APC/C^{Cdh1} modulates the ER stress response via Gadd34

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Here I declare that my doctoral thesis entitled “APC/C^{Cdh1} modulates the ER stress response via Gadd34” has been written independently and with no other sources and aids than quoted

Hannover, the 20/06/2011

Hanane Belahmer

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Zusammenfassung

APC/Ccdh1 spielt eine wichtige Rolle in der Regulation der Zellzyklusprogression durch Markierung von Proteinen für die Degradation durch das Proteosome.

In einem "Yeast-Two-Hybrid Screen" wurde Gadd34 als neuer Interaktionspartner für Cdh1 gefunden. Gadd34 ist ein bekannter Regulator der integrierten Antwort auf endoplasmatisches Retikulum Stress infolge einer Akkumulation von falsch gefalteten Proteinen im ER. Diese Bedingungen sind häufig in soliden Tumoren durch Hypoxie and Mangelversorgung zu finden.

Im Rahmen dieser Arbeit wurde die biologische Funktion der Interaktion zwischen Gadd34 and Cdh1 analysiert. Co-Immunopräzipitations-Versuche zeigten die spezifische Interaktion zwischen Cdh1 and Gadd34 in humanen Zellen nach Überexpression. Es wurde auch nachgewiesen dass, der C-Terminus von Gadd34 für die Interaktion mit Cdh1 ausreichte. Darüber hinaus wurde in His-Ubiquitinierungs-Versuche gezeigt, dass Überexpression von Cdh1 zur Ubiquitinierung von Gadd34 führt. Dieses war von der Menge des überexpremierten Cdh1 abhängig. Eine Abnahme in der Ubiquitinierung von Gadd34 erfolgte nach Überexpression von Cdh1 Inhibitoren.

Analysen zeigten, dass APC/CCdh1 die Degradation von Gadd34 unter physiologischen Bedingungen reguliert: die Menge und die Stabilität von Gadd34 war größer wenn in Zellen, die durch Glucoseentzug gestresst wurden, Cdh1 depletiert war. In diesen Zellen blieb Gadd34 länger detektierbar nachdem Abschalten des Stresses. Insbesondere konnte gezeigt werden, dass Ausschalten von Cdh1 zur Stabilisierung von Gadd34 führte. Es wurde auch nachgewiesen, dass das Ausschalten von Cdh1 die Viabilität von ER gestressten Zellen reduziert. Dieser Effekt korrelierte mit höherer Akkumulation von Gadd34. In der Tat konnte durch eine Co-Depletion von Gadd34 die Viabilität erhöht werden. Diese Daten weisen daraufhin, dass Cdh1 die Viabilität der ER gestressten Zellen durch die Modulation von Gadd34 Menge kontrolliert.

Die vorgestellten Ergebnisse deuten drauf hin, dass mit der Inhibition von Cdh1 durch seine Rolle in der ER Stress Antwort eine vielversprechende Strategie zur Eliminierung von Krebszellen entwickelt werden könnte.

Summary

APC/Ccdh1 plays an important role in regulating the cell cycle progression by marking proteins for degradation by the proteoasome.

In a yeast-two-hybrid screen Gadd34 was identified as new interacting partner for Cdh1. Gadd34 is a known regulator of the integrated response to endoplasmic reticulum stress caused by an overloaded of misfolded proteins in the ER. These conditions are often found in tumours due to hypoxia and nutrient starvation.

In this thesis the biological function of the interaction between Cdh1 and Gadd34 was analysed. Co-immunoprecipitation assays showed that Gadd34 interacts with Cdh1 in human cells after overexpression. Furthermore, the C-terminal part of Gadd34 was sufficient to promote this interaction. His-ubiquitination assays demonstrated that overexpression of Cdh1 leads to the ubiquitination of Gadd34. This ubiquitination was enhanced after increasing the level of Cdh1 and decreased after overexpressing inhibitors of Cdh1.

Analyses under physiological conditions revealed that APC/CCdh1 controls Gadd34 turnover. This was concluded after analysing the levels and the stability of Gadd34 in Cdh1 depleted HeLa cells stressed by glucose deprivation. In these cells, Gadd34 levels were higher under stress conditions and more slowly reduced after removal of stress. In particular, Cdh1 depletion resulted in the stabilisation of Gadd34.

Cdh1 depletion was seen to reduce the viability of cells undergoing ER stress, which correlated with high Gadd34 levels. Indeed, a partial rescue was observed after co-depleting Gadd34 in these cells. This argues for a role played by Cdh1 in controlling the survival of ER stressed cells through controlling Gadd34 turnover.

The results presented in this work suggest that increasing the ER stress and cell death by inhibiting Cdh1 could be a promising strategy for eliminating cancer cells.

Zellzyklus, ER Stress Antwort, ER Stress und Krebs, Cell cycle, ER stress response, ER stress and cancer

I. Introduction

A. The Eucaryotic Cell Cycle

1. General aspects of the Eucaryotic Cell Cycle

Cell proliferation is a fundamental aspect of life. Cells grow and proliferate by a series of highly coordinated events termed the cell cycle.

The cell cycle is divided into four distinct phases: G1 phase (Gap1), S phase (synthesis), G2 phase (Gap2) and M phase. The period between two M phases is also termed interphase. Throughout G1, the cell grows by producing proteins and duplicating cytoplasmic organelles. Chromosomes are replicated only during S phase. In the following G2, the cell continues growing and ensures that the conditions are favourable for entering M phase. At this stage of the cell cycle, the growth stops and the cell divides in two identical daughter cells (Vermeulen et al., 2003).

The M phase is consisting of mitosis and cytokinesis. The mitosis includes prophase, prometaphase, metaphase, anaphase and telophase. Spindle assembly and preparation of chromosomes for separation occur during prophase and prometaphase. During metaphase, all the chromosomes are fully attached to the spindle, align to the metaphase plate and await the signal to separate. In the following anaphase sister-chromatids separate and microtubules elongate, which pull the chromosomes to the opposite poles of the cell. Finally, in telophase a new nuclear envelope reforms around each set of separated chromosomes. These events are immediately followed by cytokinesis dividing the cell and marking the end of M-phase (David O Morgan).

Cell proliferation depends on the availability of growth factors. In the absence of nutrients or after induction of differentiation, the progression is restricted in G1 phase at a point termed restriction point (R) or point of non-return. At this point, cells can leave the cell cycle and enter a quiescent state termed G0. Under certain conditions, resting cells can re-enter the cell cycle and proliferate (Hulleman & Boonstra 2001). After passing the restriction point, cells become refractory to growth factors until the next G1 (Coleman et al., 2001, Foijer & Riele 2006).

To make sure that the genetic material is transmitted to the daughter cells with high fidelity, the cell cycle machinery elaborates surveillance systems termed checkpoints. These systems monitor that the initiation of later cell cycle events dependent on the proper completion of the previous one (Vermeulen 2003). Checkpoints can be activated by exogenous and endogenous insults causing DNA damage, cellular stress or in the absence of growth factors, hormones and nutrients.

The DNA damage checkpoints for example restrict the cell cycle progression at certain stages and provide time for DNA repair before entry into S phase (G1/S checkpoint), during DNA replication (intra S checkpoint) and before entry into mitosis (G2/M checkpoint) (Karsten & Bartek 2004). Progression through mitosis is inhibited by the spindle assembly checkpoint, which is activated when a single kinetechore is not properly attached to the mitotic spindle during metaphase (Flatt & Pietenpol 2000).

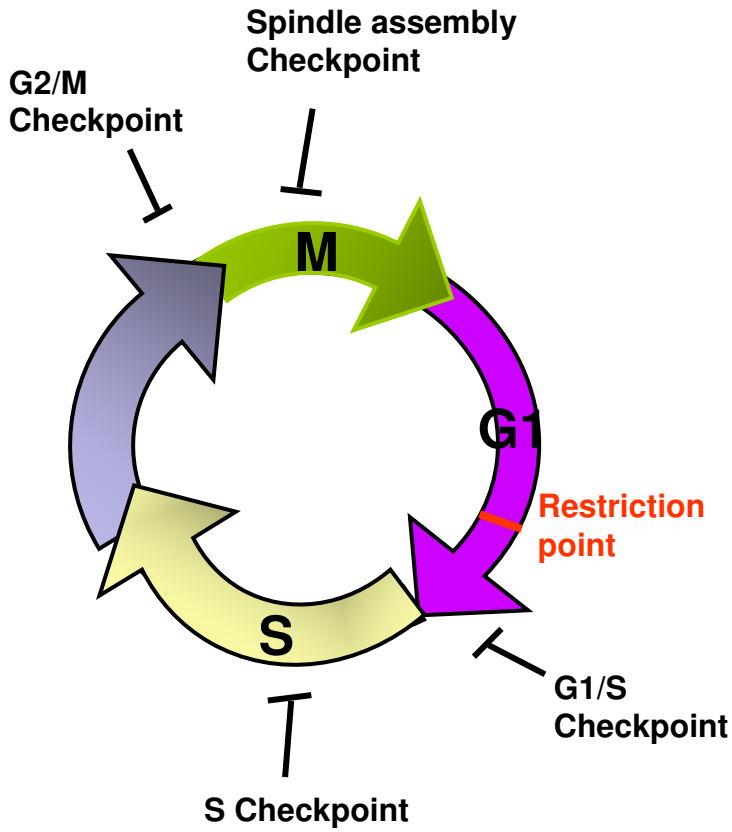

Fig. 1: The Eukaryotic Cell Cycle

Generally, the checkpoint system controls the timing and the order of the cell cycle events. Often, alteration in either checkpoint control or cell cycle regulation result in

genomic instability and increase the risk of tumour development (McGowan 2003, Negrini et al., 2010).

2. Cell Cycle Regulation by Cyclin/CDKs

The entry to a cell cycle phase, the progression and the exit from this phase are controlled by the activity of cyclin-dependent-kinases (CDKs). CDKs are serine/threonine protein kinases, which are activated and regulated through their binding to specific proteins termed cyclins at specific stages in the cell cycle. The Cyclin/CDKs phosphorylate keys regulators of the cell cycle controlling thereby crucial processes such DNA replication, chromosome condensation, spindle assembly and disassembly, nuclear division and cytokineses (Thornton 2008).

The G1 progression, the G1/S transition and re-entry from quiescence are controlled by the activity of Cyclin D-CDK2/4 and Cyclin E in combination with CDK2. Cyclin A associates with CDK2 and controls the S-phase. The progression through G2 and M phases is regulated by the activity of Cyclin A/CDK1 and Cyclin B/CDK1 (Satyanarayana & Kaldis 2009).

