Homeodomain transcription factors Six3 and Six6 have distinct protein binding characteristics

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Abstract

The goal of this study was to characterize Six3 and Six6 proteins interactions and to better understand their functions.

The Six class proteins are found in a wide variety of animals ranging from primitive invertebrate species to mammals. They belong to the class of evolutionarily conserved homeodomain-containing transcription factors, which regulate the transcription process of key genes during animal development. Mammals contain 6 Six proteins divided into three groups (Six1/2, Six3/6, and Six4/5) while fish contain more Six proteins due to extra whole genome duplication events occurred in the teleost lineage. The proteins belonging to the Six3/Six6 group are crucial for early embryonic specification of forebrain and eyes and for retinal cell proliferation and neurogenesis. However, mechanistic aspects of their actions and the functional divergence in this group have not been studied well.

Six3 proteins have been shown to act in forebrain and eye development both as transcriptional activators and repressors and to function as regulators of cell proliferation through interactions with other proteins such as the cell cycle inhibitor Geminin. Six6 proteins have been shown to be less involved in brain development and the implications of their interaction with Geminin have not been studied to date.

In order to achieve the goal of this study, the interactions of human SIX3 and SIX6 proteins to GEMININ were investigated by docking analysis, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and bimolecular fluorescence complementation (BiFC). Docking analyses showed a different interphase for the SIX3-GEMININ complex as compared to the SIX6-GEMININ complex. To verify these results, SPR was used. We observed a selective binding of the SIX domain of SIX3 (SIX3SD) relative to the full-length SIX3 protein, implying that SIX3SD is the main structure involved in SIX3 binding to GEMININ. Furthermore, SIX3SD showed

a 10-fold higher binding affinity to GEMININ compared with the SIX domain of SIX6 (SIX6SD). ITC measurements of SIX3-GEMININ interaction showed a binding ratio SIX3 to GEMININ of 1:2 and revealed, in addition, that the interaction does not occur through the coiled-coil region of GEMININ. The interaction of full-length SIX3 and SIX6 proteins to GEMININ was confirmed through a strong fluorescence signal by means of BiFC in mammalian cells. Conversely, the BiFC signal was very low for SIX3SD or SIX6SD, implying a lack of interaction of the SIX domains with GEMININ in living cells.

The zebrafish Six3 proteins Six3a and Six3b have not been characterized at the protein level although previous studies have shown spatiotemporal differences in mRNA expression patterns. We compared the predicted 3D-structures of Six3a and Six3b and their electrostatic maps and detected differences both in predicted structures and in the charge distributions between these two paralogous. Moreover, electrophoretic mobility shift assay (EMSA) showed that the full-length Six3b protein does not bind to the Six3a DNA recognition sequence. The binding affinity of Six3a and Six3b to Geminin was further measured by SPR. The SPR measurements showed that Six3b has a 3-fold higher affinity for Geminin than Six3a. In addition, the formation of strong heterodimer between Six3a and Six3b was detected in SPR. The BiFC assay in mammalian cells confirmed SPR binding results. Moreover, BiFC revealed that Six3b formed homodimers, whereas Six3a did not. Subcellular localization patterns for these two paralogous Six3 proteins were found to be different. Six3a was localized more to nucleus and nucleoli-like structures, whereas Six3b was primarily found in the cytoplasm, especially in Golgi-like structures.

As a concluding remark, the results presented in this study show that, although Six3 and Six6 proteins share very high sequence identities and may assume similar structures, they have different abilities in protein interactions and hence may assume different cellular functions.

Selected Abbvreviations

Otx2	Orthodenticle homeobox 2
Hesx1	Homeobox gene expressed in ES cells
Six3	SIX homeobox 3
Six6	SIX homeobox 6
BMPs	Bone morphogenetic proteins
FGF	Fibroblast growth factor family
Wnt	Wingless-type MMTV integration sites
Tgf-β	Transforming growth factor beta
Shh	Sonic hedgehog
Cdt1	Cdc10-dependent transcript 1
Pre-RC	Pre-replication complex
APC	Anaphase promoting complex
ORC	Origin-recognition complex
MCM	Mini-chromosome maintenance
RPCs	Retinal progenitor cells
ANP	Anterior neural plate
HD	Homeodomain
SD	Six domain
TLE1	Transducin-like enhancer protein 1
AES	Amino-terminal enhancer of split
SPR	Surface plasmon resonance
ITC	Isothermal titration calorimetry
BiFC	Bimolecular Fluorescence Complementation
EMSA	Electrophoretic mobility shift assay

1. Introduction

In the present work the homeodomain transcription factors Six3 and Six6 involved in eye and forebrain development were studied. The Introduction is divided as follows:

- Involvement of transcription factors in early eye development
- Role of Geminin in the initiation of DNA replication and differentiation
- The Six-class homeodomain proteins
- Expression patterns and functions of the Six3/Six6 group
- Six3- and Six6- interacting proteins.

1.1 The involvement of transcription factors in early eye development

Transcription factors are regulatory proteins that activate or inhibit transcription of DNA by binding to specific DNA sequences or by interacting with other DNA-binding proteins. A defining feature of transcription factors is that they contain at least one DNA-binding domain per protein.

There are two classes of transcription factors: general transcription factors and sequence-specific transcription factors. General transcription factors bind to a core promoter close to transcription initiation site and together with an RNA polymerase, assemble in an ordered way to form the pre-initiation complex (Conaway and Conaway 1997, Green 2000, Muller 2001). Sequence-specific transcription factors control the promoter by binding to regulatory DNA elements at varying distances upstream or downstream from the transcription initiation site. These transcription factors are critical for the expression of genes at the right time, at the right place and in the right quantity. Structurally, transcription factors contain the following domains: a DNA-binding domain, a trans-activating domain (includes binding sites for other proteins) and optionally a signal-sensing domain. The most common classification of

transcription factors is based on the structure of their DNA-binding domain (Latchman 1997).

Multicellular organisms evolve from a single cell (the zygote) through the process called development. Many transcription factors are involved in development. Responding to stimuli, transcription factors activate or repress the transcription of the corresponding developmental genes, which allows changes in cell morphology, or in cell fate determination and differentiation.

The retina is composed of a limited number of neuronal and non-neuronal cells and is used as a model of the brain. In vertebrates, the eye primordia starts to develop during gastrulation by the determination of the eye field in the anterior neuroectoderm under the control of some transcription factors that pattern the anterior neural plate simultaneously with the patterning of the prospective forebrain (Chow and Lang 2001) (Fig. 1). The eye field is established in three main phases: neural induction, anterior-posterior division of the neural plate and specification of the eye field (Sinn and Wittbrodt 2013). Eye development starts with the determination of the eye field. Signals that emanate from the midline split the initially single retinal anlage into two retinal primordia. These signals also establish a proximodistal pattern in the primordia.

Neural induction is triggered by members of the fibroblast growth factor family (FgF), when Wnt-signalling is suppressed (Niehrs 1999, Wilson, Graziano et al. 2000). Neural induction is assumed to take place when *Otx2* expression starts to be restricted to the anterior region of the epiblast. A few hours later in all vertebrates expression of anterior neural markers such as *Hesx1* and *Six3* becomes visible. Once the anterior neural plate has been induced (*Hesx1* and *Six3* are expressed) it is important to prevent its posteriorization. The establishment of a neural fate requires the presence of different bone morphogenetic proteins (BMPs) antagonists such as Follistatin, Noggin, Chordin and Cerberus (Bouwmeester, Kim et al. 1996, Niehrs 2001). The anterior

neural plate is further subdivided by a graded Wnt activity leading to eye anlage, telencephalon and diencephalon (Wilson and Houart 2004).

