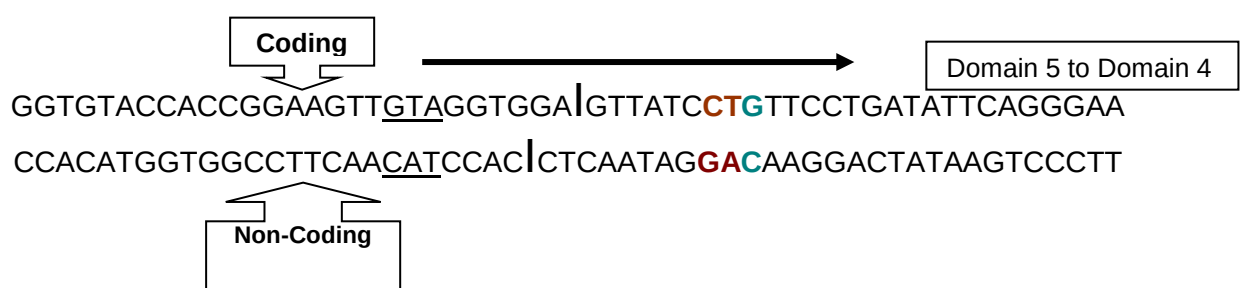
Random nucleotides before and after recognition site for restriction enzyme: TATATT and **GTTATTAC**

Recognition site: **GGCGGA**

Full matching nucleotides: GGAGTTATC**CTG**TTCCTGATATTTCAG

Restriction enzyme will cut between these two nucleotides: **AG**

Brown indicates preserved nucleotides; green indicates changed nucleotides in target codon: **CTG**



T_m (first cycles: non-italic part):
 64.05°C (calculator: mismatches ignored)*
 69.04°C (formula) **

T_m (whole primer):
 76.97°C (calculator)*

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-0.93	-6.13	
		-8.19

* http://www.finnzymes.fi/tm_determination.html

** T_m: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Primer on MfeI site on domain 4 Forward

GAGAAAGGAAACATTCAATTGTCTTATTCAGATGGTGATG (in coding sequence: Primer sequence)

T_m:
 75.23°C (calculator:)*
 78.97 °C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-0.13	-9.27	
		-8.19

* http://www.finnzymes.fi/tm_determination.html

** T_m: 81.5+0.41(%GC)-675/Nucleotide number-%mismatch

Single site directed mutagenesis on Domain 11 to disrupt IGF2

(Phenylalanine 1567 to Alanine in addition to I1572A); Selected Type 2S restriction enzyme: Eci I

GGGGGCCTGCTTTGGACAGACCAGGCTAGCGTGGGCAAGGCCAACAAGAGGCTGAGATACGT
 CCCCCGGACGAAACCTGTCTGGTCCCGATCGCACCCGTTCCGGTTGTTCTCCGACTCTATGCA

TTT: Target Phenylalanine residue

GCT: Previously mutated, I1572A

GCTAGCG: Restriction site for Nhe I

Alanine codons: GCX (GCA, GCU, GCG, GCC)

Codon preference in Homo sapiens: GCU 18.4, **GCC 27.7**, GCA 15.8, GCG 7.4

New mutated sequence

GGGGGCCTGC**GCC**GGACAGACCAGG**GCT**AGCGTGGGCAAGGCCAACAAGAGGCTGAGATACGT
CCCCCGGACG**CGG**CCTGTCTGGTCC**CGA**TCGCACCCGTTCCGGTTGTTCTCCGACTCTATGCA

StuI site

GATCAGTTTCGTGTGC**AGGCCT**GAGGCCGGGCCAACCAATAGGCCCATGCTCATCTCCCTGGACAAGCAGACATGCACTC
CTAGTCAAAGCACACG**TCCGGA**CTCCGGCCCGTTGGTTATCCGGGTACGAGTAGAGGGACCTGTTCTGTCTGTACGTGAG

BstEII site

CTTGCCTGGCCACAGCCACAGCCTGTCCCATGAAGAGCAACGAGCATGATGACTGCCA**GGTCACC**AACCCAAGCACAGG
GAAACGGACCGGTGTCGGTGTCTGGACAGGGTACTTCTCGTTGCTCGTACTACTGACGGT**CCAGTGG**TTGGGTTCTGTGTCC

