Chapter 6

General discussion

In multicellular organisms, numerous processes and reactions are required for cells to organize as a unit. Signal transduction is one of the essential processes during the whole lifespan of each multicellular organism. Sensing changes of the environment at the cellular level largely depends on signal transduction. Today, we know that signaling pathways triggered by TGF-β/BMP, Wnt, and IL-1R/TLR ligands play major roles in the molecular control of embryonic development and adult homeostasis by regulating cellular functions such as proliferation, differentiation and apoptosis. (Clevers, 2006; Dunne and O'Neill, 2003; Massague et al., 2000). The ligands of these signaling pathways are secreted by specific cell or tissue types to activate themselves, and/or neighbouring cells or tissues, resulting in changes of cell properties. The combined activation of multiple distinct signal transduction pathways endows the diversity of cells. In normal tissues, the activities of these signaling pathways are tightly regulated but in cancer and other diseases this regulation can be compromised. Therefore, the control of the activity of these signaling pathways is investigated intensively to understand the molecular mechanisms and potential cures of these diseases.

Depending on the cell type, cell localization, and cell state, activation of a specific signaling pathway can give rise to different phenotypes. These different phenotypic outcomes raise the question how the outcome specificity is regulated. One of the answers seems to be that a specific cell type can express a unique set of regulatory proteins and/or RNAs for a particular pathway in a cell context-dependent manner. These cell type-specific regulatory proteins or RNAs can target different components of signaling pathways and thereby propagate specific responses. TGF-β/BMP, Wnt and IL-1R/TLR ligands all play very important roles in the control of cell proliferation, differentiation, and apoptosis, from embryonic development to adult homeostasis. To better understand how these signaling pathways are regulated, we have used luciferase transcriptional reporter-based screens, whole proteomic protein-protein interaction screens and siRNA screens to identify novel regulators and/or modulators of TGF-β/BMP, Wnt and IL-1R/TLR-induced signals. In addition, we have investigated how the identified novel regulators modulate these signaling pathways.

1 UBE2O monoubiquitinates SMAD6 and is involved in BMP7-induced adipogenesis

TGF-β family members are secreted cytokines (*chapter 1*), including TGF-βs, bone morphogenic proteins (BMPs), activin, growth and differentiation factors (GDFs), nodal and anti-müllerian hormone (AMH) (Massaous and Hata, 1997). TGF-β was first identified by its ability to induce growth of large colonies of normal rat kidney (NRK) cells in soft agar assays when combined with small amounts of EGF (Centrella and Canalis, 1985; Roberts et al., 1982; Roberts et al., 1980). To activate signaling inside the cell, membrane-localized type II receptors heterodimerize with type I receptors upon binding of ligand, which leads amongst others to the phosphorylation and activation of R-SMADs. Activated R-SMADs will then translocate to the nucleus to initiate transcriptional responses with the

help of Co-SMAD (SMAD4). I-SMADs negatively regulate TGF-β signaling by inhibiting the phosphorylation of R-SMADs via binding to the type I receptors (Heldin et al., 1997).