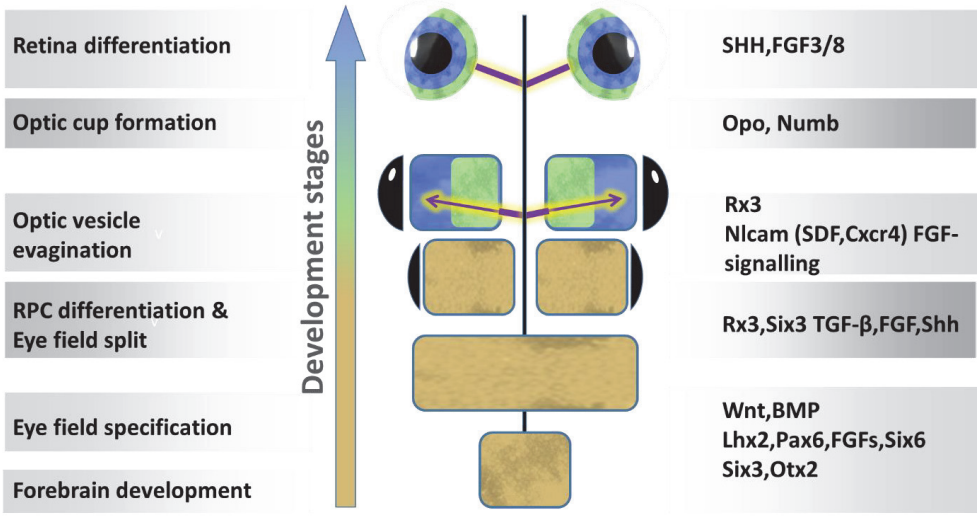

Figure 1. Christmas tree scheme of vertebrate eye development. The tissue from which the neuroectodermal part of the eye will form (retina, optic stalk) is shown in brown at the lower part of the image. Eye development starts at the end of gastrulation with the determination of the eye field; tissue that will give rise to the lens is shown in black. Concomitant with the neurulation process, optic vesicles undergo the first morphogenetic transition: they evaginate and become sub-patterned into optic stalk (violet), pigmented retinal epithelium (green) and prospective neuroretina (blue). In the subsequent second morphogenetic transition, the formation of the optic cup, retinal differentiation triggered by a FGF signalling centre, spreads over the entire retina. The lens (black) forms from surface ectodermal derivatives. Further details are presented in this chapter (Adapted from Wittbrodt et al, 2002, and Sinn and Wittbrodt, 2013).

The development of the optic vesicles (Fig. 1) begins when the Wnt activity in the eye anlage is repressed (Sinn and Wittbrodt 2013). This is partly mediated through the transcription factor Six3, which is activated in regions of low Wnt activity and supresses the Wnt signalling (Wilson and Houart 2004). Six3 is the orthologous of the *Drosophila* gene, *Optix* (Oliver, Mailhos et al. 1995, Loosli, Koster et al. 1998, Toy, Yang et al. 1998) and a key factor in early eye field specification (Lagutin, Zhu et al. 2003, Liu, Lagutin et al. 2010).

Pax6 is a homeodomain transcription factor that has been defined as the master control gene for eye development (Gehring 1996). However, recent studies have shown that eyes did not form in the absence of Six3. On the contrary, Pax6 mutant mice or rats initially developed eyes, but failed to maintain (Sinn and Wittbrodt 2013). Thu, it seems that seems that Six3, as an evolutionary conserved anterior neural plate and eye determinant (Sinigaglia, Busengdal et al. 2013), has taken the role of Pax6 as the master control gene for eye development.

In optic cup morphogenesis and photoreceptor specification the presence of the retinal homeobox transcription factors (Rx) is crucial. Rx genes are involved in proliferation control within the eye field facilitated through the regulation by Six3/Six6 (Del Bene and Wittbrodt 2005). The repression of the transcription factor Otx2 is also required for the formation of retinal identity. Otx2 is required for the formation of the eye field specific transcription factor network formed by Six3, Rx3 and Pax6.

After retinal identity is established by the overlapping expression in the centrally positioned eye field of the Six3, Rx3 and Pax6, secreted factors of the Tgf-β-, FGF and SHH-families are emanating from the axial mesoderm to split the eye anlage into two bilateral symmetric retinal primordia. If this process fails one centrally positioned (cyclopic) eye is formed. Interestingly, the graded response to SHH is morphologically mimicked by a graded loss of Six3, revealing the role of Six3 in proximo-distal patterning of the eye primordia. The repression of the Six3 activity in a complete knock-down results in the complete absence of eye and forebrain (Sinn and Wittbrodt, 2013).

Six3 activity is required not only in eye field specification but also later in eye formation by balancing proliferation and differentiation. Six3 and its paralogous Six6 function as transcriptional repressors though interactions with co-repressors of the Groucho family (Kobayashi, Nishikawa et al. 2001, Lopez-Rios, Tessmar et al. 2003). Six6, in association with Dach co-repressors, also stimulates proliferation by repressing the transcription of the cell cycle inhibitor $p27^{kip1}$ (Li, Perissi et al. 2002).

In addition, Six3 directly interacts with the DNA replication inhibitor Geminin and promotes proliferation by efficiently competing with Cdt1 on binding to Geminin (Del Bene, Tessmar-Raible et al. 2004). By binding to Geminin, Six3 stops an inhibitor and allows Cdt1 to bind to the pre-initiation complex and thus permits the licensing of the origins for the G1/S transition (Li and Rosenfeld 2004). Six3 initially stimulates proliferation during the initiation of retinal identity, and later during retinal differentiation it promotes RPCs to adopt their terminal fate as retinal neurons or glia (Sinn and Wittbrodt 2013).

1.2 Role of Geminin in the initiation of DNA replication and differentiation

Geminin could work as a master regulator of DNA replication in the cell cycle of metazoans by ensuring that DNA is replicated only once during S phase (Ballabeni, Zamponi et al. 2013).

In eukaryotic cells DNA replication occurs in the S phase of cell cycle which is flanked by two periods, G1 and G2, in which there is no replication or cell division. Cdc10-dependent transcript 1 (Cdt1) protein is essential for the formation of pre-replication complexes (pre-RCs) (Maiorano, Moreau et al. 2000, Nishitani, Lygerou et al. 2000). Its levels fluctuate during the cell cycle: it is high in G1 phase (allows pre-RC formation), low in S phase (prevents pre-RC formation and reinitiation), and then high again in G2 (Nishitani, Lygerou et al. 2000, Nishitani, Taraviras et al. 2001, Ballabeni, Melixetian et al. 2004). Geminin is absent during G1 phase, accumulates during S, G2 and M phases, and disappears at the metaphase-anaphase transition targeted for degradation by the anaphase-promoting complex (APC) (McGarry and Kirschner 1998). These expression profiles suggest that Geminin and Cdt1 may co-exist during the M/G1 transition when Cdt1 starts to accumulate and then at the G1/S transition when Geminin starts to accumulate again (Caillat and Perrakis 2012).

Geminin controls the Cdt1 levels. The binding of Geminin on Cdt1 inhibits MCM (mini chromosome maintenance) loading (McGarry and Kirschner 1998, Wohlschlegel, Dwyer et al. 2000) and affects histone acetylation (Miotto and Struhl 2010) and deacetylation (Wong, Glozak et al. 2010). Geminin promotes also the accumulation of Cdt1 during mitosis. In metaphase Geminin is degraded via APC leading to the activation of Cdt1 in early G1 for pre-RC formation (Ballabeni, Zamponi et al. 2013).

