Protein ubiquitination in auxin signaling and transport

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Chapter 1

Regulation of auxin transport and signaling through protein ubiquitination

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Introduction

Charles Darwin's observations on bending of *Avena* coleoptiles towards the light at the end of the 19th century led him to conclude that some compound synthesized in the apical part of the coleoptile is transported to the lower part to regulate its directional growth (Darwin, 1880). Years later these initial findings led to isolation of the plant hormone auxin and its characterization as the compound indole-3-acetic acid (IAA) (Went, 1937). Since its discovery, many aspects of auxin biology have been extensively studied, from its biosynthesis and metabolism to its transport-driven asymmetric distribution and the elucidation of molecular components of downstream signaling. Based on these studies we know now that auxin plays a central role in diverse developmental processes throughout a plant's life cycle, by regulating cell division, growth and differentiation. The physiological effects of auxin are wide and complex. Application of exogenous auxin to plant cells leads to immediate responses, such as an increase in intracellular calcium levels, cell wall acidification, and changes in membrane potentials and enzyme activities, which are followed by changes in gene expression. In the context of the whole plant these changes regulate patterning processes, apical dominance, and root growth, and mediate lateral root- and fruit initiation, among others (Delker *et al.*, 2008; Benjamins and Scheres, 2008). In this chapter we shortly review the current knowledge on auxin transport, auxin response and protein ubiquitination.

Auxin transport

Using radioactively labeled auxin, it was observed that IAA is transported from cell to cell in a unidirectional manner. In the 1970s different hypotheses about the mechanism converged into the chemiosmotic model for polar auxin transport (PAT). This model postulates that due to the relatively acidic extracellular pH (5.5), a portion of the free IAA in the apoplast is in its protonated form (IAAH) that can pass the plasma membrane by import carriers, or freely by diffusion. In the more basic cytoplasmic environment (pH 7.0) auxin ionizes to form the anion IAA^- that cannot freely pass the plasma membrane, and becomes trapped inside the cell. The only way these IAA^- anions can exit

the cell is by auxin efflux carriers, and polar placement of such carriers in the plasma membrane will give directionality to the transport (Rubery and Sheldrake, 1973; Raven, 1975). In the past decade, the molecular elements in this model have been identified. The AUX1/LAX family of auxin permeases were shown to act as auxin import carriers (Swarup *et al.*, 2004; Yang *et al.*, 2006) that were first predicted and later shown to be important enhancers of PAT (Kramer, 2004; Swarup *et al.*, 2008; Bainbridge *et al.*, 2008). On the other hand, PIN FORMED (PIN) proteins were identified along with several ABC transporter-like phosphoglycoproteins (PGPs) to act as the auxin efflux carriers (Petrasek *et al.*, 2006; Bandyopadhyay *et al.*, 2007; Mravec *et al.*, 2008). The PIN proteins were named after mutants of the *PIN FORMED/PIN1* gene, which form pin-like inflorescence that develop only few or no flowers or other lateral organs (Okada *et al.*, 1991). The Arabidopsis PIN protein family comprises 8 members, six of which contain two transmembrane domain regions intervened by a large central hydrophilic loop (HL). The role of the HL-containing proteins PIN1, PIN2, PIN3, PIN4 and PIN7 in plant development has been well established, and apart from their specific function there is also considerable functional redundancy between the corresponding genes (Tanaka *et al.*, 2006; Vieten *et al.*, 2007). All five proteins show tissue-specific polar distribution at the plasma membrane (Tanaka *et al.*, 2006) that dictates of the direction of auxin flow through their asymmetric subcellular localization (Wisniewska *et al.*, 2006a). The function of PIN6 is still elusive, and also for PIN5 and PIN8 that lack a large HL no function has been reported.

Of all the Arabidopsis *pin* loss-of-function mutants, *pin1* is most severely affected in development with the needle-like inflorescences as most striking phenotype (Okada et al, 1991). This already indicated a crucial role for PIN1 in shoot development, and more detailed analysis has shown that PIN1-driven auxin transport in the epidermis of the shoot apical meristem generates auxin maxima that are responsible for the initiation of new organs and thus for phyllotactic patterning (Reinhardt et al., 2003; Heisler et al. 2005). An extensive screen for pin-formed mutants has revealed two allelic groups with a similar phenotype, and besides new *pin1* alleles the screen identified *pinoid (pid)*

mutants that all carry mutations in a gene coding for a protein serine-threonine kinase (Bennett *et al.*, 1995; Christensen *et al.*, 2000; Benjamins *et al.*, 2001). In *pid* loss-of-function plants PIN1 proteins were found at the basal, instead of apical, side of epidermal cells in the shoot meristem, explaining the defective organogenesis leading to the pin-formed phenotype (Friml *et al.*, 2004). The fact that in PID overexpressing roots PIN1, PIN2 and PIN4 were found at the apical side of cells, confirmed that PID is a central regulator of PIN polarity and auxin transport (Friml *et al.*, 2004). Recently, the PID kinase has been shown to directly phosphorylate the HL of PIN proteins, and to act antagonistically with the PP2A protein phosphatases on the phosphorylation status of PIN proteins (Michniewicz *et al.*, 2007). The current model defines that PID regulates polar auxin transport by controlling PIN localization, and thereby determines the direction of auxin flow (Benjamins *et al.*, 2001; Friml *et al.*, 2004). The role of calcium as a second messenger in modulating auxin responses and PAT is well established. Evidence that calcium is one of the early signals in auxin response came from experiments on maize coleoptile cells (Gehring *et al.*, 1990; Felle *et al.*, 1991), parsley cells, maize and pea roots (Gehring *et al.*, 1990). A rapid increase in the cytosolic calcium concentration is detected within minutes after auxin application. Early studies on sunflower stem sections showed that PAT was abolished by the presence of calcium chelators and restored by application of calcium solutions, which suggested an important role for calcium in the regulation of PAT (la Fuente and Leopold, 1973). A molecular link connecting calcium and PAT was found by the identification of the calcium-binding proteins PINOID BINDING PROTEIN1 (PBP1) and TOUCH3 (TCH3) as interacting proteins of PID (Benjamins *et al.*, 2003). Neither of the calcium-binding proteins is a phospho-target of PID but both regulate PID kinase activity. TCH3 is a negative regulator of PID activity, whereas PBP1 positively regulates the kinase *in vitro* (Benjamins *et al.*, 2003; Robert-Boisivon, 2008). TCH3 is a Calmodulin-like protein with six EF-hand domains encoded by a touch-responsive gene while PBP1 is a small protein with a single EF-hand (Braam and Davis, 1990; Sistrunk *et al.*, 1994). PBP1 was also named KRP2 (for KIC-related protein2), as it is part of a small protein family that includes KIC (KCBP-interacting

Calcium binding protein) and the close PBP1 homolog PBP1H/KRP1 (Reddy *et al.*, 2004). PID belongs in the AGC3 clade of the AGCVIII (cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C) kinase family together with three other members: WAG1 (WAVY ROOT GROWTH1), WAG2 and AGC3-4 (Galvan-Ampudia and Offringa, 2007). Functional analysis of WAG1 and WAG2 has indicated that these kinases play roles in root growth. The enhanced root growth sensitivity of *wag1wag2* loss-of-function seedlings to the PAT inhibitor NPA (Santner and Watson, 2006), together with the fact that WAG kinases, like PID, are membrane-associated suggests that they may also be involved in the regulation of polar targeting of PIN proteins (Galvan-Ampudia and Offringa, 2007). In fact WAG1 and WAG2 are able to phosphorylate PINs *in vitro* more efficiently than PID in some cases (Galvan-Ampudia, C.; unpublished). Many components have been identified regulating auxin transport and polarity maintenance in plants, from the auxin efflux carriers PINs and PGPs (Wisniewska *et al.*, 2006b; Mravec *et al.*, 2008), influx facilitators like AUX1 (Swarup *et al.*, 2001) to regulators of PIN polarity through phosphorylation/dephosphorylation like PID, WAGs, D6PK and RCN1 (Friml *et al.*, 2004; Michniewicz *et al.*, 2007; Galvan-Ampudia and Offringa, 2007; Zourelidou *et al.*, 2009) or cycling/stability processes regulated by GNOM, COP9 and the 26S proteasome (Geldner *et al.*, 2003; Abas *et al.*, 2006; Laxmi *et al.*, 2008). A complete understanding of phosphorylation- and cycling-dependent polarity maintenance explaining the way these processes proceed and interact *in planta* is still lacking The components discussed above are responsible for transporting auxin to the cells were it activates the responses essential for plant development. The way auxin is perceived by plant cells will be discussed below.