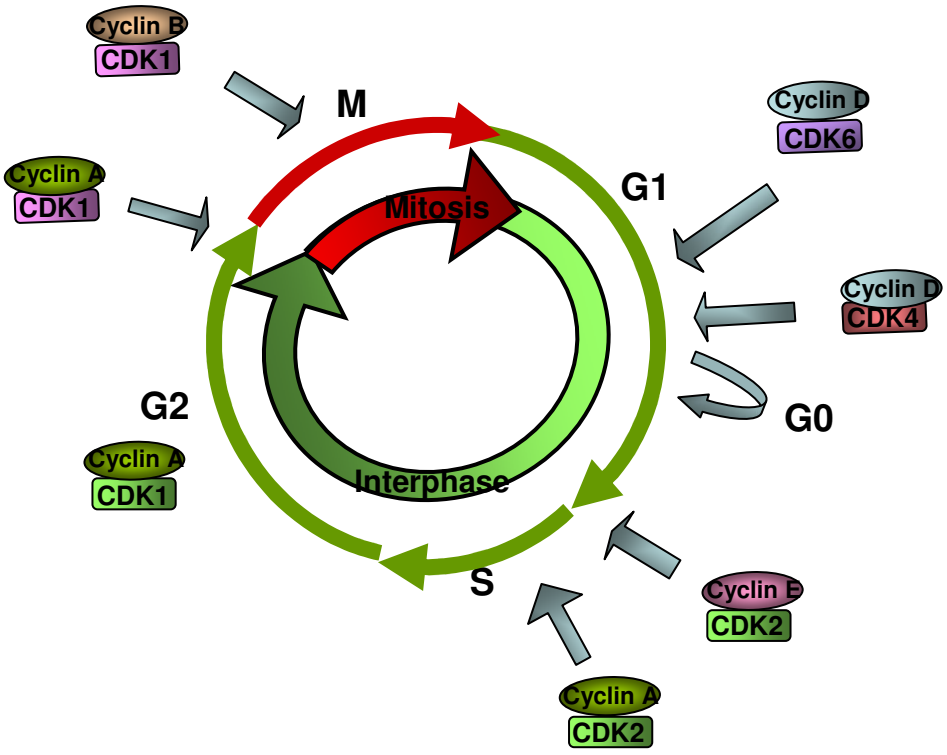

Fig. 2: The cell cycle progression is controlled by Cyclin/CDK activity

Cyclin D/CDK4-CDK6 promote the phosphorylation of the retinoblastoma protein (RB) and the subsequent release of the transcription factor E2F. The activity of E2F controls the expression of genes encoding regulators of the cell cycle progression including Cyclin E and Cyclin A. (Fu et al., 2004, Scherr & Roberts 2004). Cyclin E and Cyclin A are synthesised sequentially. Late in G1, Cyclin E binds CDK2 and continues the phosphorylation of RB resulting in a complete release of E2F and expression of genes required for S phase. Cyclin E/CDK2 regulates the G1/S entry by regulating the first event of DNA replication including phosphorylation of Cdc6 and the subsequent recruitment of the replicative helicase MCM4 (Mini Chromosome Maintenance proteins) to the pre-replication complexes assembled at the origin of DNA replication (Hwang & Clurman 2005). During S phase, Cyclin A activates CDK2 and promotes the initiation of DNA synthesis by phosphorylating for example proteins involved in DNA replication (Hulleman & Boonstra 2001). Moreover, the Cyclin A/CDK2 activity inhibits re-replication by phosphorylating Cdc6 and Cdt1 preventing thereby reloading of MCM4 to the origin of DNA replication (Yam et al., 2002, Coller 2007).

At the end of interphase, Cyclin A activates CDK1 and promotes the entry and the progression through prophase. Cyclin A/CDK1 activity is essential for chromosomes condensation, for the disassembly of the nucleoli, and for activation of Cyclin B/CDK1 (Furuno et al., 1999). At mitotic entry, Cyclin B/CDK1 activity is required for chromosome condensation, nuclear envelope breakdown, mitotic spindle assembly, and in controlling the reorganisation of golgi apparatus (Hochegger et al., 2008, Satyanarayana & Kaldis 2009).

A variety of signals act to inhibit the activity of CDKs and thereby the cell cycle progression. These inhibitory effects are mediated by negative regulators termed CDK inhibitors (CDKIs), which inhibit the CDK activity through binding either directly to the CDKs or to the Kinase-Cyclin complexes. Two families of inhibitors have been identified: firstly, the INK4 family including, $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4C}$ and $p19^{INK4d}$, which bind to G1 CDKs (CDK4 and CDK6) and inhibit their binding to Cyclin D and secondly, the Cip/Kip family includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which bind and inhibit the Cyclin D- E-A-B-/CDK complexes (Besson et al., 2008).

In addition, the activity of CDKs is regulated both positively and negatively by cell cycle specific phosphorylation. Whereas the levels of CDKs remain constant, the levels of cyclins oscillate during the cell cycle. This is achieved by transcriptional regulation and by cell cycle dependent degradation by the ubiquitin-proteasome pathway (Hochegger et al., 2008).

3. The ubiquitin- proteasome pathway

The ubiquitin is a highly conserved 76-amino acid protein. Covalent attachment of multiple ubiquitin molecule targets proteins for degradation by the proteasome. This transfer is directed by the activity of 3 enzymes (E1 ubiquitin activating enzyme, E2 a ubiquitin conjugating enzyme and a ubiquitin ligase E3 (Burton et al., 2007, Dye & Schulmann 2007). The first step in the ubiquitin pathway is an ATP-dependent reaction, which catalyses the activation of ubiquitin through creation of high-energy thioester bond between the active cysteine of the E1 and the C-terminal residue of the ubiquitin (Baker et al., 2005). In the next step the ubiquitin is transferred to the E2, which catalyses in combination with the E3 ligase the transfer of the ubiquitin to the target protein resulting in an isopeptide bond between the carboxyl group of the C-terminal Gly of ubiquitin and an amino group of lysine residue in the target substrate (Bie & Ciechanover 2011).

The E3 ligase provides selectivity in substrate recognition and can be divided into 2 groups, namely the HECT domain E3 ligases and RING finger domain E3 ligase. This classification was based on the presence of HECT (Homologous to the Carboxy Terminal of the proteolytical member of the family E6-Associated Protein) and a RING finger domain, respectively. They are functionally different: whereas activated ubiquitin is transferred first from the E2 to a conserved cystein residue within the HECT domain, in the second group the activated ubiquitin is directly transferred to the substrate. The RING-finger E3 ligases serve as adaptor, which facilitate the positioning and the transfer of the ubiquitin from an E2 onto the substrate (Glickman & Ciechnover 2001).

The sequential attachment of ubiquitins to each other by E3 ligase occurs via one of the seven-lysine residue present in the ubiquitin (including K6, K11, K27, K29/33,

K48 and K63). However the proteasome recognises only lysine11 and lysine-48-linked formed chains (Hass & Wilkinson 2008, Glickman & Ciechanover 2001 and Rape 2011), whereas ubiquitination through the other lysine residues serves as signal for non-proteolytic functions (Glickman & Ciechanover 2001, Bie & Ciechanover 2011).

The ubiquitination reaction is reversible. The ubiquitin is removed from the protein through the action of cystein protease termed deubiquitinatting enzymes (DUBs). In addition, DUBs play a role in recycling free ubiquitin to the ubiquitin pathway (Glickman & Ciechnover 2001, Komander et al., 2009).

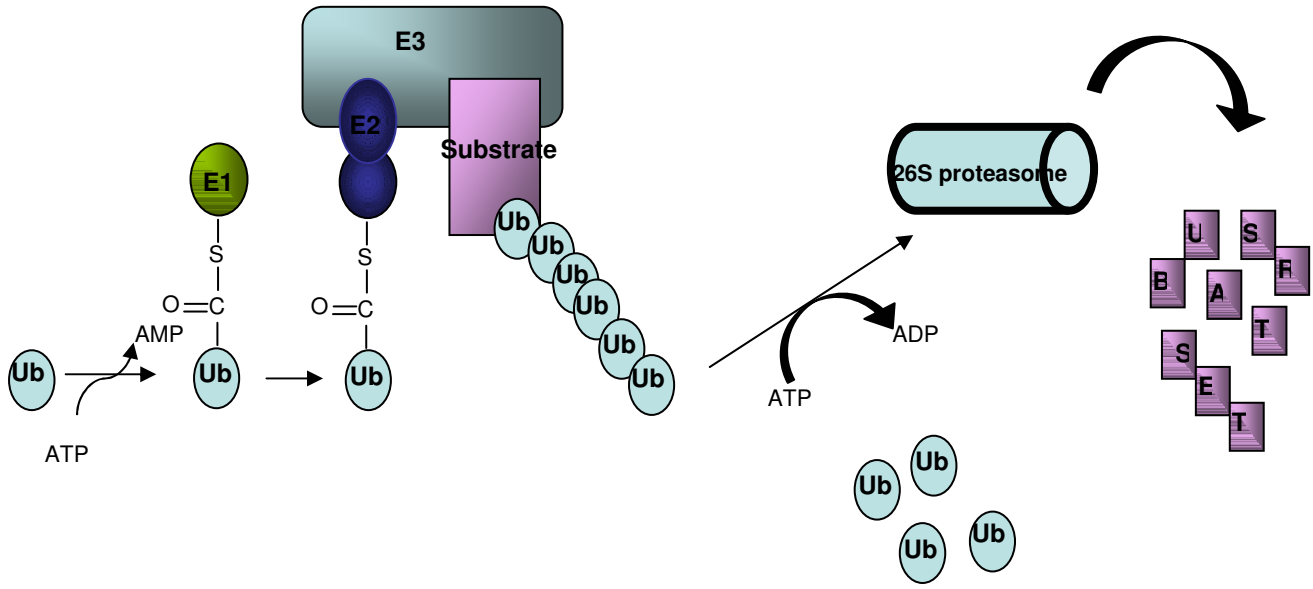

Fig. 3:The Ubiquitin-Proteasome Pathway (according to Nakayama 2006)

Together, the ubiquitin pathway regulates different cellular processes through both proteolytic and non-proteolytic mechanisms (Ciechanover 2005, Lecker et al., 2006). This depends on the number of the ubiquitin attached to the target protein and the lysine residues involved in the formation of the ubiquitin chain (Glickman & Ciechanover 2001, Robinson & Ardley 2004).

Two E3-ligases are involved in the regulation of the cell cycle progression, namely the SCF (Skp1-Cul-F box protein) and the APC/C (anaphase promoting complexes or cyclosome). Both of them are members of the RING-Finger family of E3 and contain subunits with both cullin and RING finger domains.

Despite the similarity in the structure SCF and APC/C are functionally different. The activity of the SCF complexes is mainly needed in the regulation of G1/S transition, during G2 and early M phase, while the activity of APC/C is required for progression through mitosis and during G1 (Vodermaier 2004).

4. Cell Cycle Regulation by the APC/C

4.1 APC/C and activators

The APC/C is a 1,5-MDa protein complex. Genetic and biochemical analysis identified 13 subunits. Apc2, Apc11, Apc10 (Doc1) and Apc3/Cdc27 are the well-characterised subunits, whereas the exact biological function of the other subunits is not well understood and still under investigation (Harper et al., 2002, Foe & Toczyski 2010).

Analysis of the APC/C structure in yeast reveals that APC/C is consisting of 3 subcomplexes; a platform region connected to the catalytic and to the TRP subcomplexes (Schreiber et al., 2011, Izawa & Pines 2011). The TRP core is composed of Cdc16, Cdc23 and Cdc27 subunits containing a conserved tetratricopeptide repeats (Foe & Toczyski 2010). Whereas Cdc23 connects TRP to the platform, Cdc27 is involved in the binding of the two coactivators Cdc20 (Fizzy) and the Cdh1 (fizzy related protein) to the APC/C thereby regulating the activity of this complexe and substrate recruitment (Peters 2006, Foe & Toczyski 2011). The binding to Cdc27 subunit requires the presence of a motif called C-Box and a conserved Isoleucine-Arginine (IR-tail) within Cdc20 and Cdh1 (Peters 2006). Both coactivators are characterised by the presence of c-terminal WD40 repeat, which recognises and binds APC/C substrates through specific motifs termed the D-box (destruction Box) and the KEN-box (consensus KENXXXE/D/N). While the D-Box (RXXLXXXN) is recognised by Cdc20 and Cdh1, substrates containing the KEN-Box are only targeted for ubiquitination by APC/C^{Cdh1}. Other destruction motifs have also

been identified such as the A-box (RxLxPSN), O-box, and CRY box (CRYxPS) (Leuken et al., 2006).