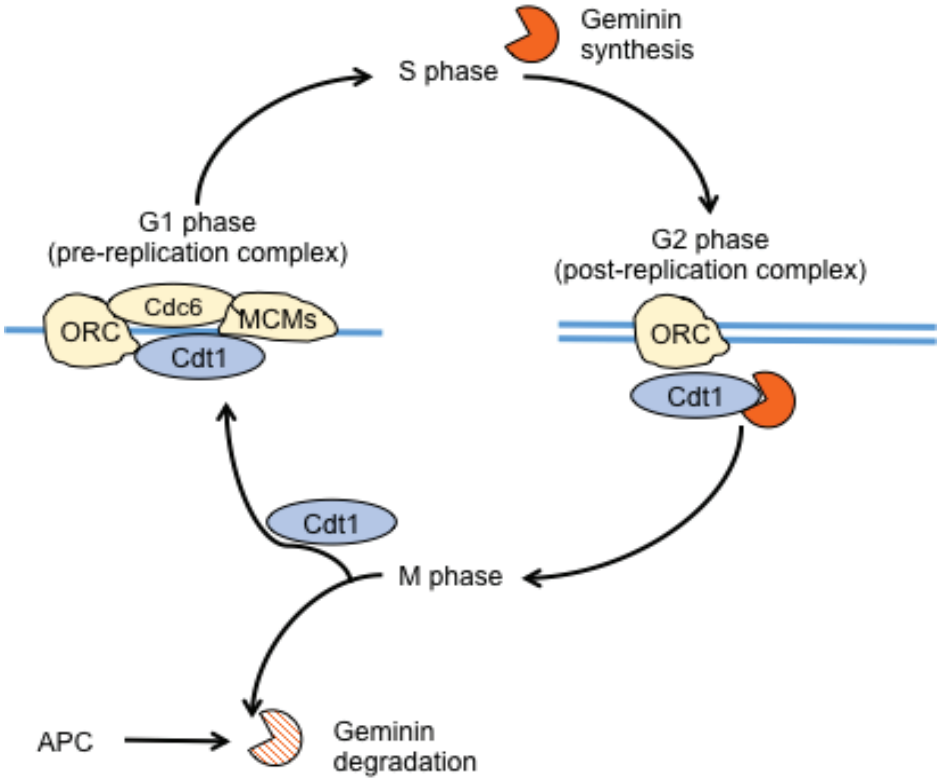

Figure 2. Model illustrating how Cdt1 and Geminin limit DNA replication to exactly one round per cell cycle. The origin-recognition complex (ORC) remains bound throughout the cell cycle. During mitosis Cdt1 is sequestered by geminin; upon exit from metaphase, geminin is degraded, releasing Cdt1. Cdt1 and Cdc6 bind to DNA, allowing the mini-chromosome maintenance (MCM) complex to bind to DNA during G1 phase, thereby 'licencing' DNA for a single round of replication. Newly synthesized geminin binds to Cdt1 during S, G2 and M phases, preventing re-licencing of DNA within the same cell cycle. (Adapted from http://www.nature.com/ncb/journal/v3/n2/pdf/ncb0201_e49.pdf).

The Cdt1-Geminin complex can switch between a licensing permissive heterotrimer to a licensing inhibitory heterohexamer state depending on the concentration of Geminin (Lutzmann, Maiorano et al. 2006, De Marco, Gillespie et al. 2009).

Geminin has also a role in cell differentiation (Seo and Kroll 2006). Degradation of Geminin switches the hematopoietic stem cells from an undifferentiated to a proliferative and differentiated state (Ohtsubo, Yasunaga et al. 2008, Ohno, Yasunaga et al. 2010). Geminin maintains also chromatin in an accessible and hyperacetylated state promoting neural fate acquisition (Yellajoshyula, Patterson et al. 2011).

Geminin can interact with several homeodomain transcription factors such as Six3 (Del Bene, Tessmar-Raible et al. 2004) and Hox proteins (Luo, Yang et al. 2004, Zhou, Liu et al. 2012, Zhou, Liu et al. 2014). Geminin also interacts with other proteins including the SWI/SNF remodelling complex catalytic subunit Brg1 (Seo, Herr et al. 2005), the coiled-coil proteins ERNI and BERT (Papanayotou, Mey et al. 2008) and the Geminin-related protein Idas (Pefani, Dimaki et al. 2011, Caillat, Pefani et al. 2013).

1.3 The Six-class homeodomain proteins

1.3.1. Phylogeny of the Six-class proteins

The eye regulatory gene *sine oculis* (*so*) in *Drosophila* (Cheyette, Green et al. 1994) was the first isolated *Six* gene. Two additional *so*-like genes, *optix* (also known as *D-six3)* and *D-six4*, were identified and subcloned subsequently (Toy, Yang et al. 1998, Seo, Curtiss et al. 1999).

The *Six* genes are present in many species ranging from simple invertebrate to humans (Serikaku and O'Tousa 1994, Oliver, Mailhos et al. 1995, Bovolenta, Mallamaci et al. 1998, Loosli, Koster et al. 1998, Seo, Drivenes et al. 1998, Zhou, Hollemann et al. 2000), suggesting that they originate very early in evolution (Pineda, Gonzalez et al.

2000, Dozier, Kagoshima et al. 2001, Bebenek, Gates et al. 2004, Stierwald, Yanze et al. 2004, Hoshiyama, Iwabe et al. 2007, Sinigaglia, Busengdal et al. 2013).

The three *Six* genes in *Drosophila* have probably arisen through the duplication of an ancestral *Six* gene prior to the evolution of Bilateria around 500 million years ago (Seo, Curtiss et al. 1999). Due to the whole genome duplication event occurred in the gnathostomes, genetic diploid vertebrates have two members per each *Drosophila Six* gene (Dineva G.K., 2013). Based on gene structures and expression patterns *Six* genes are classified into three groups: Six1/2, Six3/6 and Six4/5, with corresponding *Drosophila* genes *so*, *optix/D-six3, D-six4*, respectively (Seo, Curtiss et al. 1999). Fish contain more *Six* genes because of extra whole genome duplications and in zebrafish there are a total of 13 *Six* genes (Dineva G.K., 2013).

1.3.2. Structure of Six proteins

Six proteins have two characteristic evolutionarily conserved domains: a Six-type homeodomain (HD) and a Six domain (SD). The HD is 60 amino acids in length and contains 3 α helices forming a helix-turn-helix motif (Qian, Billeter et al. 1989). Comparisons of various HDs for sequence similarities and the distinctiveness of several critical residues suggested grouping of Six proteins in the subgroup of Otd (Orthodenticle) in the Prd (Paired) class (Kumar 2009).The Six-type HD also belongs to the K50 class, in which the DNA-contacting asparagine at position 50 is replaced by a lysine (Ades and Sauer 1994).

The Six-type homeodomains differ from the typical HDs for they lack a characteristic arginine at position 5 and a glutamine at position 12 in helix 1. The arginine residue is mainly responsible for the binding at the TAAT core sequence. Both Arg-5 and Glu-12 are replaced by serine in Six1/2 and threonine in Six3/6, respectively. In Six4/5 valine replaces arginine and serine replaces glutamic acid. Reflecting these, different members of the Six proteins bind different DNA sequences (Kawakami, Sato et al.

2000, Zhu, Dyer et al. 2002, Berger, Badis et al. 2008, Suh, Ellingsen et al. 2010). The number of positive charges on the N-terminal region of HD is important for proper HD function and the net charge of +3 or +4 in the seven amino acid region gives the best affinity (Palena, Tron et al. 2001). However, for the Six proteins, the net charges for this region is either negative or zero (Both Six1/2 and Six4/5 have a net charge of -2, while in Six3/6 it is zero). This implicates that the Six-type HDs have low intrinsic sequence specificity and therefore the DNA-binding specificity should be partially provided by their binding partners, either by in cooperation with the Six domain (SD), or by extension into the C-terminal region (Harris, Winchester et al. 2000, Kawakami, Sato et al. 2000, Weasner, Salzer et al. 2007, Hu, Mamedova et al. 2008, Patrick, Cabrera et al. 2013).

The Six domain is adjacently situated at the N-terminus and consists of about 115-119 amino acids which forms 6 α helices (Patrick, Cabrera et al. 2013). This domain plays an important role in protein-protein interactions (Kawakami, Sato et al. 2000, Christensen, Patrick et al. 2008, Kumar 2009, Patrick, Cabrera et al. 2013).

Both the N- and C-terminal region of Six proteins have variable length and are less conserved and unstructured. The C-terminal region of Six2, Six4 and Six5 contains an activation domain, thus these proteins are able to activate transcription independent of cofactors (Fig. 3). Also, the C-terminal region in Six1 mediates cell cycle-specific degradation of the protein (Christensen, Patrick et al. 2008). For the Six3/Six6 group, the first 7-14 amino acids of the C-terminal segment serve as an extension of the HD and modulate DNA binding (Weasner, Salzer et al. 2007, Hu, Mamedova et al. 2008).