In order to find novel regulators for TGF-β signaling, we used a whole proteomic protein-protein interaction screen for 19 members of the TGF-β signaling cascade, including receptors, SMADs and known regulators such as Ski and SnoN. Among the candidates identified, we found UBE2O, a putative E2 enzyme, to interact with both SMAD6 and SMAD7 (*chapter 2*). It has been shown that many components of the TGF-β signaling pathway are regulated by ubiquitination, such as TGF-β receptor I (ALK5) and various SMAD proteins (Dupont et al., 2009; Gao et al., 2009; Kavsak et al., 2000; Koinuma et al., 2003; Zhang et al., 2012). However, to our knowledge previous reports only identified the E3 enzymes and not the E2 enzymes. This drew our attention since UBE2O could be the first E2 enzyme identified that regulates TGF-β signaling through ubiquitination. Indeed, we could show that UBE2O alone is sufficient to monoubiquitinate SMAD6 in *in vitro* assays. Lysine mutation of SMAD6 indicated that lysine 174 is the site modified by UBE2O. However, we still observed some ubiquitin-modified SMAD6 when lysine 174 is mutated, suggesting that ubiquitin might attach to neighboring lysine residues or other amino acids, when lysine 174 is unavailable (Hagai et al., 2012; Tokarev et al., 2011; Wang et al., 2007). Consistent with the concept that monoubiquitination changes the localization or activity of the substrate rather than its stability (Hicke, 2001), we observed that the localization of monoubiquitinated SMAD6 is more cytoplasmic compared to non-modified SMAD6. Moreover, ubiquitinated SMAD6 has less affinity for the type I receptor ALK2, leading to less SMAD6 inhibitory ability and resulting in activation of signaling. The fact that MH2 domain in C-terminal of SMAD6 mediates interaction with receptor and UBE2O monoubiquitinates SMAD6 only influence ALK2-mediated BMP7 signaling (*chapter 2*) suggests that a conformation change of monoubiquitinated SMAD6 could affect its interaction with ALK2. Therefore, crystal structure differences of SMAD6 and monoubiquitinated SMAD6 could help us to answer this question.

For a long time UBE2O was a functionally unknown protein although it was hypothesized to be an E2-E3 hybrid (Berleth and Pickart, 1996). We proved for the first time that UBE2O can act as an E2 enzyme and found that cysteine 885 is a critical residue in its catalytic domain. In addition, we showed UBE2O to function as an E2-E3 hybrid in the ubiquitination of SMAD6. An N-terminal deletion mutant of UBE2O still containing the C-terminal E2 ubiquitin-conjugating domain could partially monoubiquitinate SMAD6 with the help of the remainder of UBE2O. This suggests that the N-terminus of UBE2O might contain the E3 activity, yet further investigations are needed to confirm this hypothesis.

Next, we demonstrated that UBE2O is involved in BMP7-mediated signaling and adipogenesis. Importantly, both SMAD6 and UBE2O are upregulated during the BMP7-induced commitment of mesenchymal stem cells to preadipocytes, but with different kinetics (*chapter 2*). The earlier presence of UBE2O might be responsible for counteraction of the inhibitory effects of upregulated SMAD6, leading the cells to commit to preadipocytes. And the downregulation of UBE2O in differentiating adipocytes could be

explained by the fact that the level of BMP signaling is lower in these cells (Suenaga et al., 2010). Therefore, checking the ratio of adipose tissue in UBE2O knockout mice would be helpful to investigate its role in adipogenesis *in vivo*.

Our ongoing projects are trying to investigate the role of DUBs in SMAD6 ubiquitination. We screened all DUBs in UBE2O-mediated SMAD6 ubiquitination assays and found that one DUB might be involved in removing ubiquitin from monoubiquitinated SMAD6. It will be of great interest to determine the coordinate roles of SMAD6, UBE2O and this DUB in BMP7-induced signaling and adipogenesis. Also, combining the facts that E2s are responsible for determining the type of ubiquitination and that UBE2O monoubiquitinates SMAD6, TRAF1 and TRAF3 (*chapter 3*), we speculate that UBE2O might largely function as a monoubiquitination-inducing E2 enzyme. Therefore, further experiments such as checking by mass spectrometry-aided approaches the whole proteome ubiquitination status in UBE2O-overexpression or knockdown cells would be very attractive.