The catalytic core is implicated in recruiting the E2 ubiquitin ligase and is consisting of the cullin protein Apc2, the RING protein Apc11 and Apc10 (Schreiber et al., 2011). Apc10 is implicated in binding to and reducing the dissociation of the APC/C substrates (Foe & Toczyski 2010). A recent study demonstrates that Apc10 binds the Cdc27 subunits and contributes in combination with Cdh1 to the recognition of substrates containing D-Box (da Fonseca et al., 2011).

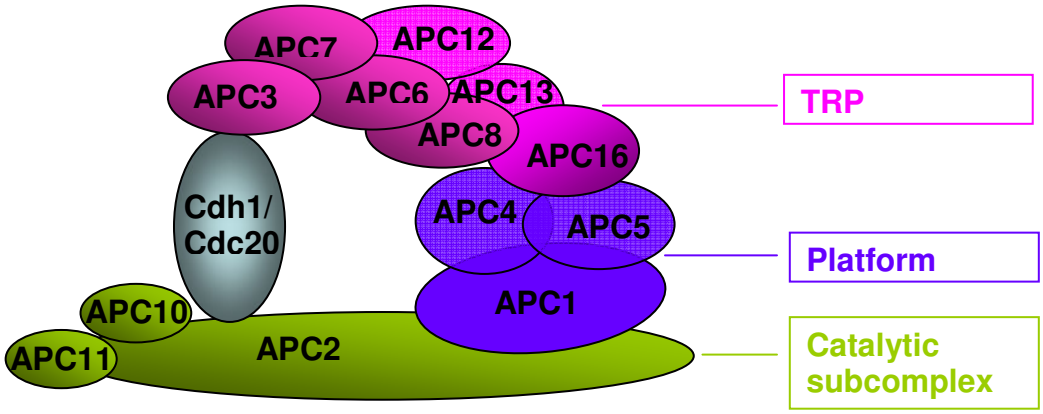

Fig. 4: APC/C Structure (modified from Smolder & Teodoro 2011). The organisation of APC/C in 3 subcomplexes is shown according to the study performed by Schreiber et al., 2011 in budding yeast.

Additional mechanisms control the APC/C activity including the phosphorylation of APC/C subunits and coactivators and the interaction with regulatory proteins (Schreiber et al., 2011).

CDC20 and Cdh1 bind and activate the APC/C at different stage of the cell cycle, which provides selectivity in the substrate recognition and specificity in cell cycle regulation.

4.2 APC/C^{Cdc20} regulation and functions

Cdc20 starts accumulating at S phase and peaks at M phase, but it can bind and activate APC/C only in early M phase (Yu 2007).

Several mechanisms exist to control the APC/C^{Cdc20} activity. Emi1 (early mitotic inhibitor1) is an inhibitor of the APC/C^{Cdc20} activity. This inhibition is achieved by interacting with newly synthesized Cdc20 as pseudo-substrate (Baker et al., 2007, Thornton & Toczyski 2008), and binding to the APC core (Pesin & Orr-Weaver 2008). Activation of APC/C by Cdc20 is promoted after phosphorylation of Emi1 by Polo-Like Kinase1 (PLK1) and its subsequent degradation by the $SCF^{\beta TrCP}$ complexe (Baker et al., 2007). The association of Cdc20 to the APC/C is also enhanced by phosphorylation of Cdc20 and several APC subunits by mitotic kinases including Cdk1 and PLK1 (Peters 2002, Eckerdt & Strebhardt 2006).

In prometaphase, APC/C with it co-activator Cdc20 initiates the destruction of Cyclin A and Nek2 (NIMA-related-kinases). Nek2 is implicated in the regulation of centrosome structure (Yam et al., 2002) and separation at the onset of mitosis (Nakayama 2006). APC/C^{Cdc20} initiates the metaphase anaphase transition. This function can be inhibited by the spindle checkpoint (Peters 2006). Mediator of the spindle checkpoint binds Cdc20 and forms the Mitotic Checkpoint Complexe (MCC) (acquaviva & Pines 2006, Hardwick & Shah 2010). However, the mitotic checkpoint is not affecting the activity of APC/C^{Cdc20} towards Cyclin A and NeK2, which continue to get ubiquitinated and degraded by the proteasome (Manchado et al., 2010, van Zon & Wolthuis 2010, Izawa & Pines 2011). The MCC disassembles once all chromosomes are properly attached to the microtubules. Active APC/C^{Cdc20} targets securin and cyclin B for degradation resulting in activation of separase. This protease cleaves the Scc1 subunit of cohesion responsible for holding sister chromatids together allowing thereby the sister chromatids separation and the anaphase onset (Peters 2006). Later in mitosis, Cdc20 becomes subject to APC/C^{Cdh1} dependent degradation (see 4-3).

4.3 APC/C^{Cdh1} regulation and functions

Although Cdh1 accumulates starting from G2, its binding to the APC/C is inhibited during G2 and early M phase by CDK phosphorylation (Skaar & Pagano 2008). A decrease in the CDK activity late in M phase leads to dissociation of Cdc20,

dephosphorylation of Cdh1, and activation the APC/C^{Cdh1}. It has been reported that CDC14 is the phosphatase involved in dephosphorylating Cdh1 in budding yeast. Human homologue of CDC14 (h CDC14a) is able to dephosphorylate Cdh1 and to activate APC/C^{Cdh1} *in vitro*, but its involvement *in vivo* requires further investigation (Peters 2002, Bembenek & Yu 2001).

APC/C with its coactivator Cdh1 continues targeting cyclins for degradation and promotes also the degradation of Cdc20. APC/C^{Cdh1} targets other mitotic regulators for degradation by the proteasome. This includes Plk1 and Aurora B controlling the timing of cytokinesis and driving thereby cells out of mitosis (Li & Zhang 2009, Sigl et al., 2009).

Cdh1 is considered as a master regulator of G1. During this phase, the APC/C^{Cdh1} activity is required for keeping the activity of CDKs activity at a low level by targeting Cyclin A and Cyclin B for degradation. APC/C^{Cdh1} limits also the level of Cyclin D through targeting the transcription factor Ets2 for degradation allowing thereby the maintenance of G1. Consistent with this function, APC/C^{Cdh1} targets Skp2 (the substrate recognition subunit of the SCF ubiquitin ligase) and its cofactor Cks1 for degradation (Liu et al., 2007). This allows the stabilisation of its targets including p21 and p27 (Bashir et al., 2004, Cunliffe 2004) and results in the inhibition of Cyclin E/CDK2 the main regulator of the G1/S transition (Liu et al., 2007, Engelbert et al., 2008, Bashir & Pagano 2004).

An additional function contributed to APC/C^{Cdh1} during G1 phase is the regulation of DNA replication through controlling the assembly of the pre-replicative complex on origin of replication. This function is achieved by ensuring a low level of CDKs activity and by promoting the degradation of geminin (an inhibitor of DNA replication factor Cdt1) (Peters 2006). In parallel, APC/C^{Cdh1} prevents an early DNA synthesis and unscheduled entry into S phase by targeting Cdc6 for degradation (Sakai et al., 2010, Smolders & Teodoro 2011). Furthermore, APC^{Cdh1} targets Cdc25A (cell cycle division 25A) a regulator of Cyclin E/CDK2 and of Cyclin A/CDK2 activity for degradation by the proteasome (Skai 2010).

Whereas APC/C^{Cdh1} activity promotes the formation of the pre-replication complex in early G1 phase (Sigl 2009, Ayad 2005), inactivation of APC/C^{Cdh1} prevents re-

replication during S phase. At the G1/S boundary, APC/C^{Cdh1} activity is inhibited by several mechanisms. Emi1 inhibits APC/C^{Cdh1} as a pseudosubstrate. This inhibition results in the stabilisation of Cyclin A and geminin required for controlling the processes involved in DNA replication (Sivaprasad et al., 2007). Increase in CDKs activity leads to phosphorylation and dissociation of Cdh1 (Li & Zhang 2009, Smolders & Teodor 2011). At this stage, APC/C^{Cdh1} mediates its own degradation (Listovsky et al., 2004). During S phase, the abundance of Cdh1 is also controlled by the SCF ubiquitin ligase complexe (Benmaamar & Pagano 2005).

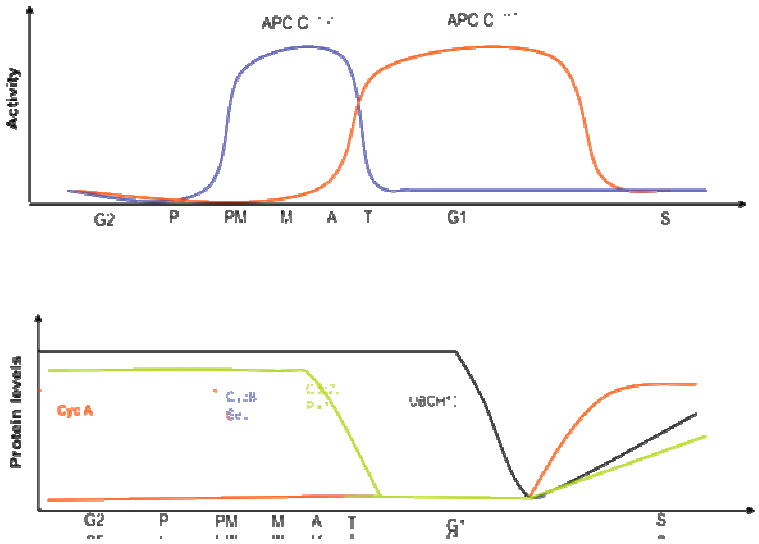

Fig. 5: Correlation between the APC/CCdh1 activity and Cyclins level (according to Peter 2006).

5. Other functions of the APC/C^{Cdh1}

5.1 During DNA damage

In response to DNA damage, distinct mechanisms cooperate to initiate and to maintain cell cycle arrest. Several studies implicate Cdh1 in activating the G2/M checkpoints and in promoting G2 arrest upon genotoxic stress (Sudo et al., 2001, Bassermann et al., 2008). Under these conditions, APC/C^{Cdh1} becomes transiently active. Cdc14B is the phosphatase involved in dephosphorylating Cdh1 and in the subsequent reactivation of APC/C^{Cdh1} (Wulf & Visintin 2008, Bassermann et al., 2008). Activated APC/C^{Cdh1} targets Plk1 for proteasome-dependent degradation. This results in the stabilisation of two targets of Plk1, namely claspin and the Cdk1 inhibitor Wee1. Claspin phosphorylates and activates the kinase Chk1, which is

involved together with Wee1 in phosphorylating and inactivating CDK1. Attenuation of CDK1 activity promotes the G2 arrest and inhibits mitosis (Bassermann et al., 2008).

Interestingly, reactivation of Cdh1 at G2 upon genotoxic stress was only affecting the stability of Plk1. Suggesting the existence of other mechanisms involved in regulating APC/C^{Cdh1} activity and in protecting other Cdh1 targets from degradation in G2 DNA damaged cells (Bassermann et al., 2008).

5.2 During G0 and in differentiated cells

APC/C^{Cdh1} activity is not restricted to proliferating cells but covers as well non-proliferating cells. Several observations point to the implication of APC/C^{Cdh1} in cell cycle exit and differentiation. It has been reported that depletion of the APC2 subunit of the APC/C results in an inactivation of the APC/C^{Cdh1}, accumulation of Skp2 and Ets2 and a re-entry of this cell into the cell cycle (Wirth et al., 2004). Therefore, APC/C^{Cdh1} activity is most likely necessary to maintain the quiescence state of hepatocytes and to prevent a spontaneous re-entry to the cell cycle (Wäsch et al., 2010).