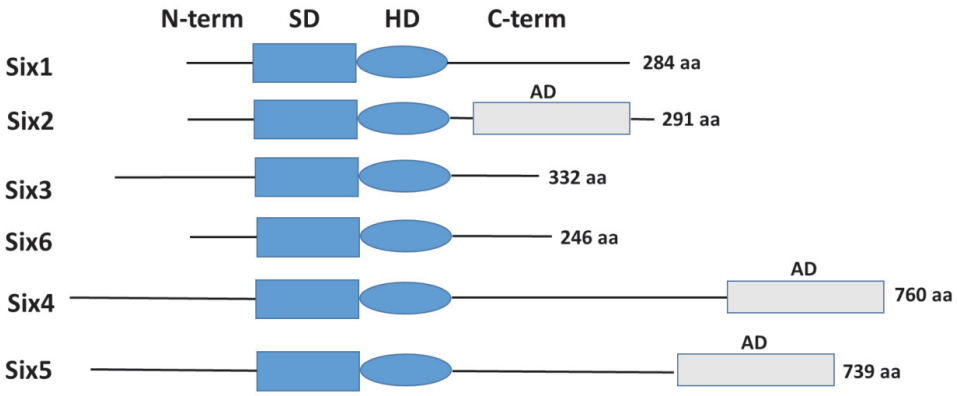

Figure 3. Schematic representation of the structure of the Six family of Homeoproteins. Structural elements of the Six family members include a well-conserved Six domain (SD) that is important for protein interactions, and a homeodomain (HD) that is responsible for DNA binding. The N- terminus is variable in length. The C-terminus is more divergent and also contains an activation domain in the case of Six2, Six4, and Six5. (Adapted from Christensen et al., 2008).

1.4 Expression patterns and functions of the Six3/Six6 group

Both *Six3* and *Six6* genes have partially overlapping expression patterns. Expression studies of *Six3* in medaka, zebrafish, *Xenopus*, chick and mouse illustrate an early expression localized in the anterior neuroectoderm and later maintained in the forebrain, eye anlage, lens placode, olfactory placode, and hypothalamus (Oliver, Mailhos et al. 1995, Bovolenta, Mallamaci et al. 1998, Loosli, Koster et al. 1998, Seo, Drivenes et al. 1998, Zhou, Hollemann et al. 2000). Studies in zebrafish, *Xenopus*, chick and mouse confirm that the expression of *Six6* begins later than *Six3* and that it is in a more restricted pattern detected in the hypothalamus (circadian temporal expression pattern), pituitary gland, optic stalk and neural retina (Seo, Drivenes et al. 1998, Toy, Yang et al. 1998, Lopez-Rios, Gallardo et al. 1999, Zuber, Perron et al. 1999, Ghanbari, Seo et al. 2001, Clark, Gorman et al. 2013). In the central nervous system, *Six3* is generally the dominant transcript of the two (Aijaz, Allen et al. 2005).

The importance of the *Six3/6* genes in eye and forebrain development was proven by multiple gain- and loss-of-function analyses in different species. In vertebrates Six3 coordinates cell proliferation and differentiation and its overexpression induces enlargement of the forebrain, expansion of the normal optic vesicles and formation of the ectopic optic vesicles in the neural tube region (Kobayashi, Toyama et al. 1998, Loosli, Winkler et al. 1999, Bernier, Panitz et al. 2000, Kobayashi, Nishikawa et al. 2001, Lagutin, Zhu et al. 2001, Zhu, Dyer et al. 2002, Ando, Kobayashi et al. 2005, Gestri, Carl et al. 2005). In the same way, Six6 overexpression in *Xenopus* induces ectopic retinal tissue and expands the opical vesicles (Zuber, Perron et al. 1999, Bernier, Panitz et al. 2000). In chicken embryos Six6 overexpression induces trans-differentiation of dissociated pigment epithelium cells into neural retina phenotypes (Toy, Yang et al. 1998). The inactivation of Six3 results in the loss of forebrain structures including the retina (Kobayashi, Toyama et al. 1998, Loosli, Winkler et al. 1999, Bernier, Panitz et al. 2000, Lagutin, Zhu et al. 2001) and a loss of the patterning of the optic vesicle and the forebrain (Carl, Loosli et al. 2002, Lagutin, Zhu et al. 2003). Loss-of-function mutations in Six3 causes holoprosencephaly in mice and humans (Wallis, Roessler et al. 1999, Pasquier, Dubourg et al. 2000, Pasquier, Dubourg et al. 2005, Domene, Roessler et al. 2008, Lacbawan, Solomon et al. 2009, Solomon, Lacbawan et al. 2009, Paulussen, Schrander-Stumpel et al. 2010, Savastano, El-Jaick et al. 2014) . Furthermore, Six3 was down-regulated in lung adenocarcinoma (Mo, Okamoto et al. 2013). In humans, deletions at 14q22.3-23 harboring the *Six6* locus have been associated among other effects with dysmorphic face, moderate developmental delay (Martinez-Frias, Ocejo-Vinyals et al. 2014) . Six6 missense mutations have been associated with glaucoma (Carnes, Liu et al. 2014), macular atrophy and optic disc anomalies (Yariz, Sakalar et al. 2014). Moreover, Six6 was required in mouse for proper reproductive function through the control of the hypothalamo-pituitary gonadal axis (Larder, Clark et al. 2011).

1.5 Six3/Six6 interacting proteins

Six3 and Six6 interaction with other proteins is important in modulating their function. Therefore, a major part of this study was devoted to the characterization of protein interactions and especially the interaction with Geminin, which was previously shown to have an important role in cell cycle regulation.

Six3 and Six6 have both shared and distinct expression patterns and functions. Thus, some of the natural binding partners are common to both Six3 and Six6. Six3 interacts with transcriptional co-repressor such as Groucho family: Grg1, Grg3, Grg5, TLE1, AES (Kobayashi, Nishikawa et al. 2001, Zhu, Dyer et al. 2002, Lopez-Rios, Tessmar et al. 2003), transcriptional co-activator Eya4 (Abe, Oka et al. 2009), transcription factors like NeuroD, Ath1, NOR-1, NR4A3 (Tessmar, Loosli et al. 2002, Laflamme, Filion et al. 2004), and with the chromatin remodelling protein MTA1 (Manavathi, Peng et al. 2007) (Fig. 5). Some of these proteins also interact with Six6. Both Six3 and Six6 interact strongly through the Six domain with members of the Groucho family TLE1 and AES (Lopez-Rios, Tessmar et al. 2003). Six6 interacts strongly with the conserved N-terminal region of Dach1 and Dach2 and represses the gene expression of p27kip1 (Li, Perissi et al. 2002) (Fig. 4).

Yeast two-hybrid and pull-down experiments showed that both Six3 and Six6 interacted with Geminin. Furthermore, Geminin binding to Six3 did not affect Six3 binding to DNA and the importance of this interaction in proliferation and differentiation of eye and forebrain was proven by gain- and loss- of function studies (Del Bene, Tessmar-Raible et al. 2004) (Fig. 4).