Perception: auxin-responsive gene expression

The polar transport-generated auxin maxima and gradients are instructive for plant cell growth and differentiation. At the cellular level, auxin concentrations are translated into a gene expression response by the complex and dynamic interaction between two large families of transcriptional regulators: the Auxin

Response Factors (ARFs) and the labile Aux/IAA proteins (Guilfoyle *et al.*, 1998b; Ulmasov *et al.*, 1999; Tiwari *et al.*, 2001).

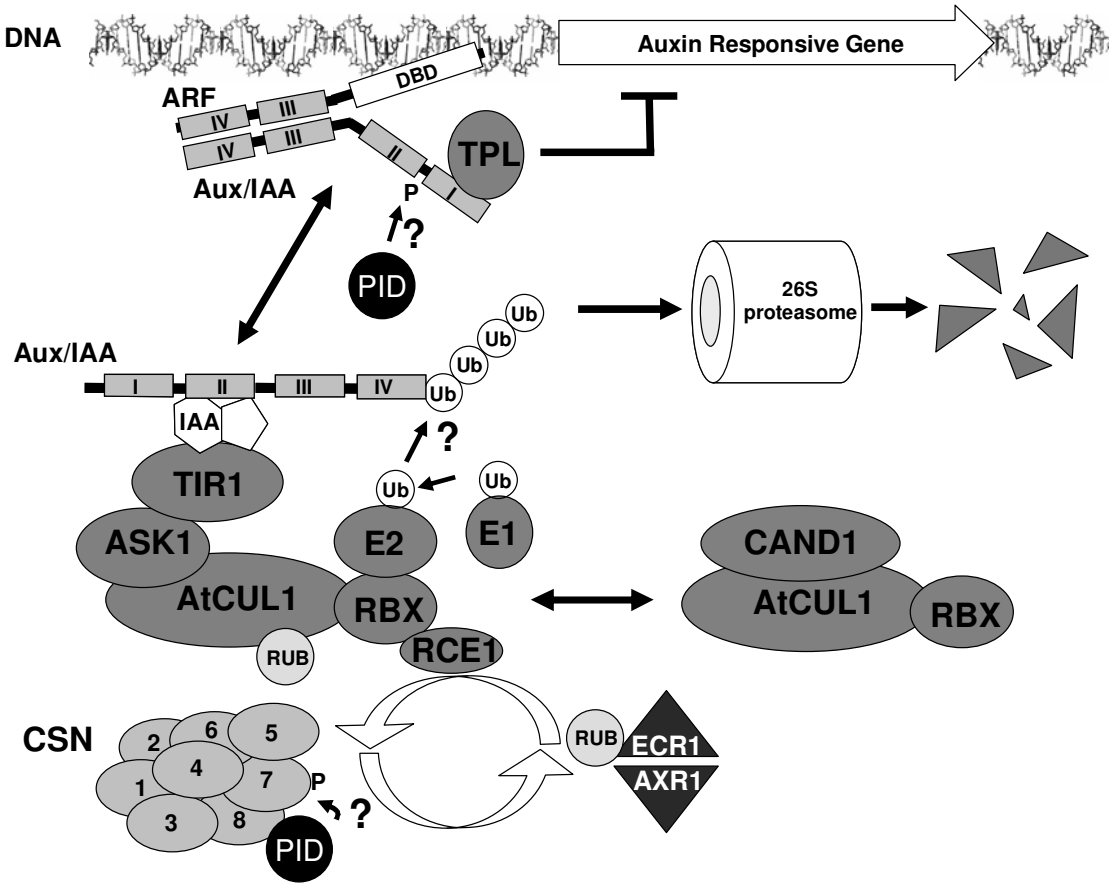

Figure 1: Mechanism of auxin perception by the SCF^{TIR1} E3 ubiquitin ligase in *Arabidopsis thaliana*. Aux/IAA proteins are labeled for proteolysis by ubiquitination. This process is mediated by the ubiquitin activating enzyme E1, the ubiquitin conjugating enzyme E2 and the ubiquitin ligase E3. Under low auxin concentrations Aux/IAAs proteins heterodimerize with the ARF transcription factors, thereby repressing auxin-inducible gene expression trough association with the co-repressor TPL. Auxin binding to TIR1 stimulates its interaction with the domain II of Aux/IAAs which leads to their proteasomal degradation, presumably preceded by Aux/IAA ubiquitination, releasing ARF-dependent transcription. PID-dependent phosphorylation of BDL/IAA12 close to domains I and II might impair TIR1 binding and/or TPL association. The CSN complex can cleave the RUB modifier from CUL1, thus facilitating CAND1 binding to CUL1 and SCF disassembly. PID interacts with the CSN8 subunit of COP9 and phosphorylates CSN7 *in vitro*. Conjugation of RUB to CUL1 by the AXR1-ECR1 and RCE1 enzymes might free CUL1 from CAND1, promoting re-assembly of the active complex. DBD, DNA-binding domain; Ub, ubiquitin; IAA, indole-3-acetic acid. For other abbreviations see text.

ARF proteins bind to specific sequences in the promoters of auxin-responsive genes through their N-terminal DNA-binding domain, and either activate or repress transcription. At the C-terminus they share the conserved domains III and IV with the Aux/IAA proteins, through which they homo- or heterodimerize with other ARFs or with the Aux/IAA proteins (Figure 1) (Guilfoyle *et al.*, 1998a; Guilfoyle *et al.*, 1998b). Several lines of evidence indicate that Aux/IAA proteins do not bind DNA directly, but function as transcriptional repressors by heterodimerizing with activating ARFs (Ulmasov *et al.*, 1997; Kim *et al.*, 1997; Guilfoyle *et al.*, 1998a). Most Aux/IAA proteins are short-lived and degradation of Aux/IAA proteins is essential for auxin signaling. Their half-lives and abundance are dramatically reduced by auxin as a primary response and this process can be blocked by treatment with proteasome inhibitors (Worley *et al.*, 2000; Ramos *et al.*, 2001). Aux/IAA proteins act as transcriptional repressors through the EAR motif present in the conserved domain I (Tiwari *et al.*, 2004) that was shown to mediate the interaction of BDL/IAA12 with the co-repressor TOPLESS (Szemenyei *et al.*, 2008). This interaction seems to be essential for the repressive activity of BDL/IAA12, as the *tlp-1* mutation is able to rescue the rootless *bdl* phenotype. BDL/IAA12 is known to interact and inhibit the activity of the MP/AFR5 transcriptional activator (Hamann *et al.*, 2002). A translational fusion of TOPLESS with domains III and IV of BDL/IAA12 resulted in *bdl/mp*-like phenotypes (Szemenyei *et al.*, 2008). These results indicate that one of the functions of BDL/IAA12 is to bridge the ARF-TLP interaction, which is disrupted upon BDL/IAA12 degradation (Figure 1).

It is clear now that transcriptional and developmental responses to auxin are sensitive to the levels of Aux/IAA proteins (Worley *et al.*, 2000; Ramos *et al.*, 2001; Zenser *et al.*, 2003; Dreher *et al.*, 2006). Several Arabidopsis mutants displaying diminished auxin responses were found to have gain-of-function mutations in *Aux/IAA* genes (Figure 2). Strikingly, all these mutations affect specific sites in domain II, and lead to extended protein half-life and presumably much greater abundance of the respective Aux/IAA proteins (Worley *et al.*, 2000; Ouellet *et al.*, 2001). The conserved domain II of Aux/IAA proteins contains a 13 amino acid sequence that functions as a transferable degradation

signal, and it is necessary and sufficient to define Aux/IAA protein stability (Ramos *et al.*, 2001). This motif was defined as QVVGWPPVRSYRK, underlined residues indicate those conserved among all the domain II-containing Arabidopsis Aux/IAAs.