The importance of Cdh1 during quiescence is thought to be related to the ability of APC/C^{Cdh1} to inhibit proliferation through targeting Cyclins for degradation (Coller 2007, Alexandrow & Hamlin 2004). Skp2 is also a target of APC/C^{Cdh1} during G0. This allows the accumulation of p27 and the subsequent inhibition of Cyclin E and Cyclin A- associated kinase activity (Leuken 2008). Furthermore and in contrast to the above mentioned APC/C^{Cdh1} function concerning the control of the formation of the pre-replicative complexe during G1 (see 4-3), APC/C^{Cdh1} prevents the assembly of the pre-replicative complex during quiescence. This function is accomplished through targeting components and regulators of this complex for degradation by the proteasome. This includes Cdc6, geminin and Cyclin A (Coller 2007).

Once quiescent cells are stimulated to re-enter the cell cycle, Cyclin E and Cyclin E/CDK2 activity increase. Cyclin E/CDK2 phosphorylates Cdc6 resulting in its stabilisation and binding to origin of DNA replication initiating thereby DNA synthesis and promoting a cell cycle re-entry (Ayad 2005).

Cdh1 as well as subunits of the APC/C are highly expressed in postmitotic neurones (Kim & Bonni 2007). Recent studies revealed that APC/C^{Cdh1} regulates different aspects of neuronal function and development. This function is achieved by targeting specific substrates for degradation during neuronal growth and differentiation (Harmey et al., 2008). APC/C^{Cdh1} inhibits the axonal growth through targeting the transcriptional regulators Sno1 and Id2 for degradation (Huynh et al., 2009, Stegmüller et al., 2008). Other studies pointed to the importance of APC/C^{Cdh1} in controlling neuronal survival. This function is achieved through targeting Cyclin B. Degradation of cyclin B inhibits a re-entry to the cell cycle and apoptotic cell death (Almeida et al., 2005). It has been reported that inhibition of APC/C^{Cdh1} by phosphorylation by Cdk5 stabilises Cyclin B and triggers apoptosis in neurons (Maestre et al., 2008).

Comparative electrophysiological and behavioural studies between Cdh1 heterozygous knockout mice and the wild type implicate APC/C^{Cdh1} in learning and memory by controlling synaptic plasticity (Li et al., 2008, Garcia-Higuera et al., 2008). Targets of APC/C^{Cdh1} required for establishing this functions are still unknown (Li et al., 2008). Studies in worms and flies give the first evidence for the involvement of the APC/C^{Cdh1} in the regulation of synapse morphogenesis and function. This is provided by targeting liprin-α (regulator of synaptic button numbers and activity) for degradation and by controlling indirectly the abundance of the post-synaptic glutamate receptors GLR-1 in the ventral nerve cord (Stegmüller & Bonni 2005, Kim & Bonni 2007, Yue et al., 2010). In this regard, it will be interesting to investigate whether homologues of Liprin-α and GLR1 exist in mammals. This will give most likely more insight into the importance of APC/C^{Cdh1} in controlling synapse morphogenesis and thereby learning and memory (Li 2009).

6. Life without Cdh1

Although Cdh1 is involved in regulating the mitotic exit, the function of APC/C^{Cdh1} function in mitosis is not essential (Skaar & Pagano 2008). Evidence suggests that Cdh1-deficient cells are able to exit mitosis and to enter G1, most likely due to the compensatory effect played by the stabilisation of Cdc20 in the absence of Cdh1

(Garcia-Higuera et al., 2008, Li et al., 2008, Li & Zhang 2009). In contrast, G1 phase is more concerned in its absence. It has been shown that absence of Cdh1 or deregulation of its functions results in an early accumulation of Cyclin A, a stabilisation of Skp2 and destabilisation of p27. This results in shortening G1 and elongation of S phase (Li 2009), most likely due to activation of checkpoint in response to an early onset of and impaired progression through S phase (Wei et al., 2004, Engelbert et al., 2008, Sigl el al., 2009).

To further analyse the importance of Cdh1 *in vivo* and in an attempt to generate a cdh1 deficient homozygous mice, the Zhang group realised that Cdh1 deficiency is embryonic lethal and that the homozygous embryos die at around E9,5 due to placenta defect. This was due to inhibition of the endoreplication resulting in a failure in the formation of placental giant cells (Li et al., 2008, Garcia-Higuera et al., 2008). Malumbres and colleagues gave more evidence for the direct implication of the placentas deficiency with lethality observed Cdh1 deficient embryos. By developing an embryo model, in which Cdh1 was expressed only in the placenta, they were able to rescue these embryos at different stage of developments and 3 days after birth. But for unknown raisons the Cdh1 knockout mice die later on, and the survival of Cdh1 heterozygous mice was reduced at later age compared to wild type (Garcia-Higuera et al., 2008, Skaar & Pagano 2008).

Mouse embryonic fibroblasts (MEF´s) derived form Cdh1 knockout embryos are able to grow and to divide in culture. But they show a slower and defective exit from mitosis (Li et al., 2008).

In contrast to Cdh1 deficient cells, Cdh1 deficient MEF´s show a slower proliferation rate compared to wild type MEF´s and become senescent. This was due to the stabilisation of the transcription factor Ets2 and the subsequent upregulation of p16 in the absence of Cdh1 (Li & Zhang 2009), whereas in the case of Cdh1 deficient cells accumulation of Ets2 promotes proliferation by inducing Cyclin D expression (see 4-3) (Li 2009). These observations implicate Cdh1 in maintaining the replicative life span of the MEF´s.

Cdh1 deficient MEF's show also a high degree of genomic instability manifested by multinucleated cells, misaligned metaphase chromosomes and multipolar spindles (Skaar & Pagano 2008). Deregulation in the turnover of Cdh1 substrates including Plk1 and Aurora A could be a trigger for centrosomal abnormalities, cytokinesis defects and aneuploidy (Engelbert et al., 2007, Wäsch et al., 2008).

Genomic instability can lead also to tumour development during proliferation in the absence of Cdh1 (Garcia-Higuera et al., 2008). Accumulating evidences point to the tumour suppressor function played by Cdh1 (Wäsch et al., 2008, Garcia-Higuera et al., 2008). In fact, many targets of Cdh1 are highly expressed in tumours (Nakayama 2006). This includes Aurora A, Plk1, Cyclin A, B and Cdc20 and other APC/C^{Cdh1} substrates such as the potential oncogenes Skp2 (Gao el al., 2009), Ets2 (Li & Zhang 2009, Li 2009) and Cdc25A (Sakai et al., 2010).

Although Jung Cdh+/- heterozygous mice develop smaller induced tumours compared to wild type, Cdh1 heterozygous mice were more susceptible to spontaneous tumours compared to wild type at later ages. They can develop epithelial tumours, which were not detectable in wild type mice. A reduced expression of Cdh1 has been also detected in various types of cancer cells (Engelbert et al., 2008, Wäsch & Engelbert 2005, Skaar & Pagano 2008). However, the mechanism involved in reducing Cdh1 expression in tumour cells is still unclear (Garcia-Higuera et al., 2008).

All these evidences suggest that Cdh1 could be a haplo-insufficient tumour suppressor (Garcia-Higuera et al., 2008) and point to the importance of regulating the APC/C^{Cdh1} activity in preventing genomic instability and tumourogenesis.

B. General aspects of the Endoplasmic Reticulum Stress Response

1. ER function

The endoplasmic reticulum is a central organelle in eukaryotic cells. The ER is considered to be the first compartment in the secretory pathway (DuRose et al., 2006). It is the place of biosynthesis of steroids, cholesterol and many lipids. The ER

is involved also in regulating calcium homeostasis, in the synthesis, modification and delivery of proteins to their target sites (Schröder & Kaufmann 2005). Proteins are translated into the ER lumen by ribosomes localised on the cytosolic surface of the ER (Rasheva & Domingos 2009), where they fold to their native conformation by undergoing different translational, co-translational and post-translational modifications (Shröder & Kaufmann 2005, Schröder 2006). Folding requires two classes of proteins namely folding enzymes and chaperones. Folding enzymes accelerate the kinetics of reactions involved in the folding process, whereas chaperones facilitate proper folding (Hebert & Molinari 2007). Bip/GRP78 is the most abundant chaperones in the ER. Bip is involved in controlling many aspects of the ER function by facilitating the translocation of newly synthesised proteins to the ER, by assisting protein folding and by preventing aggregation of proteins within the ER (Kaufman 2002, Hebert & Molinari 2007). The ER function is surveyed by a process called Quality Control. This process allows only properly folded proteins to be exported to the golgi complex and retains incompletely folded or missfolded proteins in the ER in order to complete the folding (Schröder & Kaufmann 2005). Missfolded protein can be also exported into the cytosol and become targets for the ubiquitin-mediated proteasomal degradation in a process termed ER-associated degradation (ERAD) (Römish 2005).

2. ER stress and ER stress response

Perturbation in the ER functions by changes in calcium concentration, nutrient deprivation, increase in the secretory protein synthesis and alterations in the oxidation-reduction balance, for example, cause ER stress manifested by an accumulation of unfolded proteins in the ER lumen (Schröder & Kaufman 2005).

To cope with this perturbation and to re-establish homeostasis the cell elaborates a system termed the ER stress response or the unfolded protein response (UPR).

The UPR consists of three signalling pathways, mediated by the ER transmembrane protein IRE1 (inositol-requiring kinase1), ATF6 (activating transcription factor 6) and PERK (double strand RNA-activated protein kinase-like ER kinase).

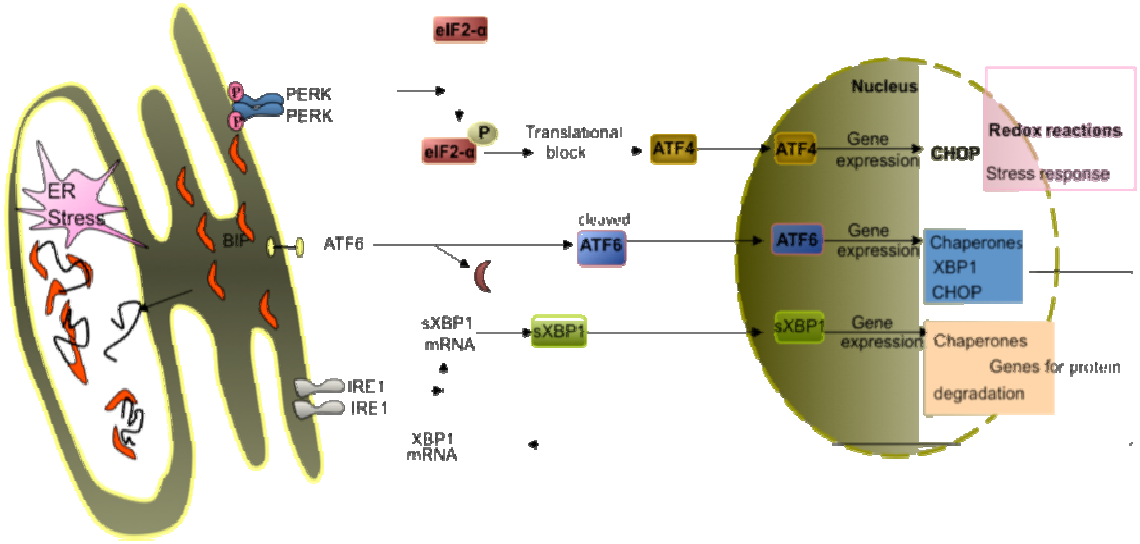

Fig. 6: Pathways activated during the UPR (according to Szegezdi et al., 2006)

Under normal conditions, the three signalling pathways are inactive through association of the ER stress sensors with the ER chaperone Bip (Binding IgG protein). ER stress conditions induce dissociation of Bip from the ER transmembran proteins to bind to missfolded proteins accumulated in the ER. This allows the dissociation of ATF6 from the ER membrane, the demerization, autophosphorylation of the ER transmembrane proteins PERK and IRE1 and thereby the activation of all three ER signalling pathways (Fribley et al 2009). Additional mechanisms controlling the ER stress sensing and the subsequent activation of the UPR signalling pathways are still under discussion and investigation (Lai et al., 2007).