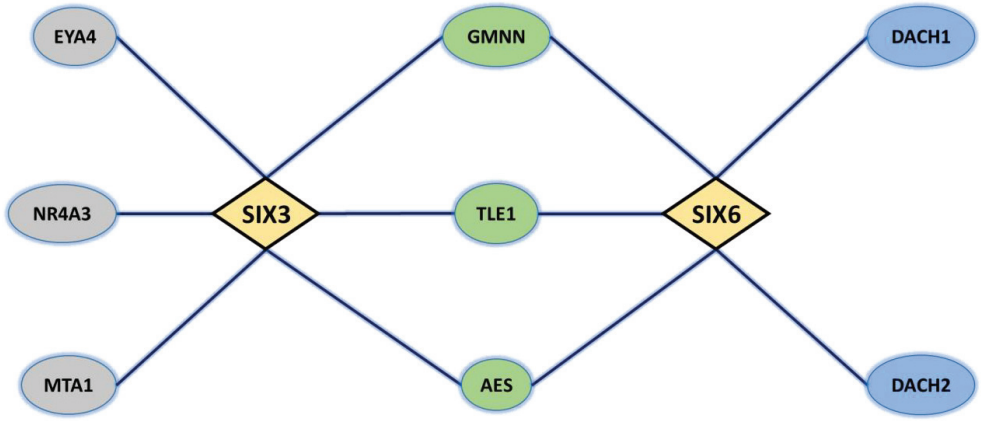

Figure 4. Schematic representation of the protein interacting network for the human SIX3 and SIX6 proteins. Both SIX3 and SIX6 interact with the members of the Groucho family TLE1 and AES and with GEMININ (GMNN). Additionally, SIX3 interacts with EYA4, NR4A3 and MTA1, while SIX6 interacts with DACH1 and DACH2.

As a concluding remark, the available data demonstrate the involvement of Six3 and Six6 proteins in development and proliferation/differentiation of eye and forebrain in a transcriptional way, as well as through interactions with other proteins. Earlier studies have shown a difference in the expression pattern of Six3 and Six6, in knock-out/ knock-down studies and inborn defects. However, the 3D structure of the Six3 and Six6 proteins is still unknown. In addition, information on the interacting domains and biophysical parameters of the Six3 and Six6 proteins interactions is still missing.

2. Aims of study

Six proteins are a group of evolutionary conserved specific homeodomain transcription factors that control the development of nervous system and visual organs. We have studied the Six3 and Six6 proteins, which are essential for eye and forebrain formation. Earlier studies have shown similarities and differences between these two paralogous proteins in expression pattern and in gain- and loss-of-function studies. Furthermore, there are two orthologous of the mammalian Six3 in zebrafish, with different spatiotemporal expression pattern. However, there is limited knowledge about how Six3 and Six6 interact with other proteins (for example Geminin) or with DNA. The binding of Six3 to Geminin is important for regulating the cell cycle, while the implications of the Six6-Geminin interaction are not known. In addition, the binding mechanism of these interactions is still unknown.

Therefore, we aimed at better understanding of the mechanism of Six3 and Six6 proteins interaction. In addition, for zebrafish Six3 proteins, the EMSA studies have been previously performed with the homeodomains alone.

Consequently, it was important to:

- Identify apparent regions and residues involved in the Six3-Geminin and Six6-Geminin interactions.
- Determine the stoichiometry of the Six – Geminin complex.
- Establish new biophysical parameters of the Six proteins – Geminin binding.
- Confirm the Six-Geminin interactions in living cells.
- Characterize the zebrafish Six3 proteins (Six3a and Six3b) using computational, biophysical and biochemical methods.
- Study the interaction of the full-length zebrafish Six3 proteins with the Six3 DNA recognition sequence.
- Biophysically and biochemically detect and characterize the interaction of zebrafish Six3 proteins (Six3a and Six3b) with Geminin.
- Describe Six3a and Six3b localization patterns in living cells.

3. Three main methods used in this study

3.1 Biacore: Surface Plasmon Resonance (SPR) technology

The Biacore system monitors in real-time, in a label-free manner, biomolecular interactions and provides specificity, kinetics and affinity of the interaction. It is a powerful tool largely used in life science research and biotherapeutics.

The Biacore technology is based on the optical phenomenon of surface plasmon resonance (SPR) which occurs when plane-polarized light hits a metal film under total internal reflection conditions (McDonnell 2001). The Biacore system consists of a light source emitting a near infrared light, a sensor microchip, an automated liquid handling system with constant flow and a diode-array position-sensitive detector (http://www.biaffin.com/techniques/biacore). The sensor chip consists of a glass surface covered by a thin gold layer. A carboxymethylated dextrane is coupled onto this gold layer providing a hydrophilic environment with low specific binding. The sensor chip is connected to a flow system in which the analyte flows over the ligand immobilized on the chip. When the analyte concentration at the surface changes, it induces a change in the refractive index, and this correlates with the change of mass (Fig. 5). The rate at which the change occurs provides readout for the association rate constant (k_a) and the dissociation rate constant (k_d) of the intermolecular interaction:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

The association rate constant, k_a, describes the rate of complex formation while the dissociation rate constant, k_d, describes the dissociation (the fraction of complexes that decays per second). Equilibrium dissociation constant, K_D, describes the system at equilibrium and is calculated from:

$$K_D = \frac{k_a}{k_d}$$

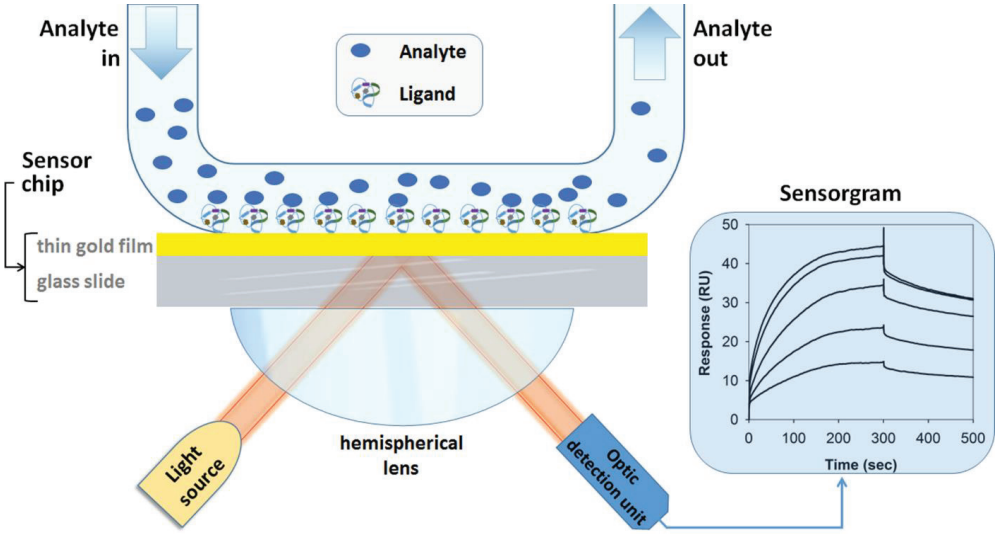

Figure 5. Illustration of the SPR principle. Surface plasmon resonance (SPR) detects changes in the refractive index in the immediate vicinity of the surface layer of a sensor chip. SPR is observed as a sharp shadow in the reflected light from the surface at an angle that is dependent on the mass of material at the surface. The SPR angle shifts when biomolecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored non-invasively in real time as a plot of resonance signal (proportional to mass change) versus time.

3.2 Bimolecular Fluorescence Complementarity (BiFC)

Bimolecular Fluorescence Complementarity (BiFC) has emerged as a key technique to study protein-protein interactions in different organisms. The BiFC assay is based on reconstituting an intact fluorescent protein when two complementary non-fluorescent fragments are brought together by the interaction of the two proteins (Fig. 6). Thus, the reconstituted fluorescence signals reflect the interaction of the two studied proteins (Hiatt, Shyu et al. 2008).