Aux/IAA gene	Domain II mutations	References
AXR2/IAA7	PAKAQVV**GWPPV**RN	
arx2-1	**S**	Nagpal *et al.*, 2000
AXR3/IAA17	PAKAQVV**GWPPV**RS	
axr3-1	**L**	
axr3-3	**G**	Rouse *et al.*, 1998
axr3-101	**E**	Okushima *et al.*, unpub.
SHY2/IAA3	PPRKAIV**GWPPV**RS	
shy2-1, -2	**S**	
shy2-3	**E**	Tian and Reed, 1999
shy2-6	**L**	Fukaki *et al.*, unpub
SLR1/IAA14	PPAKAVV**GWPPV**RN	
slr1-1, -4	**S**	Fukaki *et al.*, 2002
slr-2	**S**	
slr-3	**A**	Fukaki *et at.* unpub
IAA28	VEVAPVV**GWPPV**RS	
iaa28-1	**L**	Rogg *et al.*, 2001
MSG2/IAA19	PAAKASV**GWPPV**CS	
msg2-1	**S**	
msg2-2	**R**	
msg2-3	**L**	
msg2-4	**L**	Tatematsu *et al.*, 2004
BDL/IAA12	PPRSSVV**GWPP**IGL	
bdl	**S**	Hamann *et al.,* 2002
IAA13	PPRSSVV**GWPP**IGL	
iaa13	**S**	Weijers *et al.*, 2005
IAA18	TAPGPVV**GWPPV**RS	
crane-1	**R**	
crane-2	**E**	Uehara *et al.* 2008
SHY1/IAA6	PVVKSAV**GWPPV**CS	
shy1-1	**R**	Reed, 2001
ARX5/IAA1	PPAKTQIV**GWPPV**R	
iaa1-GR	**L**	Park *et al.*, 2002
axr5-1	**S**	Yang *et al.*, 2004

Figure 2: Amino acid substitutions in domain II that stabilize Aux/IAA proteins

This domain interacts with the F-box-protein TIR1 and the interaction is promoted by auxin in a concentration-dependent manner leading to Aux/IAA proteasomal degradation (Gray *et al.*, 2001; Dharmasiri *et al.*, 2003). For a long time it was thought that the degron was modified upon auxin treatment. Recently, however it was uncovered that TIR1 binds auxin and this enhances the interaction with the Aux/IAA proteins (Figure 1). The Aux/IAA proteins bind

TIR1 in the absence of auxin, but with low affinity (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a). Crystallographic studies showed that the auxin molecule acts as "molecular glue" between TIR1 and its substrate, binding both proteins and facilitating hydrophobic packing between TIR1 and its substrate (Tan *et al.*, 2007). The conserved central GWPPV motif is the hallmark of the Aux/IAA degron. Two amino acids in the motif, tryptophan and the second proline, interact with the surrounding hydrophobic wall of the TIR1 pocket and stack against the auxin molecule lying underneath, packing against the auxin indole ring and the auxin side chain, respectively. In the structure, the glycine residue is located at a critical position, where flexibility of the peptide is required for the N-terminal region of the substrate peptide to take a sharp turn and continue interacting with TIR1, indicating that the integrity and hydrophobicity of domain II is crucial for TIR1 recognition. Aux/IAA domain II mutants were identified with these core amino acids changed into the acidic residue glutamic acid (Tian and Reed, 1999; Uehara *et al.*, 2008; Ploense *et al.*, 2009), indicating that the acidic modification (i.e. phosphorylation) of domain II is a plausible mechanism for reducing TIR1-Aux/IAA interaction (Figure 2).

TIR1 is the first true auxin receptor described, acting alongside other members of the AFB (Auxin F-box protein) family to form $SCF^{TIR1/AFB}$ complexes that control auxin-dependent degradation of Aux/IAA proteins (Dharmasiri *et al.*, 2005b). The dependence on SCF^{TIR1} and the 26S proteasome suggests that Aux/IAA proteins are degraded via the ubiquitin-dependent pathway, although direct evidence for this post-translational modification is lacking. Ubiquitin dependency of proteasomal degradation will be discussed in more detail below.

Proteasomal degradation and ubiquitination.

Much of cellular physiology, growth, and development are controlled by the selective removal of regulatory proteins. Like all macromolecular components of an organism, the proteome is in a dynamic state of synthesis and degradation. In eukaryotic organisms, ubiquitin conjugation to target proteins and

subsequent degradation by the proteasome plays an important role in diverse cellular processes.

Ubiquitin is a highly conserved 76 amino acid (~9 kDa) protein that is abundantly expressed in all eukaryotic cells. Protein ubiquitination is a multistep process, involving at least three types of enzymes and generally results in the covalent attachment of poly-ubiquitin chains to target proteins. A prominent role of poly-ubiquitin chains is that they label proteins for degradation by the proteasome (Figure 1). As a first step in the ubiquitination process, an ubiquitin-activating enzyme (also known as E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent process. Then, a ubiquitin-conjugating enzyme or ubiquitin-carrier enzyme (UBC, also known as E2) accepts ubiquitin from the E1 by a *trans*-thiolation reaction, again involving the glycine at the carboxy-terminus of ubiquitin. Finally, an ubiquitin protein ligase (E3) catalyses the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the substrate (Glickman and Ciechanover, 2002). Chains containing at least four glycine-76 to lysine- 48 isopeptide-linked ubiquitins are necessary for efficient binding to the component S5a/Rpn10 of the proteasome (Baboshina and Haas, 1996; Thrower *et al.*, 2000). The quaternary structure of ubiquitin polymers and the exact spatial relationship between each ubiquitin molecule is also critical for their ability to target substrates for degradation by the proteasome.

The 26S proteasome is a 2.5-MDa ATP-dependent proteolytic complex that mostly degrades ubiquitin conjugates (Voges *et al.*, 1999). It contains 32 principal subunits arranged into two subcomplexes, the 20S core protease (CP) and the 19S regulatory particle (RP). The 20S core subunit bears a broad spectrum ATP- and Ub-independent protease activity. The active sites of the CP are very sensitive to the proteasome inhibitors MG115, MG132, lactacystin, and epoxomycin (Yang *et al.*, 2004). The 19S RP associates with one or both ends of the CP and confers both ATP-dependence and specificity for Lys48-linked polyubiquitin chains to the particle. ATP-ase and de-ubiquitinase (DUB) activities associated to 19S RP subunits are responsible for protein unfolding and directing the unfolded de-ubiquitinated polypeptides into the lumen of the

CP for breakdown. Posttranslational modification of proteins by covalent attachment of ubiquitin is a reversible process and the processed ubiquitins are recycled to be re-used by the cell. All known deubiquitinating enzymes (DUBs) are cysteine proteases that specifically hydrolyze the amide bond immediately after the COOH-terminal residue. Based on their molecular size, sequence homology, and active site residues, DUBs are categorized as UCHs (ubiquitin COOH-terminal hydrolases) or UBPs (ubiquitin-specific proteases) (Nijman *et al.*, 2005). UCHs are generally small enzymes (20–30 kDa) that remove short or flexible peptide chains from the COOH terminus of ubiquitin. UBPs on the other hand belong to a larger and a more diverse group of enzymes and have a larger molecular mass, typically in the range of ~100 kDa. UBPs can cleave the isopeptide bond linking Ub-Ub or Ub-protein. Despite the common active site residues, the UBP and UCH families do not share sequence homologies with one another or with cysteine proteases (D'Andrea and Pellman, 1998; Chung and Baek, 1999). DUBs play several roles, both in maintaining the levels of free ubiquitin and in regulating the stability of Ub-conjugated proteins (Nijman *et al.*, 2005), including Aux/IAAs in Arabidopsis (Yang *et al.*, 2007).

Ubiquitin was first identified as a covalently attached signal to proteins targeted for degradation (Hershko *et al.*, 1982). Polyubiquitin chains linked *via* Lys-48 are the principal signals recognized and degraded by the proteasome. It has, however, now been realized that ubiquitination not only regulates intracellular proteolysis, but also diverse processes such as transcription, receptor-mediated signal transduction and endocytotic sorting (Mukhopadhyay and Riezman, 2007). Ubiquitination on Lys-63 of ubiquitin appears to play a role in a variety of processes not involving proteolysis including endocytosis of cell surface receptors (Hicke, 1999), post-replicative DNA repair (Spence *et al.*, 1995), stress response (Arnason and Ellison, 1994), mitochondrial DNA inheritance (Fisk and Yaffe, 1999), ribosomal function (Spence *et al.*, 2000), and activation of the IκBα signaling complex (Wang *et al.*, 2001). Mono-ubiquitination also plays important non-proteolytic roles such as endocytosis (Terrell *et al.*, 1998) and control of gene transcription (Pham and Sauer, 2000).