3. The UPR signalling and cell survival

The ER stress response is initially cytoprotective and aims to re-establish cellular homeostasis. This function is achieved by increasing the protein folding capacity of the ER, inhibiting the protein synthesis and activating the ER associated degradation (ERAD) to reduce the level of missfolded proteins accumulated in the ER (Szegezdi et al., 2006).

3.1 Increase in protein folding capacity of the ER

Activation of IRE1 and ATF6 signalling pathways promotes the transcription of gene involved in increasing the folding capacity of the ER. IRE1 is a protein kinase endoribonuclease. Activation of IRE1 signalling results in the cleavage of an intron

sequence from the mRNA of the X-box DNA binding protein (XBP1), which inhibits its translation. Once translated, XBP1 translocates to the nucleus and promotes the expression of genes encoding chaperones. XBP1 regulates also the biosynthesis of membrane proteins and phospholipids in order to induce membrane proliferation and to expand the ER (Schröder 2006). The expression of XBP1 can also be regulated by ATF6 signalling pathway. ATF6 is transported from the ER to the golgi after its dissociation from Bip. In the golgi, ATF6 becomes subjected to cleavage by specific proteases. After translocation to the nucleus, ATF6 promotes the expression of genes encoding chaperones, folding enzymes and protein disulfide isomerases (Schröder 2006, Kim et al., 2006). ATF6 is also able to promote phosphatidylcholine synthesis supporting thereby ER biogenesis independent of XBP1 signalling pathway (Tsai & Weissman 2011).

3.2 Inhibition of protein synthesis

The third UPR transmembrane sensor is PERK, which is a serine/threonine kinase. Activation of the PERK signalling pathway results in the phosphorylation of eIF2-α (eukaryotic translation initiation factor 2α) at serine-51. eIF2-α is a translation factor, which is in its GTP-bound form responsible for bringing methionine charged tRNA (Met-$tRNA_i^{Met}$) to the small ribosomal subunit and the subsequent formation of the pre-initiation complexe. This complexe scans the 5´ end of the mRNA for the initiation codon AUG. Before initiating the elongation step in the protein synthesis, the GTP is hydrolysed to GDP to release the translation initiation factor. To start another round of translation, the guanine nucleotide exchange factor, eIF2B, converts the inactive form GDP-eIF2-α to active GTP- eIF2-α. Phosphorylation of eIF2-α prevents the exchange of GDP-bound eIF2-α for GTP and thereby the formation of a new pre-initiation complex. This results in the inhibition of general protein synthesis (Koumenis & Wouters 2006, Kim et al., 2008).

Phosphorylation of eIF2-α can be induced by other forms of cellular stress (Holick & Sonnenberg 2005). GCN2 (general control non depressible 2), HRI (heme regulated inhibitor of translation) and PKR (double-stranded-RNA-activated protein kinase) are other kinases involved in this phosphorylation respectively during amino acid

deprivation, heme deficiency and by double-stranded-RNA species produced by viral infection for example (Brush et al., 2003, Jiang & Wek 2005, Lee 2009).

Inhibition of general protein synthesis under stress conditions is thought to play a cytoprotective function by reducing the protein load. However, specific genes are expressed and translated selectively and preferentially during the ER stress. This includes the transcription factor ATF4 (activating transcription factor 4). ATF4 in turn induces genes involved in amino acid transport, glutathiones biosynthesis, and redox regulation (Hussain & Ramaiah 2007, Brush 2003) promoting thereby cell survival under ER stress conditions (Fribley 2009). The translation machinery involved in the translation of these genes is different from the one involved in the translation of genes expressed under normal conditions. Stress inducible genes are translated in a specific mechanism termed cap-independent translation or IRES-mediated translation, which requires the existence of internal ribosome entry site (IRES) in the 5´ untranslated region (UTR) (Holcik & Sonenberg 2005, Jackson 2010).

The Shutoff of protein synthesis induced by the ER stress is transient. The translational recovery is regulated in a feedback loop: after activation of the PERK pathway, ATF4 induces the expression of GADD34 (growth arrest and DNA damage). GADD34 contributes as a subunit of the phosphatase type 1 (PP1) in dephosphorylating the eIF2-α and thereby in the resumption of protein synthesis (Shröder & Kaufman 2005, Boyce & Yuan 2006). Gadd34 was first identified in a screen for gens, which were upregulated during UV irradiation in Chinese hamster ovary cells (Yagi el al., 2003, Hasegawael el al., 2000). It is induced by other factors including DNA damaging agents, growth arrest treatments (Hasegawa et al 2000), heat shock, exposure to agents inducing apoptosis (Grishin et al., 2001) and by interleukine 6 in myeloid differentiation (Liebermann & Hoffman 2002).

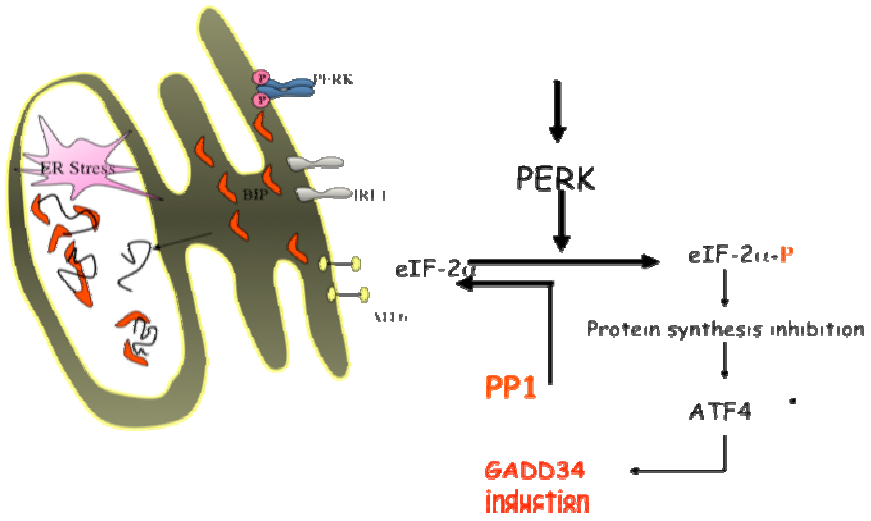

Fig. 7: Gadd34 modulates the ER stress response

GADD34 deficient MEF´s show a delay in the resumption of protein synthesis during ER stress (Yagi et al., 2003, Kojima et al., 2003) and reduced activation of genes induced during ER stress (Bruch & Shenolikare 2008). This suggests that the negative-feedback loop played by the GADD34/PPI activity is an important mechanism in the regulation of stress-induced gene expression (Novao et al., 2003).

3.3 Removal of missfolded Proteins

ER-associated degradation (ERAD) is an additional mechanism activated under ER stress in order to eliminate missfolded proteins accumulated within the ER (Römish 2005). ATF6 and IRE1 – XBP1 signalling pathways are involved in the expression of genes coding proteins required for the ER-associated degradation (ERAD), especially the membrane protein EDEM (ER degradation-enhancing 1, 2-α-mannosidase-like protein) (Zhang & Kaufman 2004). EDEM in combination with the ER chaperone calnexin recognises missfolded proteins and promotes their translocation to the cytoplasm. These proteins then become subject to ubiquitination and proteasomal degradation (Vembar & Brodsky 2008).

Besides the ubiquitin-proteasome system, autophagy or lysosomal degradation is another mechanism involved in degradation of missfolded proteins, cytoplasmic components and damaged subcellular organelles (Ogata et al., 2006). Autophagy started by the formation of double membrane vesicle termed autophagosomes

surrounding a part of the cytoplasm. Fusion of autophagosomes with lysosomes allows the release of the inner vesicle into the lysosome and the degradation of the vesicles by lysosomal hydrolase followed by a release of digested components into the cytoplasm (Singletary & Milner 2008).

Both PERK and IRE1 Signalling can promote the expression of autophagy genes (ATG). Activation of autophagy can serve many functions. By promoting the degradation of macromolecules for example, autophagy provides nutrients and ATP helping thereby cells to survive under and to adapt to extreme conditions during ER stress (Vicencio et al., 2008, Ogata et al., 2006, Ding 2006).

4. The UPR signalling and cell death

In prolonged ER stress, the three axes of the UPR can switch from the pro-survival to the cytotoxic response. This function is achieved by activating different genes controlling apoptotic and non-apoptotic cell death.

The pro-apoptotic protein Chop (CCAAT/enhancer binding protein (C/EBP) homologous protein) also called GADD153 is one of the pivotal effectors of this switch. Chop is a transcription factor, which can be induced by PERK, ATF6 and IRE1 signalling pathways under severe ER stress (Schröder 2006). It has been reported that deletion of Chop protects cells from death induced during severe ER stress (Rutkowski et al 2006). The mechanism by which Chop induces death in ER-stressed cells was the topic of many investigations. Evidences suggest that induction of GADD34 by Chop and the subsequent resumption of protein synthesis in stressed cells can result in apoptosis (Zhang & Kaufman 2008). Chop/GADD135 can activate caspase-3 (Zhang & Kaufman 2004, Bateman). It can suppress the anti-apoptotic protein Bcl2 allowing thereby the release and the activation of the apoptotic proteins BAK and BAX (Lai et al., 2006). Chop can also promote cell death by inducing TRB3 (tribbles homologues 3) (Ohaka et al 2005, Verfaillie et al., 2010) and DR5 (death receptor 5) (Zhang & Kaufman 2008).

Chop induction is not only the mechanism regulating ER stress-induced apoptosis. IRE1 signalling can also eliminate damaged cells by activating other apoptotic pathways. IRE1 can recruit TRAF2 (tumor necrosis factor receptor-associated factor 2 to the ER membrane. The interaction between IRE1 and TRAF2 is mediated by JIK (cJun N-terminal inhibitory kinase) and results in the activation of ASK1 (apoptotic signal regulating kinase) and the downstream JNK pathway leading to cell death. JNK activation induces apoptosis during the ER stress through phosphorylation of Bcl2 family proteins resulting in the release and the subsequent activation of the pro-apoptotic proteins Bax and Bid (Lai et al., 2007, Lamara & Scorrano 2009). Furthermore, the phosphorylation of Bcl2 by JNK promotes its dissociation from the autophagic protein Beclin-1 resulting in increased autophagy followed by increased susceptibility to apoptotic and non-apoptotic cell death (Verfaillie el al., 2010).

Recruitment of TARF2 by IRE1 results also in cleavage and activation of caspase-12 (Kim et al., 2006, Szegezdi et al., 2003). Activated caspase-12 activates caspase-9, which in turn activates caspase-3 and leads to apoptosis (Zhang & Kaufmann 2006).