2 UBE2O and USP4 mitigate TRAF6-mediated NF-κB and AP-1 activation

TRAF6 is a very important adaptor protein and E3 ligase that mediates IL-1β/LPS-induced transcription factor NF-κB activation and plays an important role in regulating the innate and adaptive immune defense (Loiarro et al., 2010; Sims and Smith, 2010; Wu and Arron, 2003). Ligand binding to the IL-1R and TLR4 receptors triggers interaction of the receptor complex with MyD88, IRAKs and TRAF6, which then recruit the E2 enzyme Ubc13/Uev1a to polyubiquitinate TRAF6 (Deng et al., 2000). Lysine 63 polyubiquitinated TRAF6 activates NF-κB by forming a complex with TAK1-TAB2-TAB3 to phosphorylate IKKs and subsequently phosphorylate, ubiquitinate and degrade IκBα, allowing NF-κB proteins to activate transcription in the nucleus (Karin and Ben-Neriah, 2000; Takaesu et al., 2000; Wang et al., 2001). Ubiquitin modification appears to be a key regulatory mechanism for NF-κB activation in IL1R/TLR4 signaling. Many components such as IRAKs, TRAF6, TAB2/3, TAK1 and IκBα have been described to be regulated by ubiquitination (Adhikari et al., 2007; Chen et al., 1995; Ishitani et al., 2003; Newton et al., 2008; Ordureau et al., 2008; Yamin and Miller, 1997). Normally, to ubiquitinate a substrate, three different types of enzymes (E1, E2 and E3) are required for ubiquitin conjugation (Ye and Rape, 2009). Interestingly, a recent publication identified TRAF6 among more than hundred E3 proteins that could interact with UBE2O in a yeast two hybrid E2-E3 interaction screen (Markson et al., 2009). It should be noted here that although UBE2O can function as an E2-E3 hybrid when monoubiquitinating SMAD6, UBE2O might still need E3 enzymes to ubiquitinate other substrates.

To answer whether the reported interaction between UBE2O and TRAF6 is relevant for TRAF6-mediated NF-κB activation, we first verified the screen results in primary mouse fibroblast and HEK293T cells (*chapter 3*). As expected, ectopic UBE2O or TRAF6 interacts with endogenous TRAF6 or UBE2O in both cell lines. Unexpectedly, UBE2O decreases rather than increases the polyubiquitination of TRAF6, suggesting that UBE2O does not function as an E2 enzyme in TRAF6 polyubiquitination. Consistent with the

change of TRAF6 polyubiquitination we found overexpression of UBE2O to inhibit and depletion of UBE2O to increase TRAF6-mediated NF-κB activation. In support of the TRAF6-specificity, UBE2O had no effect on poly (I:C)- and TRIF-induced NF-κB activation which is TRAF6-independent. Moreover, IL1β/LPS-induced phosphorylation and target gene expression, including JNK, p38 and AP-1 activity were changed upon misexpression of UBE2O. Based on these results, we concluded that UBE2O is a negative regulator for TRAF6-mediated NF-κB and AP-1 activation. However, we cannot exclude the possibility that some effects of UBE2O on NF-κB signaling might be mediated through other TRAFs, as we found that UBE2O could trigger the monoubiquitination of TRAF1 and TRAF3 and polyubiquitination of TRAF2 and TRAF4.

How could an E2 enzyme inhibit polyubiquitination of TRAF6? Since UBE2O has no known deubiquitinase enzymatic site, we hypothesized that UBE2O might function upstream of TRAF6 ubiquitination. To ubiquitinate TRAF6, ligand-induced interaction of IRAKs, MyD88 and TRAF6 is essential for recruitment of the E2 complex Ubc13/Uev1a to TRAF6. We found that the TRAF domain of TRAF6, which mediates TRAF6-interaction with upstream proteins, also interacts with UBE2O (*chapter 3*). Therefore, we checked the effect of UBE2O on complex formation between IRAKs, MyD88 and TRAF6. Interestingly, we found that the interaction of TRAF6 and MyD88 is interrupted by UBE2O ectopic expression. Moreover, the inhibitory effect of UBE2O on TRAF6 and MyD88 interaction depends on its TRAF6 interaction domain, which strongly supports our hypothesis that UBE2O functions upstream of TRAF6 ubiquitination.