The necessity of ubiquitin modification as a signal for proteasomal degradation was challenged by the discovery of ornithine decarboxylase (ODC) which is degraded via an ubiquitin-independent process (Figure 3). ODC is a rate-limiting enzyme in polyamine biosynthesis and accumulation of polyamines stimulates the synthesis of the antizyme protein. Antizyme in turn was found to negatively regulate ODC by binding, which is sufficient to trigger ODC degradation by the 26S proteasome (Coffino, 2001). Non-ubiquitinated ODC monomers are degraded by 20S proteasomes in a process regulated by NAD(P)H Quinone Oxidoreductase1 (NQO1) (Asher *et al.*, 2005a). The REGγ alternative lid complex of the proteasome was shown to mediate the ubiquitin-independent degradation of the mammalian cell cycle regulator $p21^{CIP21}$ (Chen *et al.*, 2007). Moreover, the tumor suppressor proteins p53 and p73 are degraded by the proteasome in an ubiquitination-independent manner, and association with the 20S proteasome gatekeeper NQO1 blocks this degradation (Asher *et al.*, 2005b). The regulation of inherently unstable proteins like ODC, p53 and p73 was proposed to follow a "degradation by default" mechanism (Asher *et al.*, 2006) where degradation occurs unless specific intervention with NQO1 or homodimerization, in the case of ODC, prevents it. What is clear from these examples from the animal research field is that proteasomes have diverse ways for target recognition (Figure 3).

Considering the amount of examples of different ubiquitin modifications and the downstream effects of these processes, it is now clear that ubiquitination is much more than a proteasomal targeting signal. How it mediates responses to DNA damage, facilitates endosomal transport, and increases the efficiency of translation are all open questions. The genome of *Arabidopsis* encodes more than 1400 (or >5% of the proteome) ubiquitin pathway components, illustrating the importance of the ubiquitin pathway in the regulatory plasticity of plants (Lechner *et al.*, 2006).

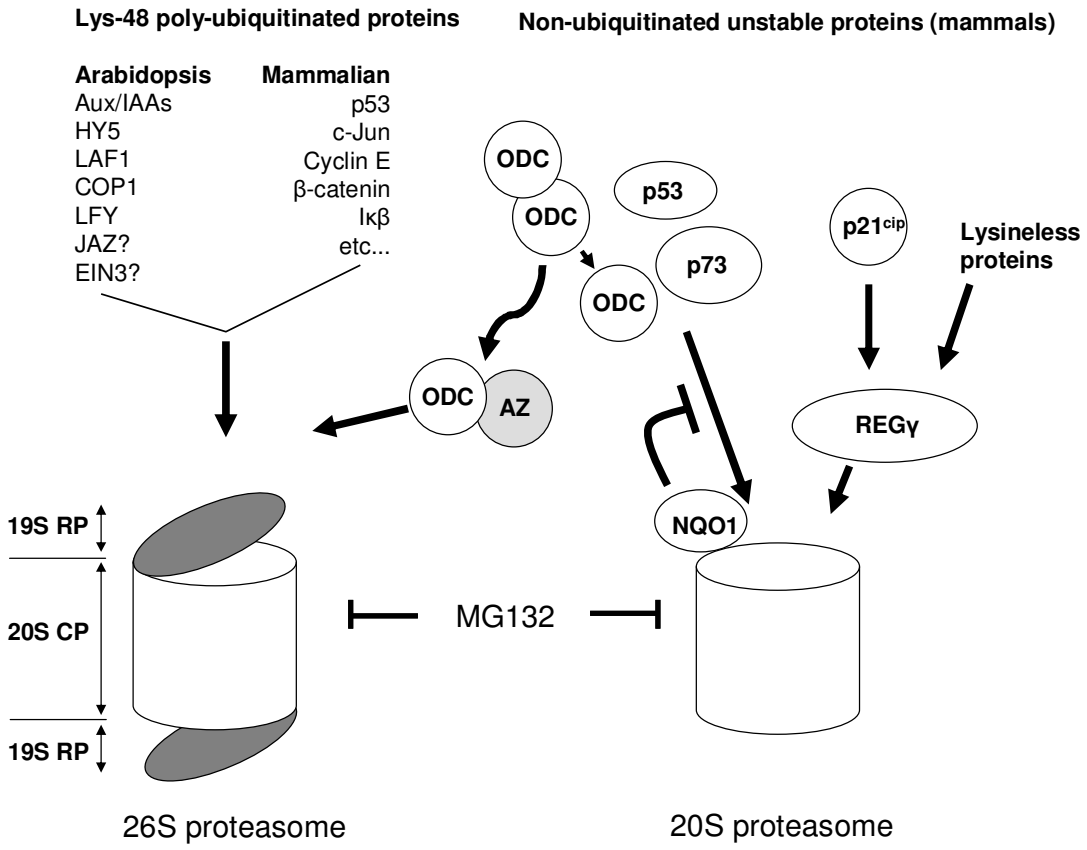

Figure 3: Schematic representation of ubiquitin-dependent and -independent pathways of proteasomal degradation. Proteins containing Lys-48 linked poly-ubiquitin chains are recognized by the 19S regulatory particle (RP) of 26S proteasomes and degraded in the proteolytically active 20S core particle (CP). Examples of *Arabidopsis* proteins experimentally demonstrated to be ubiquitinated are shown together with the presumably Ub-modified JAZ and EIN3 proteins. Classical examples of mammalian ubiquitinated proteins degraded by the 26S proteasome are shown. Some unstable proteins are degraded via ubiquitin-independent processes in mammals. Binding of antizyme (AZ) to ODC disrupts ODC homodimers and induces ubiquitin-independent 26S proteasomal degradation of ODC. Free ODC monomers are also degraded by 20S proteasomes without ubiquitination. NQO1 functions as a gatekeeper of 20S proteasomes and interacts with p53, p73 and ODC in a NADH-dependent manner to protect them from 20S proteasomal degradation. The REGγ complex acts as an alternative lid of 20S proteasomes and controls the degradation of $p21^{cip}$ and other lysine-less proteins. Both 20S and 26S degradation pathways are inhibited by 20S CP proteasome inhibitors like MG132.

CULLIN1 containing (SCF) E3 ligases and hormone responses in plants

Ubiquitin is a widespread cellular signal and, as described above, ubiquitin conjugation is achieved through an ATP-dependent reaction cascade involving the sequential action of three enzymes, E1, E2s, and E3s. E1 activates

ubiquitin, E2s catalyze covalent attachment of ubiquitin to target proteins which are recognized by associated E3s. As the final enzyme in the cascade, the E3s or Ubiquitin-protein ligases are responsible for recognizing the substrate and facilitating Ub transfer, determining the specificity of the response. Different types of E3s have been identified that differ according to their subunit organization and/or mechanism of Ub transfer (Deshaies, 1999). One important E3 type comprises the SCF complexes which are composed of four major subunits: CULLIN 1 (CUL1), SUPPRESSOR OF KINETOCHORE PROTEIN 1 (SKP1), RING-BOX 1 (RBX1)/REGULATOR OF CULLINS 1 (ROC1) and an F-box protein (Figure 1). Structure–function studies in yeast and mammals have demonstrated that CUL1 functions as a scaffold in assembling the different subunits of the complex. CUL1 interacts at its carboxyl terminus with the RING-domain protein RBX1 (forming the core catalytic domain) and, at its amino terminus, with the adaptor protein SKP1, which links to one of multiple F-box proteins. F-box proteins, in addition to the loosely conserved F-box motif that binds to SKP1, usually carry one of a variety of typical protein–protein interaction domains that confer substrate recognition specificity to the SCF complexes. The large number of F-box proteins in plant genomes, nearly 700 in Arabidopsis (Gagne *et al.*, 2002), is thought to allow for the specific ubiquitination of a large number of functionally and structurally diverse substrates.