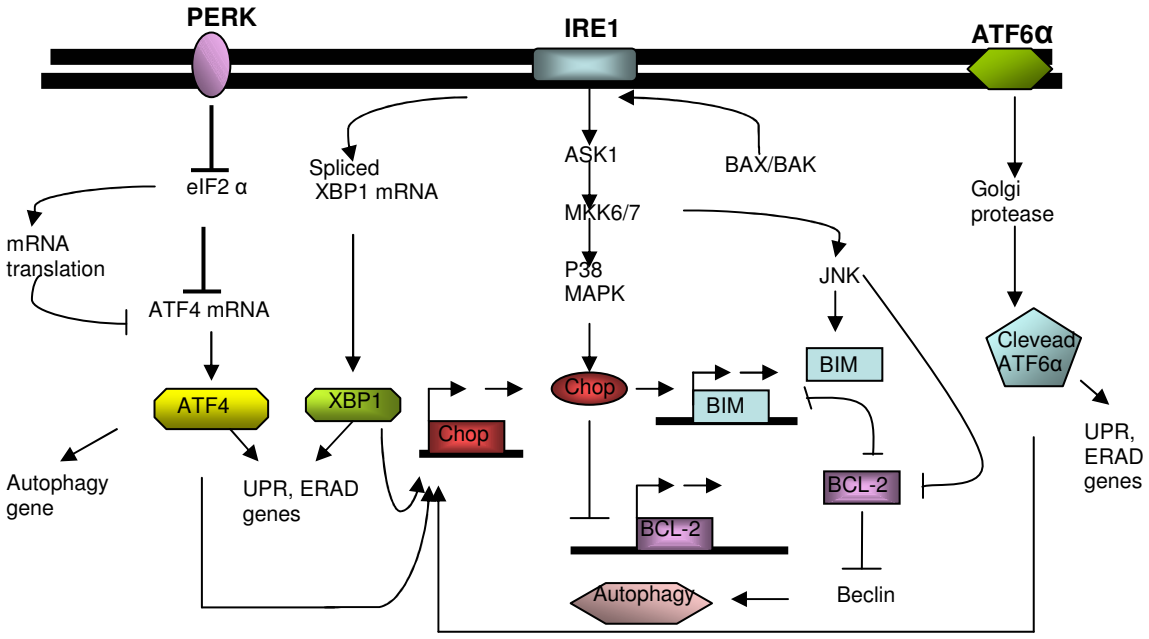

Fig. 8: Connections between the UPR and the cell death machinery (according to Kim et al., 2008).

5. UPR and cell cycle

There is evidences pointing to the connection between the ER stress and the regulation of cell cycle progression. This connection is mediated by the activation of PERK signalling pathway. Inhibition of protein synthesis by this signalling results in a rapid degradation of short lived proteins including the G1 regulator Cyclin D. Loss of Cyclin D results in a decrease in Cyclin D/CDK4 activity and release of CKIs. This leads to inhibition of the Cyclin E/ and Cyclin A/CDK2 complexes and in arresting the cell cycle at G1 (Brewer et al., 1999, Brewer & Diehl 2000). Diehl and colleagues suggested also the existence of other mechanisms implicated in the G1/S arrest observed upon ER stress. They have demonstrated that ER stress caused by glucose deprivation or tunicamycin treatment leads to the activation of p53, which is involved as transcription factor in regulating the transcription of gene activated during growth arrest and apoptosis. Activation of p53 results in the induction of p21, which arrests stressed cell at G1/S (Diehl 2006). p53 is also induced during metabolic stress. Another study describes the mechanism linking glucose availability to the regulation of growth proliferation and cell cycle progression by activating p53 pathway. This connection is provided by the activation of AMP-activated protein kinase. AMPK kinase is a metabolic sensor, which senses the increase in the intracellular AMP: ATP ratio in response to ATP depletion and initiates the cell cycle arrest. AMPK promotes the cell cycle arrest by phosphorylating p53 at serine-15 and the subsequent induction of p21 (Jones et al., 2005). A recent study suggested that ER stress can promote also a G2 arrest. The arrest is mediated by p53/47 an isoform of p53. p53/47 lacks the first 39 amino acids of the full-length p53 and contains IRES entry sites, which allows its translation in a cap-independent manner after activation of PERK-signalling pathway (Bourougaa et al., 2010).

Besides reducing the protein load, the cell cycle arrest can be considered as an additional cyto-protective aspect of the activated PERK signalling pathway (Brewer & Diehl 2000).

6. UPR and cancer

The microenvironment of solid tumours is characterised by Hypoxia, low pH and heterogeneities in nutrient supply due to impaired vascularisation. Through all these

conditions, tumour cells experience ER stress and respond by activating the UPR. Several studies reported that the UPR is activated in different type of cancers (Zhao & Ackerman 2006, Wang et al., 2008). Consistent with the cytoprotective role played by the UPR, activation of this mechanism might allow cancer cells to adapt to the stressful conditions and thereby promote tumour growth (Tsai & Weissman 2010). The first evidence for the interconnection between cancer development and UPR come from the observation that GRP78-knockdown fibrosarcoma cells injected into mice do not form or quickly regress (Jomara et al., 1996). It has also been reported that chaperones including Grp78 and Grp94 are highly expressed in a variety of cancers. Induction of Grp78 promotes cells proliferation (Wang et al., 2010), adaptation to tumour microenvironment (Verfaillie et al 2010) and protects tumour cells from immune surveillance (Lee 2001). Several other studies confirm the importance of the activation of PERK and IRE1 signalling pathways in promoting tumours growth and in the adaptation to hypoxia. Both PERK and XBP1 deficient MEF's show an increased cell death under hypoxic conditions and attenuated tumour growth in xenograph model (Tsai & Weissman 2010, Verfaillie et al., 2010). There is evidences that PERK and IRE1 promote the upregulation of the vascular endothelial growth factor VEGF-A. This suggests the role of the UPR in favourising angiogenesis and thereby tumours growth (Verfaillie et al., 2010).

Other studies revealed that activation of the UPR promotes dormancy and even survival of dormant tumours. This is achieved by inducing G1 arrest due to inhibition of protein synthesis after activation of the PERK signalling pathway. The ATF6 signalling pathway has been shown to be involved in promoting survival during long period of tumour dormancy (Tsai & Weissman 2010). Autophagy can also promote survival of cancer cells under stressful conditions by recycling macromolecules and providing energy and metabolite in hypoxic regions of the tumours (Rosenfeldt & Ryan 2009). The evidence for its requirement came from the observation that inactivation of autophagy results in increased apoptotic cells death in tumours cells (Ding et al., 2007).

All these evidences detailed above indicate the involvement of the UPR in promoting the growth and the survival of tumour under hypoxic conditions and low nutrients. The connection between UPR and cancer suggests the possibility of treating cancer

by modulating the UPR through pharmaceutical intervention. This can be mediated either by inhibiting components of the UPR which provide tolerance to these conditions or by activating the pro-apoptotic function of the UPR. Targeting the pro-survival function played by autophagy during ER stress can eliminate aggressive tumours (Li et al., 2011). Inhibition of ATF6 signalling pathway can also be an effective tool in blocking survival of dormant tumours (Ranganathan et al., 2006, Tsai & Weissman 2010). It has been also shown that suppression of chaperone enhances apoptosis and can be an effective target for cancer therapy (Lee 2001, Wang et al., 2010). Versipelostatin (Kim et al., 2008) and temozolomide are examples of compounds showing an antitumor activity by antagonizing Grp78 (Wang et al., 2010). UPR-inducer drugs that cause severe ER stress either by inhibiting the proteasome (Bortezomib) or by blocking the ERAD (Eeyarestatin1) were also effective in eliminating tumour cells (Wang et al., 2010, Verfaillie et al., 2010, Li et al., 2011). δ-Tetrahydrocannabinol, an inducer of autophagic cell death derived from marijuana, was effective in turning autophagy against tumours and in treating different type of cancer cells (Salazar et al., 2009).

All these considerations suggest that manipulation of the ER stress response could be an attractive tool in treating cancer cells. However, other observations implicate the UPR in the resistance to chemotherapy. By promoting dormancy or growth arrest, PERK signalling for example can allow tumours to resist and survive stress during cancer treatment (Koumenis & Wouters 2006, Ranganathan et al., 2006). The pro-survival effect of the chaperones can also provide resistance to chemotherapy (Ying-yan et al., 2008, Andrieu et al., 2010). It has been also reported that activation of the UPR provides resistance to topoisomerase poisons such as etoposide, an effective anticancer agent, which inhibits relegation of DNA strands during replication and activates DNA checkpoints. Most likely the UPR antagonises the effect of etoposide by decreasing the level of DNA topoisomerase due to inhibition of translation (Ma & Hendershot 2004).

Therefore, inhibition of the pro-survival or activation of the pro-apoptotic functions of the ER stress response could be useful to overcome the problem of the resistance of cancer cells to chemotherapy.

In summary, the UPR is biphasic and can either promote or inhibit tumour growth. It can also provide resistance or sensitise tumours to chemotherapy. Further investigations are necessary to explore and to exploit the mechanism controlling the balance between cytoprotective and cytotoxic response of the UPR, which is a big challenge and might be an effective way to overcome drug resistance and to cure cancer.

II. Aim of the thesis

In order to define new functions of APC/C^{Cdh1}, a Yeast-two-hybrid screen was performed in our Group. In this screen Gadd34 was identified as a new interacting partner for the cell cycle regulator Cdh1. The connection between the cell cycle and the ER stress response suggested that the response of cell to ER stress might be controlled by Cdh1.

The ER stress response can promote cell survival and cell death. It was therefore aimed to find out whether Cdh1 is involved in one or the other outcomes of ER stress response.

ER stress is often involved in cancer development and therapy. These investigations should not only contribute to deeper understanding of ER stress homeostasis but might also open the road for potential cancer treatment.

III. Material and Methods

1. Expendable items

Cover slip	Menzel-Gläser, Braunschweig
Freezing tube	Sarstedt, Nümbrecht
Microscope slides	Menzel-Gläser, Braunschweig
Transfer membrane	PerkinElmer, Rodgau
Hyperfilm TM ECL	Amersham Bioscience, Braunschweig
Whatman	Omnilab, Elbingeröder
Centrifuge tubes	Beckmann, Palo Alto
Dialysis tubing	Pierce, Rockford
6 wells plate	Sarstedt, Nümbrecht
12 wells plate	Sarstedt, Nümbrecht
96 wells plate	Sarstedt, Nümbrecht
Cell culture dishes 1000x15 mm	Sarstedt, Nümbrecht
Cell culture dishes 60x15 mm	Greiner Bio-One, Frickenhausen
Micro tube 1,5 ml	Sarstedt, Nümbrecht
Filter Tips 0,1-10 ul	Sarstedt, Nümbrecht
Filter tips 20 ul	Sarstedt, Nümbrecht
Filter tips 200 ul	Sarstedt, Nümbrecht
Filter tips 1000 ul	Greiner, Solingen
15 ml tube	Sarstedt, Nümbrecht
50 ml tube	Sarstedt, Nümbrecht

2. Equipments

Agarose Gelelectrophoresis systems	Bio-RAD, Munich
Centrifuges:	Eppendorf, Hamburg
Centrifuge 5415 D	Roth Karlsruhe
Centrifuge Mikro 220 R cooled	Heraeus, Osterode
Megafuge 1.0	Hettich, Tuttlingen
Centrifuge “Rotina 38R”	Beckman GmbH, Düsseldorf
Centrifuge “L8-55M”	GFL, Burgwedel
Chaker vibramax 110	Heidolph, Kelheim
Clean Bench	Hera Safe, Kendro, Osterode
Easypet 4420	Sartorius, Göttingen
Electrophoresis power supply	Leica DM5000, Leica, Wetzlar
Fluoresence microscope	Bio-RAD, Munich
Gel chambers for proteins	Bio-RAD, Munich
Heater	Heraeus Instruments GmbH, Osterode
Incubator	Heidolph, Kelheim
Magnetic stirrers	Bio-RAD
MicroPulser ™	Tecan Deutschland GmbH, Crailsheim
Microtiterplate luminometer	Bauknecht, Stuttgart
Microwave	Bio-RAD, Munich
Mini-Protein Electrophoresis System	Bio-RAD, Munich
Mini Trans-Blot cell	Bio-RAD, Munich
Mixer 5432	Eppendorf, Hamburg
Multipette® plus	InoLab, Weilheim

pH-meter	Eppendorf, Hamburg
Photometer	Eppendorf, Hamburg
Pipett	Hirschmann, Eberstadt
Pipetman	Bio-RAD, Munich
Power supplies	Gilson, Villiers le Bel
Sonifier UP 200H	Bio-RAD, Munich
Thermo cycler	Hielscher, Stahnsdorf
Thermomixer 5436	MWG-Biotech AG, Ebersberg
ThermoStat plus	Eppendorf, Hamburg
Vortex-Genie	Eppendorf, Hamburg
Water baths	Janke & Kunkel, Staufen