StuI site-Reverse

CTCACGTACAGACGAACAGGTCCCTCTACTCGTACCCGGATAACCAACCGGGCCGGAG**TCCGGA**CGTGTGCTTTGACTAG
GAGTGCATGTCTGCTTGTCCAGGGAGATGAGCATGGGCCTATTGGTTGGCCCGCCTC**AGGCCT**GCACACGAACTGATC

Second Pair

Primer prior to NheI site on domain 11 Forward

GCTTTGGACAGACCAGGGCT (in coding sequence: Primer sequence)

Tm:

68.09°C (calculator)*

72.35°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
0.62	No	
		-6.02

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675 / \text{Nucleotide number} - \% \text{mismatch}$

Primer after to Stu I site on domain 11 Reverse

CAGGGAGATGAGCATGGG (reverse in non-coding sequence: Primer sequence)

Tm:
67.64°C (calculator)*
71.31°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-6.38	
		-6.02

* http://www.finnzymes.fi/tm_determination.html

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \% \text{mismatch}$

TGCATAGAGTCGGAGAACAACCGGAACGGGTGCGATCGGGACCAGACAGG**CCG**CGTCCGGGGG
ACGTATCTCAGCCTCTTGTTGGCCTTGCCACGCTAGCCCTGGTCTGTCCG**GCG**CAGGCCCCC

GAATGAAACTGCCCTCCTGGCGTGGGGGCCTGC**GCC**GGACAGACCAGGGCTAGCGTGGGCAAGGCCAACAAGAGGCTGAG
TTACTTTTGACGGGAGGACCGCACCCCGGACG**CGG**CCTGTCTGGTCCCGATCGCACCCGTTCCGGTTGTTCTCCGACTC

TTATATTGGCGGATATAAGGGGGCCTGC**GCC**GGACAGACCA

TTATATTGGCGGATATAATGGGGCCTGC**GCC**GGACAGACCA

Type 2 BspQI introducing mutation

GCTCTTC**GCC**GGACAGACCAGGGCTAGC

CGAGAAG**CGG**CCTGTCTGGTCCCGATCG

After STUI

CGAACAGGTCCCTCTACTCGTACC

Mutagenesis strategy to introduce F1567A and K1631E

TTT: Target Phenylalanine residue

GCT: Previously mutated, I1572A

F1567A

Alanine codons: GCX (GCA, GCU, GCG, GCC)

Codon preference in Homo sapiens: GCU 18.4, **GCC 27.7**, GCA 15.8, GCG 7.4

K1631E

Glutamic acid codon: GAA, GAG

Codon preference in Homo sapiens: GAA 29.0, **GAG 39.6**

Type 2 BspQI introducing mutation

GCTCTTCGCCGGACAGACCAGGGCTA (F1567A)

Sequence of the region coding Lys1631

ORIGINAL

GGGCCAACCAATAGGCCCATGCTCATCTCCCTGGAC**AAG**CAGACATGCACTCTCTTCTCTCCTGGCACACGCCGCTGGCCTGCGAGCAA
CCCGGTTGGTTATCCGGGTACGAGTAGAGGGACCTG**TTC**GTCTGTACGTGAGAGAAGAAGAGGACCGTGTGCGGCGACCGGACGCTCGTT

ALTERED

CCAACCAATAGGCCCATGCTCATCTCCCTGGAC**GAIG**CAGACATGCACTCTCTTCTCTCCTGGCACACGCCGCTGGCCTGCGAGCAA
GGTTGGTTATCCGGGTACGAGTAGAGGGACCTG**CTC**GTCTGTACGTGAGAGAAGAAGAGGACCGTGTGCGGCGACCGGACGCTCGT

REVERSE-ALTERED

GTCGCCGCACACGGTCTCTTCTCTCTCACGTACAGAC**GIAG**CAGGTCCCTCTACTCGTACCCGGATAACCAACCGGGCCGGAGTCC
AGCGGCGTGTGCCAGGAGAAGAAGAGAGTGCATGTCTG**CTC**GTCTTICAGGGAGATGAGCATGGGCCTATTGGTTGGCCCGGCCTCAGG