Besides the role of the $SCF^{TIR1/AFB}$ E3 ligases in auxin responses, SCF complexes also regulate other phytohormone signaling pathways, including those for jasmonate, gibberellin and ethylene. The jasmonate perception mechanism involves the F-box protein, COI1. Since its discovery, the *coi1* mutant was regarded as the strongest jasmonate-insensitive mutant (Feys *et al.*, 1994; Xie *et al.*, 1998). The F-box protein COI1 was shown to assemble into an active SCF complex (Devoto *et al.*, 2002; Xu *et al.*, 2002) but its targets remained unknown until the discovery of JAZ repressors. JAZ (Jasmonate-ZIM domain) proteins represent a family of labile proteins which are postulated to negatively regulate the expression of jasmonate-responsive genes via their interaction with the activator MYC2. (Chini *et al.*, 2007). Their stability is

regulated by SCF^{COI1} and the 26S proteasome (Thines *et al.*, 2007; Chini *et al.*, 2007). Jasmonate-Isoleucine (JA-Ile) was shown to stimulate the interaction of certain members of the JAZ family with COI1 *in vitro* and in yeast (Thines *et al.*, 2007; Melotto *et al.*, 2008). This is postulated to lead to JAZ proteasomal degradation supposedly via ubiquitination. COI1 is the closest relative to the TIR1/AFB clade of F-box proteins and their mechanism of action is very similar since COI1 (or the COI1-JAZ complexes) was found to be the receptor for Jasmonate-Isoleucine/Coronatine analogous to TIR1 for auxin (Spartz and Gray, 2008; Katsir *et al.*, 2008a; Katsir *et al.*, 2008b). The gibberellin (GA) signaling pathway is regulated in Arabidopsis by the F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNE) (McGinnis *et al.*, 2003; Strader *et al.*, 2004) and in rice by the F-box protein GID2 (Sasaki *et al.*, 2003). Like TIR1 and COI1, these F-box proteins are involved in the degradation of negative regulators, which in the case of GA responses are the DELLA proteins which belong to the GRAS superfamily of putative transcriptional regulators. DELLA proteins directly or indirectly repress the expression of GA-induced genes (Feng *et al.*, 2008; Daviere *et al.*, 2008). In Arabidopsis, the gibberellin molecule is recognized by the soluble receptor GID1. The interaction leads to a conformational change in the GID1 protein that traps the GA molecule inside a receptor pocket forming a closing lid. This induced modification allows DELLA proteins to interact with the upper surface of the lid, and it is hypothesized that this interaction may cause a change in the shape of the DELLA protein that allows it to associate with the ubiquitin ligase $SCF^{SLY/SNE}$. Thus, GA functions as an allosteric activator of GID1, causing structural changes that allow the receptor to associate with DELLA proteins but GA does not interact directly with DELLAs (Murase *et al.*, 2008; Shimada *et al.*, 2008).This is significantly different from the TIR1-Aux/IAA and COI1-JAZ interaction, where the signaling molecule does not induce conformational changes but rather acts as a molecular glue, in the case of TIR1, between F-box protein and the repressor. SCF-dependent degradation of transcriptional regulators is emerging as a common feature in plant developmental and adaptive responses with several examples described of interactors for the many Arabidopsis F-box proteins. Despite the emerging

importance of SCF complexes and protein degradation in plants the demonstration that the interacting partners of SCF complexes are actually ubiquitinated is missing for the vast majority of these targets.

Regulation of SCF E3 ubiquitin ligases by RUB: a role for the COP9 signalosome.

Given their importance in cellular functions, it is not surprising that SCF assembly and activity are highly regulated. So far, three proteins or protein complexes have been implicated in SCF regulation. These are the ubiquitin-related protein RUB/Nedd8 (for Related to Ubiquitin1 or Neural precursor cell expressed developmentally down-regulated 8), the COP9 signalosome (CSN), and CAND1 (for Cullin Associated Neddylation Dissociated1) (Figure 1). RUB conjugation to CUL1 is achieved by the activity of the ECR1 and AXR1/RCE1 complexes (del Pozo *et al.*, 2002) and phenotypes of different mutants indicate that rather than working as an on/off switch, the RUB cycling is essential for the assembly of SCF complexes in cooperation with CAND1 (Chuang *et al.*, 2004). CAND1 binds de-rubylated CUL1 and inhibits CUL1/RBX1 binding to SKP1 (Figure 1), thus preventing the formation of an active SCF complex (Feng *et al.*, 2004). Reducing the amount of CAND1 in cells leads to an increase in the number of complexes containing CUL1 and SKP1. The regulation of SCF E3 ubiquitin ligases is dependent on the activity of the COP9 (CSN) signalosome that cleaves RUB from the CUL1 subunit of SCF (Cope *et al.*, 2002b) releasing CAND1 from the CUL1/RBX1 complex allowing the formation of new SCF complexes (Zhang *et al.*, 2008). Based on these results, it has been proposed that CAND1, the RUB conjugation pathway, and the CSN together regulate a cycle of SCF assembly and disassembly (Cope and Deshaies, 2003; Pintard *et al.*, 2003; Parry and Estelle, 2006). One of the strongest auxin-resistant mutants is *axr1*, which is unable to conjugate RUB to CUL1 impairing the activity of the SCF^{TIR1} (del Pozo *et al.*, 2002). Modification of SCF^{TIR1} by RUB has been implicated as a central step in the response to the plant hormone auxin.

The COP9 signalosome (CSN) is a large nuclear-enriched multiprotein complex identified in genetic screens for *constitutive*

photomorphogenic/deetiolated/fusca (*cop/det/fus*) mutants in *Arabidopsis*. The COP9 signalosome consists of eight subunits, and shows intriguing structural and sequence homology to the 19S regulatory particle (RP) of the proteasome. A mutation in a single CSN subunit can destabilize the entire complex (Schwechheimer *et al.*, 2002; Serino *et al.*, 2003). All strong *cop/det/fus* mutations lead to seedling lethality shortly after germination, indicating that besides controlling light-dependent processes the integrity of the CSN complex is central to plant development (Kwok *et al.*, 1996). CSN-dependent RUB de-conjugation from CUL1 is accomplished by the CSN5 subunit which bears a metalloprotease activity necessary for RUB cleavage (Cope *et al.*, 2002a). CSN and SCF complexes are known to physically interact and it was shown that CSN5 reduction-of-function lines display a phenotype similar to that of the *axr1* mutant and slower degradation rates of Aux/IAA proteins (Schwechheimer *et al.*, 2001). Besides auxin, SCF-CSN processes are tightly connected to many cellular and developmental responses such as light, jasmonate, gibberellins, ethylene, floral organ formation, circadian rhythms, shoot branching and many others (Chamovitz *et al.*, 1996; Karniol and Chamovitz, 2000; Feng *et al.*, 2003; Wang *et al.*, 2003; Guo and Ecker, 2003; Han *et al.*, 2004; Cheng *et al.*, 2004; Stirnberg *et al.*, 2007). Considering the wide repertoire of SCF complexes that can be formed by the Arabidopsis proteome, the CSN is emerging as central regulator of E3 ubiquitin ligases in plant biology.

Thesis outline

Auxin biology is among the oldest fields of experimental plant research. Nowadays, auxin is one of the most extensively studied plant hormone. Most of its effects on regulating cell division, growth and differentiation are dependent on its transport driven asymmetric distribution. At the cellular level, the molecular components of downstream signaling still demand further investigation. Auxin action was found to be dependent on dynamic gradients generated by PIN efflux carriers' asymmetric distribution. Only recently, the F-box protein TIR1 was identified as a receptor for auxin and the link between synthesis, transport, perception and effects of auxin gained a molecular

framework to be explored. Being part of a SCF E3 ubiquitin ligase complex, the TIR1 receptor uses the ubiquitin system to control levels of auxin-dependent gene responses via degradation of the Aux/IAA transcriptional repressors. **Chapter 2** uses an Arabidopsis protoplast system to show the ubiquitination of the SHY2/IAA3 and BDL/IAA12 proteins by the SCF^{TIR1} E3 ubiquitin ligase complex. The mechanism of auxin perception based on auxin-induced Aux/IAA binding to the SCF^{TIR1} receptor was based on the supposition that this interaction would lead to Aux/IAA ubiquitination followed by 26S proteasomal degradation. Our results confirm that Aux/IAAs are ubiquitinated and the process is stimulated by TIR1 overexpression, whose protein levels determine the sensitivity of cells towards auxin and leads to Aux/IAA degradation even in the absence of auxin treatment. It is known that Aux/IAAs display differential activities on auxin-responsive gene expression and our experiments indicate that BDL/IAA12 acts as a stronger and less stable protein than SHY2/IAA3. It is likely that these differences observed might be linked to differential affinity of these proteins to the TIR1 receptor which reinforces our hypothesis on phosphorylation-dependent regulation of the BDL protein (Chapter 4).