Not surprisingly in view of the important role of ubiquitination in TRAF6-mediated NF-κB and AP-1 activation, deubiquitination has also been reported to be involved in TRAF6-mediated signaling. The DUBs MCPIP1, USP7, A20 and CYLD have been shown to remove polyubiquitin chains from TRAF6 to inhibit its activity and thereby regulate IL1R/TLR4 signaling (Boone et al., 2004; Daubeuf et al., 2009; Jin et al., 2008; Liang et al., 2010). Many other DUBs such as Cezanne, USP11, USP15 and USP2a also participate in the control of IL1R/TLR4-mediated NF-κB signaling (Enesa et al., 2008; He et al., 2012; Schweitzer et al., 2007; Sun et al., 2010). To screen for new DUBs for TRAF6-mediated IL-1β/TLR-induced NF-κB activation, in *chapter 4* an NF-κB luciferase reporter was co-transfected with individual DUBs. USP4 came out as a negative regulator as it decreased IL-1β- induced NF-κB and AP-1 activation. In line with this, depletion of USP4 potentiated IL-1β/LPS-induced NF-κB signaling activation in multiple cell lines. We also showed that the activity of key downstream regulators and NF-κB target genes are modulated by USP4. Furthermore, NF-κB signaling activation was found to be increased in *USP4* knockout MEF cells. Importantly, analysis of mRNA isolated from LPS-treated zebrafish showed that *USP4*-depleted zebrafish produced more TNFα, TNFβ and Il-1β mRNA than control zebrafish. Moreover, *USP4*-depleted zebrafish were more susceptible to LPS-induced lethality indicating that USP4 also functions as a negative regulator of TLR/IL-1R signaling *in vivo*.

When we investigated how USP4 regulates TRAF6-mediated NF-κB and AP-1 activation, we found that TRAF6 is a substrate of USP4 (*chapter 4*). TRAF6 was found to

interact with USP4 when we performed immunoprecipitation assays of ectopic DUBs and endogenous TRAF6. Consistent with its role as a deubiquitinase, overexpression of wild-type USP4 but not of the catalytic inactive mutant USP4-C311S strongly reduced polyubiquitination of TRAF6, which could explain why USP4 inhibits IL-1R/TLR4-mediated NF-κB activation. In addition, depletion of USP4 potentiates ubiquitination of TRAF6.

The fact that both UBE2O and USP4 decrease both lysine 48- and lysine 63-polyubiquitinated TRAF6, leaves a question about the role of UBE2O and USP4 on lysine 48 polyubiquitination of TRAF6, since endogenous TRAF6 remains unchanged upon UBE2O and USP4 misexpression.

3 LRP8 positively regulates Wnt/β-catenin signaling and promotes osteoblast differentiation

The Wnt signaling pathway is highly conserved during evolution. It has a crucial role in controlling embryonic development and adult tissue regeneration in many animal species (Cadigan and Nusse, 1997). Wnts are secreted proteins that bind to the Frizzled (Fz)/low density lipoprotein (LDL) receptor-related protein (LRP) complex at the membrane of their target cells, and have diverse roles in regulating cell proliferation, differentiation, migration and related activities (Klaus and Birchmeier, 2008). De-regulation of this signaling pathway has been implicated in many human diseases, including bone dysfunction (Krishnan et al., 2006; Rawadi and Roman-Roman, 2005; Westendorf et al., 2004).

To identify possible co-receptors for Wnt3a-induced signaling, a luciferase reporter screen for Wnt3a/β-catenin was performed. 113 siRNAs that target genes encoding membrane-associated proteins were analysed (*chapter 5*). A protein called LRP8 which shares some conserved domains with the Wnt co-receptors LRP5 and 6 was found as a Wnt3a co-receptor candidate. LRP8 was found to be an important determinant for Wnt3a-induced signaling. Interestingly, combined knockdown of both LRP5, 6 and 8 almost completely abolished Wnt3a-induced signaling compared to single knockdown, suggesting a compensation mechanism of LRP5/6 and LRP8. This might also explain why there is no strong bone disorder phenotype in LRP8 knockout mice (Robertson et al., 2009).