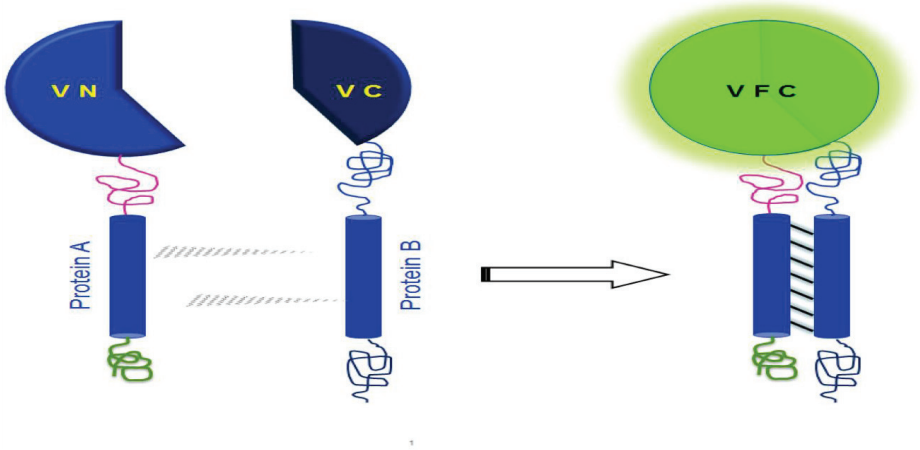

Figure 6. BiFC principle. Proteins A and B are fused to N- and C-terminal fragments of Venus protein, respectively. In the absence of an interaction between A and B, the fluorophore halves remain non-functional. Following interaction between A and B a functional fluorophore is reconstituted, which exhibits emission of fluorescence upon excitation with an appropriate wavelength.

3.3 Isothermal Titration Calorimetry (ITC) technology

Protein-protein interactions play an important role in gene regulation, immune response, signal transduction. Isothermal titration calorimetry (ITC) is the most quantitative method available for measuring the thermodynamic properties of a protein-protein interaction (Pierce, Raman et al. 1999). ITC measures the binding equilibrium directly by assessing the heat evolved on association of the two proteins. In a single experiment, the binding constant (K_a), the stoichiometry (n) and the enthalpy of binding (ΔH) are determined. In addition, the free energy and the entropy of binding (ΔS) are acquired from the association constant.

Main parts of an ITC instrument are two identical gold cells (due to high thermal conductivity) surrounded by an adiabatic shield (Fig. 7). In an ITC experiment, the reaction cell is filled with one of the interacting partners, while the reference cell contains water. Prior to the injection of the titrant, a constant power is applied to the reference cell that activates the heater located on the sample cell and gives the baseline

signal. The ITC experiment directly measures the time-dependent input of power required to maintain equal temperatures in the sample and the reference cell. During the injection of the titrant into the sample cell, heat is released or absorbed, so the system compensates by reducing or increasing the power applied to the control heater (Pierce, Raman et al. 1999). The heat absorbed or released during a calorimetric titration is proportional to the fraction of bound ligand.

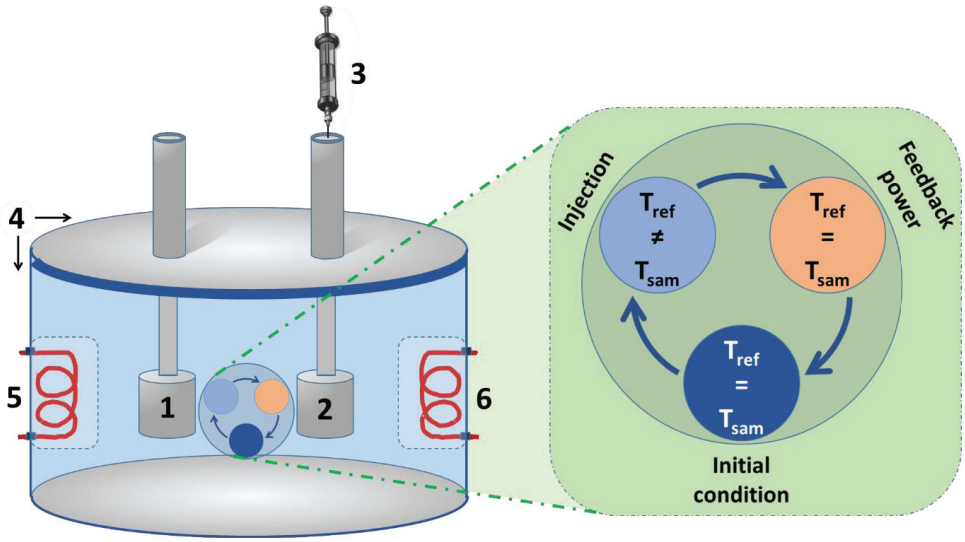

Figure 7. ITC principle. During titrant injection into the sample cell, power is applied to keep the calorimeter cell from changing temperature. The total heat exchange is calculated by integrating the heater power over time.**1.** Reference cell. **2.** Sample cell. **3.** Syringe. **4.** Adiabatic shield. **5.** Constant power. **6.** Feedback power.

4. Summary of Results

4.1 Full-length proteins of SIX3 and SIX6 are necessary for proper interaction with GEMININ in mammalian cells

The SIX3-GEMININ interaction plays an important role in regulating the balance between cell proliferation and differentiation in eye and forebrain, while the implications of the SIX6-GEMININ interaction had not been studied to date. Our focus in the first paper was to determine how SIX3 and SIX6 proteins are binding to GEMININ. Thus, we investigated complex formation between full-length SIX3 or full-length SIX6 proteins and GEMININ using surface plasmon resonance (SPR) and detected a similar apparent binding affinity. The SIX3-GEMININ binding was thermodynamically examined by isothermal titration calorimetry (ITC). We identified a 1:2 stoichiometry for this reaction and proved that the coiled-coil region of GEMININ is not involved in binding. The enthalpy driven binding detected in this case implied that electrostatic attractions are mainly responsible for the specific SIX3-GEMININ binding. Our docking analysis of the SIX3 or SIX6 proteins with GEMININ indicated that SIX3 and SIX6 are binding with different interfaces to GEMININ. While the SIX domain was the main structure in SIX3 binding to GEMININ, the SIX6 bound GEMININ mainly through its homeodomain. These observations were further confirmed in SPR studies. We observed a selective binding of the SIX domain of SIX3 (SIX3SD) relative to the full-length SIX3 protein in SPR. Also, the SIX3SD showed a 10-fold increase in apparent binding stability compared with the SIX domain of SIX6 (SIX6SD), supporting the docking studies. The shape of the binding curves is very different between these two Six domains, with a slow dissociation phase in case of SIX3SD and a very rapid dissociation phase for SIX6SD. The interaction of full-length SIX3 and SIX6 proteins with GEMININ was confirmed through a strong fluorescent signal in mammalian cell cultures using bimolecular fluorescence complementation (BiFC) method. In contrast, the co-transfection of

SIX3SD or *SIX6SD* with *GEMININ* produced a very low level of BiFC signal, suggesting a lack of interaction of the two SIX domains with GEMININ in living cells. Taken together, our results indicated SIX3 and SIX6 bind to GEMININ with different interfaces and revealed that SIX3 binds GEMININ mainly through charged amino acids residues in a 1:2 ratio in *in vitro* studies.

4.2 Differential interaction with DNA and proteins by zebrafish Six3 orthologous proteins

Zebrafish contain two orthologous of the Six3 protein, the Six3a and the Six3b proteins. Given the central role of Six3 in regulating eye and forebrain development, we analysed the Six3a and Six3b interaction to DNA and to the DNA-replication inhibitor Geminin. In 3D-structure prediction analyses, we noticed differences in the N-terminal and C-terminal arrangement of the two proteins. Electrostatic studies showed more differences in the charge distribution between the two proteins. Further, electrophoretic mobility shift assay (EMSA) was used to assess the binding of different Six3b constructs to the Six3a DNA recognition sequence. As expected, Six3a binds to its own recognition sequence. However, for Six3b, only its homeodomain (HD) bound to the DNA fragment. (Six3b-SDHD and the full-length Six3b did not bind to the DNA). Moreover, we directly compared to Six3a and Six3b for their Geminin bindings using SPR and observed an approximately 3-fold stronger binding of Six3b relative to Six3a. The apparent binding affinity of Six3b to Geminin was similar to the apparent Six3b-Six3a affinity, indicating that Six3b forms heterodimers. Our BiFC experiments confirmed the above SPR observations. A strong BiFC complex formation was detected in the *Six3b-Geminin* and *Six3a-Geminin* co-transfected cells. Furthermore, strong BiFC signals were also detected in the *Six3b-Six3a* and *Six3b-Six3b* co-transfected cell populations. In contrast, a very low BiFC fluorescence level was noticed in the *Six3a-Six3a* co-transfected cells. The Six3a

protein and the Six3a-Geminin complex had a distinct localization from the Six3b and the Six3b-Geminin complex. While Six3a was mostly localized to the nucleus and nucleoli-like structures, Six3b was mainly detected in cytoplasm and Golgi-like structures. In conclusion, our results showed that Six3a and Six3b behave differently when binding to DNA and Geminin. Six3b forms both homodimers with itself and heterodimers with Six3a, whereas Six3a does not form homodimers. Six3a and Six3b also show a different localization pattern in living cells.