Our concern on demonstrating the ubiquitination of Aux/IAAs was stimulated after the findings described on **Chapter 3** with the characterization of a calcium binding protein PBP1, first identified as an interactor of the serine-threonine kinase PINOID, a regulator of auxin transport. When expressed in Arabidopsis cell suspensions protoplasts, PBP1 was found to be highly unstable protein that is poly-ubiquitinated and degraded by the proteasome. Mutation of all the lysines on the PBP1 primary sequence abolishes ubiquitin attachment but does not affect the proteasomal degradation of the protein. All known biochemical functions are maintained in the lysine-less versions of PBP1. We believe that PBP1 is controlled by ubiquitin-independent proteasomal degradation confirming observations from the animal field that not all targets of proteasomal degradation are necessarily ubiquitinated proteins.

Chapter 4 describes the Aux/IAA protein BODENLOS (BDL/IAA12) as a putative *in vivo* phosphorylation target. From *in vitro* an *in vivo* studies there was indication that the two proteins could interact during plant development. *In*

vitro phosphorylation assays identified a PRSS motif as the site of phosphate modification by PID. Mutation of the two Serines on PRSS to PRKA abolishes *in vitro* phosphorylation of BDL/IAA12 by PID and analysis of transgenic plants carrying a phosphorylation-insensitive version of the gain-of-function *bdl* protein indicated that the phosphorylation site identified is essential for the function of the *bdl* protein *in planta.* Although the identified site seems to be important on the regulation of the BDL protein, the contribution of PID to the process is still hypothetical and the *in vivo* phosphorylation of this site still remains to be shown.

Chapter 5 describes the identification of CSN subunit CSN8/COP9 as an interacting partner of the PINOID kinase. PID phosphorylates the neighboring subunit CSN7/*COP15 in vitro* but further analysis showed that this phosphorylation is not relevant for CSN activity *in planta*. On the other hand, PID is ubiquitinated *in vivo* and overexpression of CSN8 induces PID nuclear localization and enhances its ubiquitination. Is discussed the possibility of PID to act as a CSN-associated kinase regulated by ubiquitination and that this interaction would control the association of PID with phosphorylation targets also controlled via ubiquitination such as BDL/IAA12 and PIN2.

The results presented on this thesis illustrate the wide spectrum of cellular processes in plants regulated though protein ubiquitination and proteasomal degradation. The confirmation of the TIR1-Aux/IAA model to be ubiquitin-dependent contrasts with the PBP1 ubiquitin-independent degradation and rises the discussion of how many proteasome targets are ubiquitinated. The large representation of F-box proteins in the Arabidopsis proteome and the many processes regulated by ubiquitin modification will demand careful interpretation of experimental results. The well-established TIR1 recognition of Aux/IAAs has to be challenged with other members of the diverse Aux/IAA family to assess the particularities of each protein as well as for the other TIR1/AFB proteins. Regulation of polar auxin transport through ubiquitin- and COP9-related processes seem to involve PINs and PID via a integrated mechanism using both phosphorylation and ubiquitination as targeting signals. During a long time

PID functions were analyzed based on the effects it has on its targets and now the regulators of PID, such as CSN, open field for a new exploration.

REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J. and Luschnig, C. (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat. Cell Biol.* **8**, 249-256.

Arnason, T. and Ellison, M.J. (1994) Stress resistance in Saccharomyces cerevisiae is strongly correlated with assembly of a novel type of multiubiquitin chain. *Mol. Cell Biol.* **14**, 7876-7883.

Asher, G., Bercovich, Z., Tsvetkov, P., Shaul, Y. and Kahana, C. (2005a) 20S proteasomal degradation of ornithine decarboxylase is regulated by NQO1. *Mol. Cell* **17**, 645-655.

Asher, G., Reuven, N. and Shaul, Y. (2006) 20S proteasomes and protein degradation "by default". *Bioessays* **28**, 844-849.

Asher, G., Tsvetkov, P., Kahana, C. and Shaul, Y. (2005b) A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. *Genes Dev.* **19**, 316-321.

Baboshina, O.V. and Haas, A.L. (1996) Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. *J Biol Chem.* **271**, 2823-2831.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T. and Kuhlemeier, C. (2008) Auxin influx carriers stabilize phyllotactic patterning. *Genes Dev.* **22**, 810-823.

Bandyopadhyay, A., Blakeslee, J.J., Lee, O.R. *et al.* (2007) Interactions of PIN and PGP auxin transport mechanisms. *Biochem. Soc. Trans.* **35**, 137-141.

Benjamins, R., Ampudia, C.S., Hooykaas, P.J. and Offringa, R. (2003) PINOID-mediated signaling involves calcium-binding proteins. *Plant Physiol* **132**, 1623-1630.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001) The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. *Development* **128**, 4057-4067.

Benjamins, R. and Scheres, B. (2008) Auxin: the looping star in plant development. *Annu. Rev. Plant Biol.* **59**, 443-465.

Bennett, S.R.M., Alvarez, J., Bossinger, G. and Smyth, D.R. (1995) Morphogenesis in Pinoid mutants of Arabidopsis thaliana. *Plant J* **8**, 505-520.

Braam, J. and Davis, R.W. (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. *Cell* **60**, 357-364.

Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, A.G., Staub, J.M., Matsui, M. and Deng, X.W. (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. *Cell.* **86**, 115-121.

Chen, X., Barton, L.F., Chi, Y., Clurman, B.E. and Roberts, J.M. (2007) Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. *Mol Cell.* **26**, 843-852.

Cheng, Y., Dai, X. and Zhao, Y. (2004) AtCAND1, a HEAT-repeat protein that participates in auxin signaling in Arabidopsis. *Plant Physiol* **135**, 1020-1026.

Chini, A., Fonseca, S., Fernandez, G. *et al.* (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature.* **448**, 666-671.

Christensen, S.K., Dagenais, N., Chory, J. and Weigel, D. (2000) Regulation of auxin response by the protein kinase PINOID. *Cell* **100**, 469-478.

Chuang, H.W., Zhang, W. and Gray, W.M. (2004) Arabidopsis ETA2, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF(TIR1) ubiquitin ligase. *Plant Cell.* **16**, 1883-1897.

Chung, C.H. and Baek, S.H. (1999) Deubiquitinating enzymes: their diversity and emerging roles. *Biochem. Biophys. Res. Commun.* **266**, 633-640.

Coffino, P. (2001) Antizyme, a mediator of ubiquitin-independent proteasomal degradation. *Biochimie* **83**, 319-323.

Cope, G.A. and Deshaies, R.J. (2003) COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* **114**, 663-671.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V. and Deshaies, R.J. (2002b) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608-611.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V. and Deshaies, R.J. (2002a) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608-611.

D'Andrea, A. and Pellman, D. (1998) Deubiquitinating enzymes: a new class of biological regulators. *Crit Rev. Biochem. Mol. Biol.* **33**, 337-352.

Darwin, C. (1880) The Power of Movement in Plants.

Daviere, J.M., de, L.M. and Prat, S. (2008) Transcriptional factor interaction: a central step in DELLA function. *Curr. Opin. Genet. Dev.* **18**, 295-303.

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M. and Estelle, M. (2002) AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis Cullin AtCUL1 is required for auxin response. *Plant Cell.* **14**, 421-433.

Delker, C., Raschke, A. and Quint, M. (2008) Auxin dynamics: the dazzling complexity of a small molecule's message. *Planta* **227**, 929-941.

Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15**, 435-467.

Devoto, A., Nieto-Rostro, M., Xie, D. *et al.* (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. *Plant J.* **32**, 457-466.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005a) The F-box protein TIR1 is an auxin receptor. *Nature.* **435**, 441-445.