Canonical Wnt signaling is mediated by a transcription co-factor β-catenin, which undergoes cytoplasmic-nuclear shuttling and constitutive degradation in the absence of ligand. Upon ligand binding, the Wnt receptors recruit the β-catenin degradation complex Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, and Adenomatous Polyposis Coli (APC) to the plasma membrane, resulting in inhibition of the degradation of β-catenin. Consequently, β-catenin accumulates in the nucleus and interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to affect transcription (MacDonald et al., 2009). In our assays to elucidate the mechanism by which LRP8 influences Wnt3a signaling we demonstrated that overexpression of LRP8 can induce nuclear localization of β-catenin without exogenous Wnt3a treatment. In the next step, the interaction between LRP8 and components of the Wnt pathway was checked. Interestingly, we found strong interaction

between LRP8 and Axin, which is an adapter for β-catenin degradation. However, unlike LRP5/6, LRP8 has no conserved PPPSP motifs that provide docking sites for Axin molecules (Tamai et al., 2004). Yet we found that LRP8 lacking its intracellular domain had no interaction with Axin. In addition, our unpublished observations showed that LRP8 interacts with Frizzeled and Wnt3a. Therefore, we speculate that Wnt3a induces LRP8 to mediate membrane localization of the degradation complex that inhibits the degradation of β-catenin, resulting in β-catenin-dependent transcriptional responses.

Wnt3a/β-catenin signaling has important roles in the control of osteoblast differentiation and bone formation (Krishnan et al., 2006; Rawadi and Roman-Roman, 2005; Westendorf et al., 2004). In *chapter 5* we also used mouse myoblast C2C12 and KS483 osteoprogenitor cells to investigate LRP8 function in Wnt3a-mediated osteoblast differentiation. We found that forced expression of LRP8 potentiates Wnt3a-induced expression of early and late osteoblast marker genes. Wnt3a-induced ALP activity and mineralization also showed an increase upon LRP8 overexpression. Finally, in line with the above findings, depletion of LRP8 decreased Wnt3a-induced signaling and osteoblast differentiation. Given the facts that there is no obvious bone disorder in LRP8 knockout mice and single LRP8 knockdown is not enough to fully inhibit Wnt3a-induced signaling, we speculate that LRP5/6 can function compensatively in cells lacking LRP8. Thus, investigation of bone formation, remodeling and function in LRP8 and LRP5/6 double knockout and triple knockout mice would be essential for examining the role of LRP8 in osteoblast differentiation *in vivo*.

Concluding remarks

By identifying and studying novel regulators, the studies described in this thesis give substantive insights into the molecular mechanisms and different levels of control of TGF-β/BMP, IL-1β and Wnt signaling pathways. Crucially, our work for the first time demonstrated the monoubiquitination of an I-SMAD by an E2-E3 hybrid, and added an important unraveled mechanism of how monoubiquitination could affect TGF-β/BMP signaling. We found that UBE2O participates in IL-1R/TLR4-mediated NF-κB activation via a different mechanism than in BMP signaling. Future studies of ubiquitin enzymes will benefit from our model in *chapter 3*, which shows that UBE2O disrupts the interaction between TRAF6 and its upstream adapter MyD88 to limit polyubiquitination of TRAF6. Our studies in this thesis also added a new DUB to the list of deubiquitinases that can regulate IL-1R/TLR4 signaling, emphasizing the importance of controlling ubiquitination status in regulating NF-κB activation. Also, we reported a new co-receptor for Wnt3a-induced signaling, suggesting compensating roles for co-receptors in the control of Wnt signaling. Therefore, the studies in this thesis yield more perception in understanding how TGF-β/BMP, IL-1R/TLR4 and Wnt signaling pathways can be regulated depending on the cell type, cell localization and cell state.

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