3. Softwars

Microsoft Excel 2007 (Microsoft Inc., Remond, Washington, USA)

Microsoft Word 2007 (Microsoft Inc., Remond, Washington, USA)

FlowJo Version 7.3 (Tree Star Inc., Ashland, USA)

BD Cell Quest Pro™ (BD Biosciences, Mississauga, USA)

ImageJ 1.42 (Free Software Foundation, Inc., Boston, USA)

Adobe Photoshop 7.0 (Adobe Systems, San Jose, USA)

MacVector Version 10.1 (MacVector Inc, Cambridge, United Kingdom)

4. Chemicals

Acetic acid	Baker, Griesheim

Aceton	Merck, Darmstadt
Acrylamid-solution (30%) Mi 37,5:1	AppliChem, Darmstadt
Ampicillin	AppliChem, Darmstadt
Annexin V conjugates	Invitrogen
Agar	AppliChem, Darmstadt
Agarose	Bioline, Luckenwalde
Ammoniumacetat	AppliChem, Darmstadt
Ammoniumsulfat	AppliChem, Darmstadt
Ampicillin	AppliChem, Darmstadt
Antifoam	Sigma-Aldrich, Schnelldorf
A-sepharose bead	GE Healthcare, Munich
BactoTM peptone	Becton Dickinson, Heidelberg
Benzamidine	Sigma-Aldrich, Schnelldorf
BM Cyclin	Roche, Mannheim
ß-Glycerophosphat	AppliChem, Darmstadt
ß-Mercaptoethanol	AppliChem, Darmstadt
Bromophenol bleu	AppliChem, Darmstadt
Boric acid	Merck, Darmstadt
Calcium chlorid	AppliChem, Darmstadt
Cesium chloride 99%	AppliChem, Darmstadt
DAPI	AppliChem, Darmstadt
DEPC	AppliChem, Darmstadt
DMEM	Invitrogen, Karlsruhe
dNTP	Fermentas, St. Leon-Rot
Doc	AppliChem, Darmstadt
EDTA	AppliChem, Darmstadt
EGTA	AppliChem, Darmstadt
Ethanol	Meck, Darmstadt
Ethanolamine	Sigma-Aldrich, Schnelldorf
Ethidiumbromide	AppliChem, Darmstadt
FCS	Biochrom, Berlin
Formaldehyde solution min. 37% free from acid	Merck, Darmstadt
Gelatine	Sigma-Aldrich, Schnelldorf
Glucose	AppliChem, Darmstadt
GlutaMax	Invitrogen, Karlsruhe

Glycine	AppliChem, Darmstadt
G-sepharose beads	GE Healthcare, Munich
Guanidine thiocyanate	Sigma-Aldrich, Schnelldorf
HEPS	AppliChem, Darmstadt
30% H_2O_2	Sigma-Aldrich, Schnelldorf
HiPerfect	Qiagen, Hilden
Imidazole	AppliChem, Darmstadt
Isopropanol	Merck, Darmstadt
Kanamycinesulfate	AppliChem, Darmstadt
Lauroylsarcosine	Sigma-Aldrich, Schnelldorf
Luminol min 97%, HPLC	Sigma-Aldrich, Schnelldorf
Magnesium chloride	AppliChem, Darmstadt
Methanol	AppliChem, Darmstadt
MG132	Sigma-Aldrich, Schnelldorf
Natrium	AppliChem, Darmstadt
Natriumchlorid	AppliChem, Darmstadt
Nocodazole	Sigma-Aldrich, Schnelldorf
Nonfat dried milk powder	AppliChem, Darmstadt
Nonidet P40	AppliChem, Darmstadt
p- Cumaric acid	Merck-Schuchard, Darmstadt
Penicillin- Streptomycin solution	Invitrogen, Karlsruhe
Pepstatin A	
PfuTurbo DNA polymerase	AppliChem, Darmstadt
Phenol	PfuTurbo DNA polymerase
Polyethylenimine	Roth, Karlsruhe
Ponceau S solution	Sigma-Aldrich, Schnelldorf
Potassium acetate	AppliChem, Darmstadt
Potassium chlorid	AppliChem, Darmstadt
Potassium dihydrogen	AppliChem, Darmstadt
phosphate	Merck, Darmstadt
Prolong® gold antifade reagent	
Propidium iodid	Invitrogen, Karlsruhe
Proteinase K	
Restriction enzymes	Fluka, Steinheim
SDS	AppliChem, Darmstadt
Sodium carbonate	NEB, Fermentas
Sodium chloride	AppliChem, Darmstadt

Sodiumdihydrogenphosphat	AppliChem, Darmstadt
Sodium floride	AppliChem, Darmstadt
Sodium pyrophosphate	AppliChem, Darmstadt
Talon metal affinity resins	AppliChem, Darmstadt
TEMED	Sigma-Aldrich, Schnelldorf
Thymidine 99-100%	Becton Dickinson, Heidelberg
Thermo Pol buffer	AppliChem, Darmstadt
Tris	Sigma-Aldrich, Schnelldorf
Triton X 100	Biolabs, Frankfurt
Trypsin/EDTA solution	AppliChem, Darmstadt
Trypton	AppliChem, Darmstadt
Tunicamycin	Biochrom, Berlin
Tween-20	AppliChem, Darmstadt
Urea	Sigma-Aldrich, Schnelldorf
Vanadat	AppliChem, Darmstadt
Yeast extracts	AppliChem, Darmstadt
Xylenecyanol	AppliChem, Darmstadt
	AppliChem, Darmstadt
	AppliChem, Darmstadt

5. Cell lines

-HeLa cells are a human epithelial cervical cancer. HeLa cells were derived from cervical cancer cells taken from Henrietta Lacks, a patient who eventually died of her cancer

- U2OS is a human osteosarcoma cell line cells. The U2OS cell line was cultivated from the bone tissue of a fifteen-year-old human female suffering from osteosarcoma.

- HEK293 cells or Human Embryonic Kidney 293 cells: are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. HEK 293 cells were generated by transformation of cultures of normal human embryonic kidney cells with adenovirus.

- HCT116 cells are epithelial human colorectal carcinoma cells. HCT116 cells were originally isloted from a mal patient. These cells have a wild-type P53 genotype.

The cells used in this work were cultivated in DMEM medium suplemented with:

GlutaMAX	2 mM
FCS	10%
Penicillin/Streptomycin	100 µg/ml

The cells were frozen in 10 % DMSO in FCS (=Freezing medium)

6. Buffers and solution

6.1 Buffers for the cell culture

Dialysis buffer pH 7,4

HEPS	10 mM
NaCl	150 mM

10X PBS

NaCl	137 mM
KCL	2,7 mM
Na_2HPO_4	100 mM
KH_2PO_4	2 mM

The pH was adjusted to 7,4. PBS was sterilised by autoclaving.

Annexin-binding buffer pH 7,4

HEPS	10 mM

NaCl	140 mM
$CaCl_2$	2,5 mM

Proteinase K buffer

Tris pH 8,5	100 mM
EDTA	5 mM
SDS	0,2 %
NaCL	200 mM
ddH_2O	100 ml

6.2 Buffers for bacteria

1XLB medium

Bacto-tryptone	1 %
Bacto-yeast extract	0,5 %
NaCl	170 mM

The pH was adjusted to 7,0 with 10 M NaOH and the medium was sterilised by autoclaving.

1X agar

Bacto agar	1 %

Sterilise by autoclaving

6.3 Buffers and solution for nucleic acid analysis

10x agarose loading buffer

Tris pH 7.6	150 mM
Glycerol	10 %
Bromophenol blue	0,25 %
Xylene Cyanol	0,25 %

TE buffer

Tris pH 8,0	10 mM
EDTA	1 mM

1x electrophoresis buffer TBE

Tris	89 mM
Boric acid	89 mM
Na_2EDTA pH 8.0	2 mM

Solution for Minipreps

Solution A (Resuspension solution)

25 mM Tris pH 7.5

10 mM EDTA pH 8

50 mM Glucose (add after autoclaving)

Solution B (Cell lysis solution) (fresh)

0.2 N NAOH

1% SDS

Solution C (Neutralisation solution)

3M potassium acetate

11,5 % acetic acid

DEPC H_2O

DEPC	20 µl
ddH_2O	100 ml

Mix underneath the hood!

Let sit overnight before autoclaving

Solution D for RNA extraction

Guanidinium thiocyanate	4 M
Sodium citrate pH 7.0	25 mM
ß-Mercaptoethanol	100 mM
Sodium Lauryl sarcosinate	0,5 %
Antifoam	0,1 %

DEPC H_2O

6.4 Buffers and solutions for protein extraction and analysis

6.4.1 Protein extraction

RIPA

Tris pH 8,0	50 mM
NaCL	80 mM
NaF	50 mM
$Na_4P_2O_7$	20 mM
EDTA	1 mM
EGTA	1 mM
NP-40	1 %
DOC	1 %
SDS	0,1 %

NP40

Tris pH 7,5	50 mM
NaCl	150 mM
NaF	50 mM
$Na_4P_2O_7$	20 mM
EDTA	1 mM
ß Glycerolphosphat	10 mM
NP-40	0, 5 %

4XSB

Tris pH 6,8	0,25 M
SDS	8 %
Glycerol	40 %
ß-Mercaptoethanol	10 %
Bromophenol blue	0,05 %

Protease and phosphatase inhibitors (for 10 ml buffer)

	Inhibitor of	Final concentration
Vanadat	**Tyrosin-phosphatase**	100 uM
Leupeptin	Aspartic-protease	0,5 ng/ml
Leupeptin	Serin-cystein protease	2,5 ng/ml
Benzamidin	Trypsin, thrombin, Plasmin	0,15 mM
Aprotonin	Trypsin, chymotrypsin, Kallikerin	0,5 %
PMSF	Serin-protease	0,5 mM

Table 1: List of the protease and phosphatase inhibitors

6.4.2 Protein analysis

Separating Gel

% Acrylamid	7,5 %	10 %	12 %	14 %	15 %
Acrylamid	2,5 ml	4,1 ml	3,4 ml	2,7 ml	2,45 ml
Tris pH 8,0	2,5 ml	2,5 ml	2,5 ml	2,5 ml	2,5 ml

H_2O	4,85 ml	4,1 ml	3,4 ml	2,7 ml	2,45 ml
10 % SDS	100 ul	100 ul	100 ul	100 ul	100 ul
TEMED	15 ul	15 ul	15 ul	15 ul	15 ul
10 % APS	105 ul	105 ul	105 ul	105 ul	105 ul

Table 2: Recipes of separating gels

Stacking gel

Acrylamid	1,13 ml
Tris 6,8 pH	1,75 ml
ddH_2O	3,2 ml
10 % SDS	70 ul
TEMED	7 ul
10 % APS	70 ul

Table 3: Recipe of the stacking gel

SDS running buffer (10x)

Tris pH 8,3	250 mM
SDS	1 %
Glycine	1,92 M

Western transfer buffer (10x)

Tris	250 mM
Glycine	1,92 M

TNT western blot washing buffer (10x)