Dharmasiri, N., Dharmasiri, S., Jones, A.M. and Estelle, M. (2003) Auxin action in a cell-free system. *Curr. Biol.* **19;13**, 1418-1422.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jurgens, G. and Estelle, M. (2005b) Plant development is regulated by a family of auxin receptor F box proteins. *Dev. Cell.* **9**, 109-119.

Dreher, K.A., Brown, J., Saw, R.E. and Callis, J. (2006) The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. *Plant Cell.* **18**, 699-714.

Felle, H., Peters, W. and Palme, K. (1991) The electrical response of maize to auxins. *Biochim. Biophys. Acta* **1064**, 199-204.

Feng, S., Ma, L., Wang, X., Xie, D., nesh-Kumar, S.P., Wei, N. and Deng, X.W. (2003) The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. *Plant Cell.* **15**, 1083-1094.

Feng, S., Martinez, C., Gusmaroli, G. *et al.* (2008) Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* **451**, 475-479.

Feng, S., Shen, Y., Sullivan, J.A., Rubio, V., Xiong, Y., Sun, T.P. and Deng, X.W. (2004) Arabidopsis CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein Degradation. *Plant Cell.* **16**, 1870-1882.

Feys, B., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. *PLANT CELL* **6**, 751-759.

Fisk, H.A. and Yaffe, M.P. (1999) A role for ubiquitination in mitochondrial inheritance in Saccharomyces cerevisiae. *J Cell Biol.* **145**, 1199-1208.

Friml, J., Yang, X., Michniewicz, M. *et al.* (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* **306**, 862-865.

Fukaki, H., Tameda, S., Masuda, H. and Tasaka, M. (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. *Plant J* **29**, 153-168.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M. and Vierstra, R.D. (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. *Proc. Natl. Acad. Sci U. S. A* **99**, 11519-11524.

Galvan-Ampudia, C.S. and Offringa, R. (2007) Plant evolution: AGC kinases tell the auxin tale. *Trends Plant Sci.* **12**, 541-547.

Gehring, C.A., Williams, D.A., Cody, S.H. and Parish, R.W. (1990) Phototropism and geotropism in maize coleoptiles are spatially

correlated with increases in cytosolic free calcium. *Nature* **345**, 528-530.

Geldner, N., Anders, N., Wolters, H. *et al.* (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112**, 219-230.

Glickman, M.H. and Ciechanover, A. (2002) The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol. Rev.* **82**, 373-428.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001) Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature.* **414**, 271-276.

Guilfoyle, T., Hagen, G., Ulmasov, T. and Murfett, J. (1998a) How does auxin turn on genes? *Plant Physiol* **118**, 341-347.

Guilfoyle, T.J., Ulmasov, T. and Hagen, G. (1998b) The ARF family of transcription factors and their role in plant hormone-responsive transcription. *Cell Mol Life Sci.* **54**, 619-627.

Guo, H. and Ecker, J.R. (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* **115**, 667-677.

Hamann, T., Benkova, E., Baurle, I., Kientz, M. and Jurgens, G. (2002) The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev.* **16**, 1610-1615.

Han, L., Mason, M., Risseeuw, E.P., Crosby, W.L. and Somers, D.E. (2004) Formation of an SCF(ZTL) complex is required for proper regulation of circadian timing. *Plant J.* **40**, 291-301.

Hershko, A., Eytan, E., Ciechanover, A. and Haas, A.L. (1982) Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins. *J Biol. Chem.* **257**, 13964-13970.

Hicke, L. (1999) Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* **9**, 107-112.

Karniol, B. and Chamovitz, D.A. (2000) The COP9 signalosome: from light signaling to general developmental regulation and back. *Curr. Opin. Plant Biol.* **3**, 387-393.

Katsir, L., Chung, H.S., Koo, A.J. and Howe, G.A. (2008a) Jasmonate signaling: a conserved mechanism of hormone sensing. *Curr. Opin. Plant Biol.* **.**,

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y. and Howe, G.A. (2008b) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci U. S. A.* **105**, 7100-7105.

Kepinski, S. and Leyser, O. (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature.* **435**, 446-451.

Kim, J., Harter, K. and Theologis, A. (1997) Protein-protein interactions among the Aux/IAA proteins. *Proc. Natl. Acad. Sci U. S. A.* **94**, 11786-11791.

Kramer, E.M. (2004) PIN and AUX/LAX proteins: their role in auxin accumulation. *Trends Plant Sci.* **9**, 578-582.

Kwok, S.F., Piekos, B., Misera, S. and Deng, X.W. (1996) A complement of ten essential and pleiotropic arabidopsis COP/DET/FUS genes is necessary for repression of photomorphogenesis in darkness. *Plant Physiol* **110**, 731-742.

la Fuente, R.K. and Leopold, A.C. (1973) A Role for Calcium in Auxin Transport. *Plant Physiol* **51**, 845-847.

Laxmi, A., Pan, J., Morsy, M. and Chen, R. (2008) Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in Arabidopsis thaliana. *PLoS. ONE.* **3**, e1510-

Lechner, E., Achard, P., Vansiri, A., Potuschak, T. and Genschik, P. (2006) F-box proteins everywhere. *Curr. Opin. Plant Biol* **9**, 631-638.

Leyser, H.M., Pickett, F.B., Dharmasiri, S. and Estelle, M. (1996) Mutations in the AXR3 gene of Arabidopsis result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. *Plant J* **10**, 403-413.

McGinnis, K.M., Thomas, S.G., Soule, J.D., Strader, L.C., Zale, J.M., Sun, T.P. and Steber, C.M. (2003) The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. *PLANT CELL* **15**, 1120-1130.

Melotto, M., Mecey, C., Niu, Y. *et al.* (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in

mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. *Plant J* **55**, 979-988.

Michniewicz, M., Zago, M.K., Abas, L. *et al.* (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* **130**, 1044-1056.

Mravec, J., Kubes, M., Bielach, A. *et al.* (2008) Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. *Development* **135**, 3345-3354.

Mukhopadhyay, D. and Riezman, H. (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **315**, 201-205.

Murase, K., Hirano, Y., Sun, T.P. and Hakoshima, T. (2008) Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. *Nature* **456**, 459-463.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M. and Reed, J.W. (2000) AXR2 encodes a member of the Aux/IAA protein family. *Plant Physiol.* **123**, 563-574.

Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dirac, A.M., Sixma, T.K. and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**, 773-786.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y. (1991) Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. *PLANT CELL* **3**, 677-684.

Ouellet, F., Overvoorde, P.J. and Theologis, A. (2001) IAA17/AXR3: biochemical insight into an auxin mutant phenotype. *Plant Cell.* **13**, 829-841.

Park, J.Y., Kim, H.J. and Kim, J. (2002) Mutation in domain II of IAA1 confers diverse auxin-related phenotypes and represses auxin-activated expression of Aux/IAA genes in steroid regulator-inducible system. *Plant J* **32**, 669-683.

Parry, G. and Estelle, M. (2006) Auxin receptors: a new role for F-box proteins. *Curr. Opin. Cell Biol.* **18**, 152-156.

Petrasek, J., Mravec, J., Bouchard, R. *et al.* (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**, 914-918.

Pham, A.D. and Sauer, F. (2000) Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in Drosophila. *Science* **289**, 2357-2360.

Pintard, L., Kurz, T., Glaser, S., Willis, J.H., Peter, M. and Bowerman, B. (2003) Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in C. elegans. *Curr. Biol.* **13**, 911-921.

Ploense, S.E., Wu, M.F., Nagpal, P. and Reed, J.W. (2009) A gain-of-function mutation in IAA18 alters Arabidopsis embryonic apical patterning. *Development* **136**, 1509-1517.

Ramos, J.A., Zenser, N., Leyser, O. and Callis, J. (2001) Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *Plant Cell.* **13**, 2349-2360.

Raven, J.A. (1975) Transport of indolaceticacid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. *New Phytologist* **74**, 163-172.

Reddy, V.S., Day, I.S., Thomas, T. and Reddy, A.S. (2004) KIC, a novel Ca2+ binding protein with one EF-hand motif, interacts with a microtubule motor protein and regulates trichome morphogenesis. *Plant Cell.* **16**, 185-200.