5. General Discussion

5.1 Methodological aspects

5.1.1 SPR for protein-protein interaction studies

The Six3-Geminin interaction was observed by a pull-down and by using a yeast two-hybrid system. We chose to use surface plasmon resonance (SPR) technology to analyse the association and dissociation kinetics of the interaction between Six3 or Six6 and Geminin as SPR provided several advantages (Helmerhorst, Chandler et al. 2012):

- It gives quantitative real-time data: the SPR signal is directly proportional to mass changes at the sensor surface, so that very small molecules (even 100 Da) can be observed as they bind to their target (Myszka 2004).
- It is sensitive, specific and requires a small amount of protein.
- SPR allows monitoring biomolecular interactions in a label-free environment.

The importance of the technology and its application is evident from the increasing number of SPR-related scientific articles. Several immobilization choices allows flexible assay design and analysis of a wide range of molecular interactions. One advantage of immobilizing the ligand directly to the chip is that the ligand may attach to the chip in several positions, so that there are higher chances of having a conformation accessible for the analyte. Each ligand molecule is likely available to an analyte molecule. However, for kinetic analyses, only a small amount of ligand should be immobilized to avoid steric interference in binding analyte.

One limitation of the SPR is that the ligand is physically separated from the analyte in the bulk solution. It is crucial to ensure that the transfer of the analyte to the ligand at the sensor surface is not flow-limited; otherwise, the analyte concentration near the surface will be different from the bulk concentration (mass transport limitation). A meticulous experimental design is also necessary to hinder non-specific binding effects and to avoid aggregations.

5.1.2 BiFC in protein-interactions

BiFC monitors the interaction and subcellular compartmentalization of protein complexes, making it a very good choice to validate and detect protein interactions (Wong and O'Bryan 2011). Advantages of BiFC are:

- It detects transient or weak interactions.
- BiFC has little background fluorescence.
- It requires little post processing of image data.
- BiFC does not require high-level of overexpression.

A limitation of the BiFC method is that once the fragmented fluorophore is reconstituted, the complex is irreversible, so that no kinetics analysis of the complex is possible. Another disadvantage is that the reconstituted fluorophore requires at least 30 min. for maturation, meaning that the interactions can not be observed in real time. Proper controls are important in BiFC interpretation. Empty BiFC vectors should not be used as controls, as they result in high background fluorescence. One should check the levels of protein expression, to ensure that differences in fluorescent signal are not due to different levels of protein expression. High protein expression can lead to non-specific interactions and fluorescence, hence the expression levels should be kept relatively low.

5.2 Discussion of results

The homeodomain transcription factors Six3 and Six6 play a crucial role in eye and forebrain development and are evolutionarily conserved in animals. To better understand their role in development, it is important to know how these proteins interact with other proteins (i.e., Geminin) and DNA. We have analysed association and dissociation kinetics of Geminin with Six3 and Six6 and detected variances in their binding to Geminin. Our studies showed that Six3 and Six6 proteins bind Geminin with different interfaces. Further SPR studies indicated that zebrafish Six3a and Six3b bind Geminin with different affinities. In addition, ITC experiments showed that Six3 binds Geminin in a 1:2 molar ratio and that the interaction is mainly electrostatic. We further showed in EMSA studies that the zebrafish Six3b does not bind the Six3a DNA recognition sequence. The Six3 and Six6-Geminin interactions were confirmed in living cells using the BiFC method. All findings (involving both human and zebrafish proteins) lead to the same trend of distinct behaviour in interactions with Geminin and DNA. These observations offer an explanation for the differences in expression pattern and function reported previously for these transcription factors.

5.2.1 SIX3 and SIX6 bind Geminin directly though with different interfaces

SIX3 and SIX6 compete with Cdt1 to bind to Geminin and the SIX3-GEMININ interaction is very important in proliferation and differentiation of cells in the eye and the forebrain (Del Bene, Tessmar-Raible et al. 2004). By SPR, we showed a direct interaction between human SIX3 or SIX6 and GEMININ, yielding similar apparent binding affinities for both proteins (K_D = 0.6 μM). Previous studies showed the SIX domain was involved in interaction with Eya or Groucho proteins (Zhu, Dyer et al. 2002, Patrick, Cabrera et al. 2013). Remarkably, the highly conserved SIX domains of

these two related proteins (sequence identities, 88.2%; sequence similarities, 98.4%) bind very differently to GEMININ. An approximately 10-fold stronger association between GEMININ and SIX3SD was detected (apparent K_D = 0.23 μM), whereas the interaction with SIX6SD was much weaker (apparent K_D = 2.09 μM).

Because the full-length SIX6 protein has the same apparent binding affinity for GEMININ as full-length SIX3 and SIX6SD does not bind strong, other regions of the SIX6 protein (i.e., other than SD) must contribute to GEMININ binding. Our molecular docking analyses clearly indicated that the SIX3 and SIX6 proteins bind GEMININ with different interfaces. One of the 18 residues involved in the binding, His-141 (in SIX6) is the non- conserved residue between SIX3 and SIX6. Docking studies designated SIX3SD as the main structure involved in GEMININ binding (six out of nine contact amino acids), while SIX6 bound GEMININ mainly through its homeodomain. Previous studies showed that the SIX domain modulated homeodomain binding to DNA (Kawakami, Ohto et al. 1996, Hazbun, Stahura et al. 1997, Harris, Winchester et al. 2000, Patrick, Cabrera et al. 2013). We suggest in this study that the homeodomain may modulate the SIX domain in protein interaction. Subsequently, the homeodomain of SIX6 would contribute to increase binding of SIX6 to GEMININ, while the homeodomain and C-terminal of SIX3 decreased the SIX3SD binding to GEMININ.

Site-directed mutagenesis could be used to explain the binding differences of the SIX3 and SIX6 to GEMININ. Docking studies showed that the H141S mutation made SIX6SD as the main structure in SIX6-GEMININ interaction (seven contact amino acids), while SIX6HD contributed less to the binding (one contact amino acid). The interaction analyses with H141S mutated SIX6 (one strong candidate is NMR) could confirm the results obtained by docking. Ultimately, the three-dimensional structures of the two SIX domains of SIX3 and SIX6 using NMR spectroscopy and/or and X-ray crystallography would provide an equivoval understanding of the molecular mechanisms in protein binding.

5.2.2 SIX3 binds to GEMININ in a 1:2 ratio and the coiled-coil region of GEMININ is not involved

The docking study indicated that SIX3 protein binds mainly through charge-charge contacts involving the N-terminal region of GEMININ, but not through the coiled-coil region, as CDT1 protein does. In ITC analysis, SIX3 did not bind the coiled-coil region, further supporting the docking results. The enthalpy-driven binding indicated that electrostatic attraction plays a major role in complex formation. The binding stoichiometry of SIX3 to GEMININ is 1:2, which is the same as the CDT1 – GEMININ binding ratio. Hence, although SIX3 binds a different region of GEMININ than CDT1 does, it still works as competitor/inhibitor of CDT1 and prevents the GEMININ-CDT1 binding.

5.2.3 Full-length SIX3 and SIX6 proteins, but not SIX domains alone, bind to GEMININ in living cells

By the means of BiFC, we visualized the SIX3 and SIX6 interaction with GEMININ in living mammalian cells. The BiFC signals detected in the case of full-length SIX3 and SIX6 proteins with GEMININ were strong and spotted mainly in the nucleus, but to a less degree were also detected in the cytoplasm, in accordance with protein localization detected by immunostaining.