COMPLEMENTARY-ALTERED

GGTTGGTTATCCGGGTACGAGTAGAGGGACCTG**CTC**GTCTGTACGTGAGAGAAGAAGAGGACCGTGTGCGGCGACCGGACGCTCGTT
CAACCAATAGGCCCATGCTCATCTCCCTGGAC**GAIG**CAGACATGCACTCTCTTCTCTCCTGGCACACGCCGCTGGCCTGCGAGCAA

Single site directed mutagenesis on 11 (Forward)
(Phenylalanine 1567 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATT**GCTCTTCGCC**GGACAGACCAGGGCTA

Tm:

50.39°C (calculator)*

65.14°C (formula) **

Tm (whole primer):

82.36°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-2.71		
	-9.75	-8.27

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675 / \text{Nucleotide number} - \% \text{mismatch}$

Single site directed mutagenesis on 11 (Reverse)
(Lysine 1631 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATAGCTCTTCGAGCAGGTCCCTCTACTCG

Tm:
 46.95°C (calculator)*
 65.14°C (formula) **

Tm (whole primer):
 80.72°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-2.88	-7.78	
		-8.27

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

Primers to amplify first fragment extending from Acc65I to F1567A

Single site directed mutagenesis on 11 (Reverse)
(Phenylalanine 1567 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATAGCTCTTCACGGCGCAGGCCCCAC

Tm:
 50.05°C (calculator)*
 59.28°C (formula) **

Tm (whole primer):
 83.50°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-1.71		
	-9.89	-4.55

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

Primer before Acc65I (Forward)

GACTTCATCACTCTGACCTACAAAG

Tm:

54.36°C (calculator)*

69.78°C (formula) **

Tm (whole primer):

75.52°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-4.54	
		-4,55

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \% \text{mismatch}$

After BstBI

ORIGINAL

GTTCGAATGGGAGACTCCTGTCGTCTGTCCTGATGAAGTGAGGATGGATGG

ORIGINAL-REVERSE

ORIGINAL-REVERSE COMPLEMENT

CCATCCATCCTCACTTCATCAGGACAGACGACAGGAGTCTCCCATTCGAAC

Primers to amplify first fragment extending from K1631A to after BstBI

Single site directed mutagenesis on 11 (Forward)

(Lysine 1631 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATAGCTCTTC~~TGCTC~~GTCTGTACGTGAGAGAAGAAGAG

Tm:
 54.55°C (calculator)*
 71.83°C (formula) **

Tm (whole primer):
 83.05°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-1.89		
	-10.89	-5.12

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

Primer after BstBI (Reverse)

GGAGTGAAGTAGTCCTGTCT

Tm:
 49.04°C (calculator)*
 68.25°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-0.44	No	
		-5.12

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \%mismatch$

PRIMERS FOR K1631E

GCT: Previously mutated, I1572A

F1567A

Alanine codons: GCX (GCA, GCU, GCG, GCC)

Codon preference in Homo sapiens: GCU 18.4, **GCC 27.7**, GCA 15.8, GCG 7.4

K1631E

Glutamic acid codon: GAA, GAG

Codon preference in Homo sapiens: GAA 29.0, **GAG 39.6**

Single site directed mutagenesis on 11 (Reverse)
(Lysine 1631 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATAGCTCTTCGAGCAGGTCCCTCTACTCG

Tm:
 46.95°C (calculator)*
 65.14°C (formula) **

Tm (whole primer):
 80.72°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-2.88	-7.78	
		-8.27

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675 / \text{Nucleotide number} - \% \text{mismatch}$

Single site directed mutagenesis on 11 (Forward)
(Lysine 1631 to Alanine); Selected Type 2S restriction enzyme: BspQI

TTATATAGCTCTTCTGCTCGTCTGTACGTGAGAGAAGAAGAG

Tm:
 54.55°C (calculator)*
 71.83°C (formula) **

Tm (whole primer):
 83.05°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-1.89		
	-10.89	-5.12