Reed, J.W. (2001) Roles and activities of Aux/IAA proteins in Arabidopsis. *Trends Plant Sci.* **6**, 420-425.

Robert-Boisivon, H. (2008) Calcium- and BTB domain protein-modulated PINOID kinase directs polar auxin transport. Leiden University.

Rogg, L.E., Lasswell, J. and Bartel, B. (2001) A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell.* **13**, 465-480.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M. and Leyser, O. (1998) Changes in auxin response from mutations in an AUX/IAA gene. *Science.* **279**, 1371-1373.

Rubery, P.H. and Sheldrake, A.R. (1973) Effect of pH and surface charge on cell uptake of auxin. *Nat. New Biol.* **244**, 285-288.

Santner, A.A. and Watson, J.C. (2006) The WAG1 and WAG2 protein kinases negatively regulate root waving in Arabidopsis. *Plant J* **45**, 752-764.

Sasaki, A., Itoh, H., Gomi, K. *et al.* (2003) Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. *Science* **299**, 1896-1898.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M. and Deng, X.W. (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science.* **292**, 1379-1382.

Schwechheimer, C., Serino, G. and Deng, X.W. (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell.* **14**, 2553-2563.

Serino, G., Su, H., Peng, Z., Tsuge, T., Wei, N., Gu, H. and Deng, X.W. (2003) Characterization of the last subunit of the Arabidopsis COP9 signalosome: implications for the overall structure and origin of the complex. *Plant Cell.* **15**, 719-731.

Shimada, A., Ueguchi-Tanaka, M., Nakatsu, T., Nakajima, M., Naoe, Y., Ohmiya, H., Kato, H. and Matsuoka, M. (2008) Structural basis for gibberellin recognition by its receptor GID1. *Nature* **456**, 520-523.

Sistrunk, M.L., Antosiewicz, D.M., Purugganan, M.M. and Braam, J. (1994) Arabidopsis TCH3 encodes a novel Ca2+ binding protein and shows environmentally induced and tissue-specific regulation. *Plant Cell.* **6**, 1553-1565.

Spartz, A.K. and Gray, W.M. (2008) Plant hormone receptors: new perceptions. *Genes Dev.* **22**, 2139-2148.

Spence, J., Gali, R.R., Dittmar, G., Sherman, F., Karin, M. and Finley, D. (2000) Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* **102**, 67-76.

Spence, J., Sadis, S., Haas, A.L. and Finley, D. (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell Biol.* **15**, 1265-1273.

Stirnberg, P., Furner, I.J. and Ottoline Leyser, H.M. (2007) MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. *Plant J.* **50**, 80-94.

Strader, L.C., Ritchie, S., Soule, J.D., McGinnis, K.M. and Steber, C.M. (2004) Recessive-interfering mutations in the gibberellin signaling gene SLEEPY1 are rescued by overexpression of its homologue, SNEEZY. *Proc. Natl. Acad. Sci. U. S. A* **101**, 12771-12776.

Swarup, K., Benkova, E., Swarup, R. *et al.* (2008) The auxin influx carrier LAX3 promotes lateral root emergence. *Nat. Cell Biol.* **10**, 946-954.

Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K. and Bennett, M. (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. *Genes Dev.* **15**, 2648-2653.

Swarup, R., Kargul, J., Marchant, A. *et al.* (2004) Structure-function analysis of the presumptive Arabidopsis auxin permease AUX1. *Plant Cell.* **16**, 3069-3083.

Szemenyei, H., Hannon, M. and Long, J.A. (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. *Science.* **319**, 1384-1386.

Tan, X., Calderon-Villalobos, L.I., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M. and Zheng, N. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature.* **446**, 640-645.

Tanaka, H., Dhonukshe, P., Brewer, P.B. and Friml, J. (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. *Cell Mol. Life Sci.* **63**, 2738-2754.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E. and Yamamoto, K.T. (2004) MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *PLANT CELL* **16**, 379-393.

Terrell, J., Shih, S., Dunn, R. and Hicke, L. (1998) A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cell* **1**, 193-202.

Thines, B., Katsir, L., Melotto, M. *et al.* (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature.* **448**, 661-665.

Thrower, J.S., Hoffman, L., Rechsteiner, M. and Pickart, C.M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J* **19**, 94-102.

Tian, Q. and Reed, J.W. (1999) Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. *Development* **126**, 711-721.

Timpte, C., Wilson, A.K. and Estelle, M. (1994) The axr2-1 mutation of Arabidopsis thaliana is a gain-of-function mutation that disrupts an early step in auxin response. *Genetics* **138**, 1239-1249.

Tiwari, S.B., Hagen, G. and Guilfoyle, T.J. (2004) Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell.* **16**, 533-543.

Tiwari, S.B., Wang, X.J., Hagen, G. and Guilfoyle, T.J. (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. *Plant Cell.* **13**, 2809-2822.

Uehara, T., Okushima, Y., Mimura, T., Tasaka, M. and Fukaki, H. (2008) Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in Arabidopsis thaliana. *Plant Cell Physiol.* **49**, 1025-1038.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. (1999) Activation and repression of transcription by auxin-response factors. *Proc. Natl. Acad. Sci U. S. A* **96**, 5844-5849.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T.J. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell.* **9**, 1963-1971.

Vieten, A., Sauer, M., Brewer, P.B. and Friml, J. (2007) Molecular and cellular aspects of auxin-transport-mediated development. *Trends Plant Sci.* **12**, 160-168.

Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68**, 1015-1068.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J. and Chen, Z.J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346-351.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W. and Wei, N. (2003) The COP9 signalosome interacts with SCF UFO and participates in Arabidopsis flower development. *Plant Cell.* **15**, 1071-1082.

Weijers, D., Benkova, E., Jager, K.E., Schlereth, A., Hamann, T., Kientz, M., Wilmoth, J.C., Reed, J.W. and Jurgens, G. (2005) Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. *EMBO J.* **24**, 1874-1885.

Went, F.W. (1937) SALT ACCUMULATION AND POLAR TRANSPORT OF PLANT HORMONES. *Science* **86**, 127-128.

Wisniewska, J., Xu, J., Seifertova, D. *et al.* (2006a) Polar PIN localization directs auxin flow in plants. *Science* **312**, 883-

Wisniewska, J., Xu, J., Seifertová, D. *et al.* (2006b) Polar PIN localization directs auxin flow in plants. *Science* **312**, 883-883.

Worley, C.K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A. and Callis, J. (2000) Degradation of Aux/IAA proteins is essential for normal auxin signalling. *Plant J.* **21**, 553-562.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science.* **280**, 1091-1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D. and Xie, D. (2002) The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell.* **14**, 1919-1935.

Yang, P., Fu, H., Walker, J., Papa, C.M., Smalle, J., Ju, Y.M. and Vierstra, R.D. (2004) Purification of the Arabidopsis 26 S proteasome: biochemical and molecular analyses revealed the presence of multiple isoforms. *J Biol Chem.* **279**, 6401-6413.

Yang, P., Smalle, J., Lee, S., Yan, N., Emborg, T.J. and Vierstra, R.D. (2007) Ubiquitin C-terminal hydrolases 1 and 2 affect shoot architecture in Arabidopsis. *Plant J.* **51**, 441-457.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P. and Nielsen, E. (2006) High-affinity auxin transport by the AUX1 influx carrier protein. *Curr. Biol.* **16**, 1123-1127.

Yang, X., Lee, S., So, J.H. *et al.* (2004) The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J.* **40**, 772-782.

Zenser, N., Dreher, K.A., Edwards, S.R. and Callis, J. (2003) Acceleration of Aux/IAA proteolysis is specific for auxin and independent of AXR1. *Plant J.* **35**, 285-294.

Zhang, W., Ito, H., Quint, M., Huang, H., Noel, L.D. and Gray, W.M. (2008) Genetic analysis of CAND1-CUL1 interactions in Arabidopsis supports a role for CAND1-mediated cycling of the SCFTIR1 complex. *Proc. Natl. Acad. Sci U. S. A.* **105**, 8470-8475.

Zourelidou, M., Muller, I., Willige, B.C., Nill, C., Jikumaru, Y., Li, H. and Schwechheimer, C. (2009) The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in Arabidopsis thaliana. *Development* **136**, 627-636.