Surprisingly, neither SIX3SD, nor SIX6SD interacted with GEMININ in cell culture in the BiFC assay. The levels of BiFC signal detected in these cases were much lower than those of the full-length proteins, although the protein expression levels were similar to the ones in the full-length proteins. Based on these results, we assume that the SIX domain binding to GEMININ may be regulated through an unknown mechanism when the SIX domain is part of the full-length protein. We speculate that the conformations of SIX3 and SIX6 proteins could be altered by post-translational modifications, probably by phosphorylation. The previous study of the SIX1 protein

showed this was the case (Ford, Landesman-Bollag et al. 2000). Supporting this, the NetPhosK program predicted potential phosphorylation sites at conserved motifs in the C-terminus (Ser-141 and Thr-233 in SIX3 and Ser-227 and Ser-228 in SIX6). In addition, the C-terminal regions of the *Drosophila* Six proteins were necessary for proper folding and hence renders specificity among different Six proteins (Weasner and Kumar 2009). Supporting the notion that the full-length proteins, but not SIX domains alone, are required for proper interaction, for the SIX domain alone NetPhosK predicted three very likely phosphorylation sites for SIX3SD and no such sites for SIX6SD. It is possible that phosphorylation of SIX3SD (and the lack of it in SIX6SD) prevented these domains from binding GEMININ.

5.2.4 Six3b does not bind Six3a DNA recognition sequence

In Paper I were observed differences between the human Six3 and Six6 proteins in their binding to Geminin. In Paper II, we investigated the two orthologous of Six3, zebrafish Six3a and Six3b for their binding abilities.

The two conserved domains HD and SD are very similar in 3D-predicted structures, whereas both C-termini and N-termini of Six3a and Six3b are different. More differences were detected between the two in the mapped electrostatic distributions. The groove formed between the C-terminal region and the Six domain is much smaller in Six3b than in Six3a and the charge distribution is also different.

Besides the computational modelling, further dissimilarities between Six3a and Six3b were exhibited by their DNA binding abilities. Using EMSA, we found that full-length Six3b and Six3bSDHD did not bind to the Six3a DNA recognition sequence. Only the homeodomain (HD) alone of Six3b protein binds to Six3a DNA recognition sequence. The 60 amino acid long homeodomain has a single conserved change (Ser/Gly) between these two proteins. Previous studies have shown that the HD has low specificity, and that SIX domain or C-terminus may contribute to binding (Kawakami,

Ohto et al. 1996, Hazbun, Stahura et al. 1997, Weasner, Salzer et al. 2007, Hu, Mamedova et al. 2008), supporting our interpretation. Site-directed mutagenesis could be performed again at the SIX domain starting with the two non-conserved P/A, L/Q sites (Fig. 1); this could indicate the influence of these amino acid changes on the binding process.

5.2.5 Six3a and Six3b bind Geminin with different affinities and they form heterodimers with each other

Surface plasmon resonance (SPR) analysis showed Six3b to have a three-fold higher affinity for Geminin than Six3a (K_D of 1.2 μM compared to 3.4 μM). Six3a and Six3b share extensive sequence identities (over 89%), however the predicted 3D structures and electrostatic mapping show differences that could influence their binding to Geminin. In addition, Six3a and Six3b form a strong heterodimer with an apparent K_D of 1.3 μM.

5.2.6 Validation of the detected protein interactions in living cells

The BiFC assay in living cells validated protein interactions observed in the SPR study. A slightly stronger BiFC signal was detected for the Six3b-Geminin co-transfected cell than in Six3a-Geminin co-transfected cells. However, the BiFC signal and the immunostaining showed different localization patterns for Six3a and Six3b and their complexes. While Six3a localized more to the nucleus and nucleoli-like structures, Six3b and its complexes were detected more in the cytoplasm and in Golgi-like structures. These results, together with the EMSA study, suggest that Six3a could be the more dominant protein for the transcription activity in the nucleus.

Additionally, previous studies have shown a tendency of the SIX5 protein to form homodimers by dimerization through the Six domain (Harris, Winchester et al. 2000).

We detected for the first time that Six3a and Six3b form heterodimers in *in vitro* SPR studies, and confirmed the results in living cells by a strong BiFC signal. Also, Six3b (but not its paralogous Six3a) formed homodimers in mammalian cell cultures.

The detection of non-immunostainable protein complexes suggested involvement of the Six domain in protein interaction. Consequently, it would be reasonable to conclude that the differences found in the Six domain may have resulted in differences in binding characteristics, affinity, as well as cellular localization.

Mutagenesis could address the cause of the differences in protein binding for Six3a and Six3b. The first choice would be P51A and L94Q in the Six domain, the only two non-conserved changes between these two domains. Another point to consider is that as previously reported, the C-terminal has an influence on folding and specificity (Weasner and Kumar 2009). The C-terminal region has rather different amino acid sequences and predicted 3D-structures, which could influence their binding specificities.

5.3 Concluding remarks and future perspectives

In Papers I and II was investigated the binding of Six3 and Six6 proteins to Geminin (Paper I and II) and to a DNA recognition sequence (Paper II). Although the primary sequences for human and zebrafish proteins were rather similar with each other, the modes of their bindings were quite different.

In Paper II zebrafish Six3 proteins were immunostained with antibodies raised against the Six domain epitope. A partially non-immunostainable Six3-Geminin complex was detected, suggesting an involvement of the Six domain in the Geminin interaction. This observation concurs with the results acquired by SPR and through docking experiments in Paper I, which showed the Six domain participation into Six3-Geminin interaction. No non-immunostainable Six3-Geminin or Six6-Geminin complexes were

detected when antibodies raised against the C-terminal epitope of Six3 or Six6 were used (Paper I) or when antibodies against the coiled-coil region of Geminin were used (both Paper I and Paper II). Therefore, as mentioned earlier (docking and SPR experiments), it is plausible to assume that the C-terminal region is not much, if any, involved in GEMININ binding. In addition, in accordance with the ITC experiments presented in Paper I, the experiments performed in living cells suggest that the coiled-coil region of Geminin was not involved in Six3 binding.

The SIX domain of the SIX3 protein was the main structure in SIX3-GEMININ complex formation, while the homeodomain of SIX6 protein played a major role in SIX6-GEMININ complex formation. The 141-His residue in SIX6SD (the only non-conserved residue between the two homeodomains; in SIX3, it is serine) seems to play an important part in this change. The homeodomain of Six3b also exchanged residue in the same position. However, the Ser/Gly mutation in zebrafish does not seem to affect DNA binding.

Major findings from Paper I:

- Human SIX3 and SIX6 bind GEMININ with a different interface: SIX3 uses the SIX domain as main structure in interaction, whereas the SIX domain in SIX6 plays a minor role.
- Both SIX3 and SIX6 bind GEMININ with similar affinities and the interaction could be validated in mammalian cell cultures.
- SIX3 and GEMININ form heterotrimer by binding in a 1:2 molar ratio.
- The coiled-coil domain of GEMININ is not involved in the interaction with the SIX3/SIX6 proteins.

Major findings from Paper II:

- Zebrafish Six3a and Six3b bind Geminin with different affinities.
- Six3b does not bind to Six3a DNA recognition sequence.
- Six3a and Six3b have different abilities in forming homo- and heterodimers.
- Six3a and Six3b distinct subcellular localization: the main location for Six3a is in the nucleus, whereas for Six3b it is the cytoplasm.

Our study offers some explanation to previously reported distinct functions or expression patterns for these proteins. More detailed interaction studies involving site-directed mutagenesis are warranted. Unequivocal insights on protein interaction involving Six3 and Six6 will be obtain through crystal and/or solution structures of these proteins. In addition, further EMSA studies are necessary to determine the DNA recognition sequence of the zebrafish Six3b. An EMSA study should also be employed to check if the Six6-Geminin complex still binds the common DNA sequences. Such studies will provide a basis for understanding the mechanisms of DNA and Geminin binding and their biological function.

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