Primers to amplify first fragment between Acc65I and F1567A

PRIMERS DESIGNED AT JANUARY 2014

Introducing restriction site to SphI for further mutation introduction to Domain 11 by synthetic DNA fragments.

```
GAATGTTCCGTGAGGAATGGAAGCTCTATTGTTGACTTGTCTCCCCTTATTCATCGCACTGGTGGTTATGAG
GAGTGCTCTGTTAGAAACGGCAGTTCAATCGTCGATTTATCACCTCTAATACACCGTACCGGCGGCTACGAA
      TCGGTA      GGT   TCGATAGTA      TCCCCACTGATC   CGAACGGGAGGA
      TCAGTC      GGG   TCC   GTG      TCGCCGCTC      CGGACAGGGGGG
```

SphI is the restriction enzyme

Original Sequence

```
GAATGTTCCGTGAGGAATGGAAGCTCTATTGTTGACTTGTCTCCCCTTATTCATCGCACTGGTGGTTATGAGGCTTATGATGAGAGT
CTTACAAGGCACTCCTTACCTTCGAGATAACAACCTGAACAGAGGGGAATAAGTAGCGTGACCACCAATACTCCGAATACTACTCTCA
```

Final Sequence

```
GAATGTTCCGTGAGGAATGGAAGCTCTATTGTTGACTTGTCTCCCCTTATTCATCGTACGGGTGGTTATGAGGCTTATGATGAGAGT
CTTACAAGGCACTCCTTACCTTCGAGATAACAACCTGAACAGAGGGGAATAAGTAGCATGCCACCAATACTCCGAATACTACTCTCA
```

FIRST SET: FORWARD SET

Primer on SphI Site (Forward) Name: SphI_for.Scan

CTTATTCATCGTACGGGTGGTTAT

Tm:

60.08°C (calculator)*

62.23°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-10.87	
		-5.47

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675 / \text{Nucleotide number} - \% \text{mismatch}$

Primer after BstBI (Reverse)

GGAGTGAAGTAGTCCTGTCT

Tm:

49.04°C (calculator)*

64.97°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
-0.44	No	
		-5.47

Final Sequence

GAATGTTCCGTGAGGAATGGAAGCTCTATTGTTGACTTGTCTCCCTTATTCATCGTACGGGTGGTTATGAGGCTTATGATGAGAGT
CTTACAAGGCACTCCTTACCTTCGAGATAACAACCTGAACAGAGGGGAATAAGTAGCATCGCCACCAATACTCCGAATACTACTCTCA

Final Sequence (Reverse)

TGAGAGTAGTATTCGGAGTATTGGTGGCATGCTACTTATTCCTCTGTTTCAGTTGTTATCTCGAAGGTAAGGAGTGCCTTGTAAG
ACTCTCATCATAAGCCTCATAACCACCCGTACGATGAATAAGGGGAGACAAGTCAACAATAGAGCTTCCATTCTCACGGAACATTC

Primer before Acc65I (Forward)

GACTTCATCACTCTGACCTACAAA

Tm:

56.11°C (calculator)*

62.3°C (formula) **

Tm (whole primer, without taking mismatches in account):

70.6°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	No	
		-8.52

* <http://www6.appliedbiosystems.com/support/techtools/calc/>

** Tm: $81.5 + 0.41(\%GC) - 675/\text{Nucleotide number} - \% \text{mismatch}$

Primer on SphI Site (Reverse) Name: SphI_rev.Scan

CATAACCACCCGTACGATGAATAA

Tm:

56.11°C (calculator)*

62.23°C (formula) **

Tm (whole primer, without taking mismatches in account):

70.6°C (formula) **

Net Primer programme comparison results ΔG (kcal/mol)

Hairpin	Dimer	Cross Dimer
No	-10.87	
		-8.52

Primer after Spfl (Reverse)**Sequence after the restriction site**

GAGAGTTGAGATGGAAGGCAGAGAAGGCTCCTTCTTCCCAGTCCTGGATCACCTTCTCCCTAAAG

Reverse complement of the sequence

TCTTTAGGGAGAAGGTGATCCAGGACTGGGAAGAAGGAGCCTTCTCTGCCTTCCATCTCAACTCT

Spfl (reverse primer) TTACTGGGAAGAAGGAGCC