NaCL	1,5 M
Tris pH 7,5	100 mM
Tween-20	0,5 %

Urea buffer

Urea	8 M
Imidazole	10 mM
Phosphate buffer pH 8,0	0,1 M

0,1 M phosphate buffer pH 8,0 for 100 ml urea buffer

1 M Na_2HPO_4	93,2 ml
1 M NaH_2PO_4	6,8 ml

Blocking solution

Milk powder, nonfat 5 % in TNT

ECL solution

Solution 1

Luminol	250 mM

p-cumaric acid	0,4 mM
Tris pH 8,5	0,1 M

Solution 2

Tris pH 8,5	0,1 M
30 % H_2O_2	0,061 %

7. Antibodies

7.1 Antibody for Western blot

Primary antibody

Target	Source	Working dilution	Manufacturer
Monoclonal anti-HA (12CA5)	mouse	1:1000	Roche
Monoclonal anti-Cdh1 (DCS-266)	mouse	1:10000	Sigma
Monoclonal anti-Gadd34 (D-8)	mouse	1: 500	Santa Cruz
Polyclonal anti-Gadd34 (H-173)	rabbit	1: 500:	Santa Cruz
Monoclonal anti-Actin (C-2)	mouse	1:1000	Santa Cruz
Monoclonal anti- c-Myc (9E10)	mouse	1:10000	Sigma
Polyclonal anti-Bip (3183)	rabbit	1:1000	Cell Signaling
Polyclonal anti-P-eIF2α (Ser 51) (9721)	rabbit	1:1000	Cell Signaling
Polyclonal anti-eIF2α (9722)	rabbit	1:1000	Cell Signaling
Polyclonal anti-Cyclin A (H-175)	rabbit	1:1000	Santa Cruz

Monoclonal anti-Cyclin B1 (GNS1)	mouse	1:1000	Santa Cruz
Monoclonal anti-Cyclin D1 (DSC-6)	mouse	1:1000	Neo Markers
Monoclonal anti-Chop (9C8)	mouse	1: 500	Abcam
Monoclonal anti-Cdc27 (AF3.1)	mouse	1:1000	Sigma
Polyclonal anti-LC3	rabbit	1:1000	Cell Signaling
Polyclonal anti-P53 (FL-393)	rabbit	1:1000	Santa Cruz
Polyclonal anti-P21 (C-19)	mouse	1:1000	Santa Cruz
Polyclonal anti-Cleaved Caspase-3 (Asp175) (9661)	rabbit	1:1000	Cell Signaling
Polyclonal anti-Sumo-1 (4930)	rabbit	1:1000	Cell Signaling
Monoclonal anti-Sumo-2/3 (18H8)	rabbit	1:1000	Cell Signaling

Table 4: List of primary antibodies used for western blot

Secondary antibodies

Target	Working dilution	Manufacturer
Anti-mouse IgG peroxidase conjugated	1:10000	Amersham, 1,5 mg/ml
Anti-rabbit IgG peroxidase conjugated	1:10000	Amersham, 1,5 mg/ml
Mouse TrueBlot™ HRP-conjugated anti-mouse IgG (clone eB144/7A7)	1:15000	Bioscience

Table 5: List of secondary antibodies used for western blot

7.2 Solutions and antibodies for immunofluoresence

Fixation solution Paraformaldehyd 3,7 % in PBS

Permeabilisation buffer 0,1 % triton in PBS

Blocking solution 2 % BSA in PBS

Primary antibody immunofluoresence

Target	Source	Working dilution	Manufacturer
Monoclonal anti-HA-tag (6E2) (Alexa Fluor ® 488conjugate)	mouse	1:50	Cell Signaling
Monoclonal anti-Myc-tag (4A6) (Alexa Fluor ® 555 conjugate)	mouse	1:500	Upstate
Monoclonal anti- Eset (C1C12)	rabbit	1:50	Cell Signaling

Table 6: List of fluorescent-conjugated primary antibodies used for immunofluoresence.

Secondary antibody for immunofluoresence

Alexa Fluor ® 555 goat anti-mouse	Invitrogen, Karlsruhe
Alexa Fluor ® 488 goat anti-rabbit	Invitrogen, Karlsruhe

Table 7: List of fluorescent-conjugated secondary antibodies used for immunofluoresence

8. Kits

cDNA synthesis kit	Bio-RAD
DNA isolation kit	BioLine
DC protein assay kit	Bio-RAD
DNA extraction from Agarose gel	Qiagen
TOPO TA cloning ® Kit for sequencing	Invitrogen

9. Nucleic acid

Plasmids

- pCMV vector is designed to allow transient expression in mammalian systems. The expression is driven by the human cytomegalovirus (CMV), which promotes a constitutive expression of cloned inserts in a wide variety of cell lines.

- pCS2+ is a multipurpose expression vector originally designed for expressing proteins in xenopus embryos. pCS2+ is also useful for high-level transient expression in mammalian cells.

Derivatives of this plasmid have been constructed by fusion of epitope tags

- pCS2+MT (Myc-tag) allows the expression of proteins fused to 6 copies myc-epitope tag recognised by the 9E10 monoclonal antibody. pCS2+MT was generated by Stolk and colleagues (Roth et al 1991).

- pCS2+HA (HA-tag) allows the expression of protein fused to hemagglutinin (HA) epitope tag recognised by the polyclonal HA-antibody.

Oligonucleotides

All siRNA were purchased from ambion

Cdh1 siRNA	
Sense	5'UGAGAAGUCUCCCAGUCAGtt3'
Anti-sence	5'CUGACUGGGAGACUUCUCAtt3'
Gadd34 siRNA	
Sense	5'GUCAAUUUGCAGAUGGCCAtt3'
Anti-sence	5'UGGCCAUCUGCAAAUUGACtt3'

Table 8: List of siRNA used for depleting Cdh1 and Gadd34

Primers

All primers were purchased from operon

The following primers were used for the generation of GADD34 deletion mutants:

Full length GADD34 forward	AAAAAAGAATTCAAGCCCCAGGCCAAGCACC
Full length GADD34 backward	AAAAAATCAGCCACGCCTCCCACTGAGGTC
ΔN1 forward	AAAAAAGAATTCAATATGGTGAGCGAGAGGCAACC
ΔN1 backward	AAAAAATCAGCCACGCCTCCCACTGAGGTC
ΔN2 forward	AAAAGAATTCAAGCCGAGGAAGAGGGAGTTGCTG
ΔN2 backward	AAAAAATCAGCCACGCCTCCCACTGAGGTC
ΔN3 forward	AAAAGAATTCAATCAGAAGCAGCCTTAGGAGAAG
ΔN3 backward	AAAAAATCAGCCACGCCTCCCACTGAGGTC

Table 9: List of primers used for generating deletion mutants

GADD34 forward	5'GAGGGAGTTGCTGAAGAGGAGGGA3'
GADD34 backward	5'CCTCCGTGGCTTGATTCTCTTCCT3'
HRTP forward	5'GCAGACTTTGCTTTCCTTGG3'
HRTP backward	5'GTGGGGTCCTTTTCACCAG3'

Table 10: Primers used for semi quantitative RT-PCR

10. Cell culture techniques

10.1 Culturing of adherent cell lines

Adherent cells used in this work were cultured in complete cultivation medium and in medium lacking glucose (3-5). All incubations were performed in a humidified 37℃, 5 % CO_2 incubator.

The FCS used for the cultivation medium without glucose was subjected to dialysis at 4 ℃ under steering to remove all traces of glucose. The dialysis was performed for 16 hrs and the dialysis buffer (3-6-1) was changed every 4 hrs.

10.2 Subculturing adherent cell lines

Adherent cells were subcultured after reaching 70-90 % confluency to prevent culture dying.

Culture medium was removed from the plate, and cells were washed 2 times with PBS. To dissociate adherent cells, the plate was incubated in 2 ml trypsin at 37℃ for 1-2 min. The incubation time might differ from cell line to cell line. Cell dissociation can be examined under microscope and the proteolytic reaction can be quickly terminated by the addition of pre-warmed growth medium. Cells were re-suspended with the appropriate volume of medium and 1x106 cells were split into fresh 10 cm2 plate containing complete medium. Alternatively, cells can be counted using a hemocytometer and diluted to desired density.

10.3 Quantification of cell number and viability with hemocytometer and trypan bleu staining

To perform accurate quantification and to standardise culture conditions, it is necessary to determine the cell number. The hemocytometer was the instrument used in this work to determine the cell number.

The hemocytometer consists of 4 corner squares, which are divided into 16 tertiary squares. Cells in the four corner squares were counted, and the number of cells per ml was determined according to the following equation:

Cells/ml= average count per square x dilution factor $x10^4$

(Each square is 1 m^2 and the depth is 0,1 mm)

The number of total cells was counted as follows:

Total cells= cells/ml x the volume of cell suspension from which sample were taken

To determine the viability, cells were stained with trypan blue, 20 µl of cells suspension was mixed with 20 µl trypan blue for 1 min at RT before starting the counting. The principle of this technique is based on the ability of dying cells to uptake trypan blue, whereas the membrane of living cells is not permeable to trypan blue.

The number of living cells was counted as follows:

$$\% \text{ viable cells} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100$$

10.4 Preservation of cell lines

10.4.1 Freezing

For long-term storage cells have to be frozen. FCS was used in combination with 10 % DMSO to store cells at –80°C or lower. The use of DMSO as cryoprotective agent is required to preserve cells, to reduce the freezing point and to allow a slower cooling rate. Gradual freezing is necessary to reduce the risk of ice crystal formation and cell damage.

For Freezing, cells were treated according to 4.1.2. After trypsinisation and centrifugation for 5 min at 800 rpm, the pellet was resuspended in freezing medium to obtain 1x106 cells/ml. 1 ml aliquots of suspension cells was pipetted into labeled freezing tubes. Covering the tubes with towels allows a slower freezing at –80°C.

10.4.2 Thawing

Cells were removed from frozen storage and quickly thawed in a 37°C water bath. After thawing, the cell suspension was placed into a 10 cm^2 plate containing 8 ml warm medium. The medium was changed in the next day to remove all traces of DMSO.

10.5 Preventing mycoplasma contamination

Mycoplasmas are small intracellular bacteria, which are difficult to detect visually in cultures. They multiply rapidly and affect cultures by interfering with cell metabolism, growth and eventually lead to cell death.

Several direct and indirect methods are available in detecting mycoplasma in cell cultures, which differ in specificity, sensitivity and time requirements. PCR analysis using primers, which amplify specifically and selectively a part of the mycoplasma DNA was a very sensitive methods used during this work.

To test cultures for the mycoplamsa contamination, cells were cultured for 1 week in plate without changing the medium. After scraping, 1 ml cells in suspension was pelleted for 30 min at 13000 rpm and resuspended in 100 µl proteinase K buffer containing proteinase K. The sample was incubated for 90 min at 56°C followed by 3 min at 95°C. After short centrifugation, the sample can be stored at – 20°C until testing by PCR, which was done kindly by Uta and Mona.

To treat mycoplasma contamination antibiotics like ciprobay and BM cycline were used. This method is based on cultivation of contaminated cells in the presence of antibiotics and retesting them after removal of antibiotics. Disposal of contaminated culture, aseptic techniques and thawing of clean cells were the effective methods followed in this work to reduce, to prevent mycoplasma contamination, and to avoid antibiotics resistance due to a prolonged use of antibiotics. The cells used in this work were tested and confirmed to be free from mycoplasma contamination.

10.6 Regulation of genes expression of genes expression by transfection

10.6.1 Downregulation of gene expression using RNAi

RNA interference (RNAi) is a molecular method to study the loss of gene function. Silencing target genes can be performed by the introduction of small interfering RNA (siRNA) to the cytoplasm resulting in the degradation of messenger RNA. siRNA consists of 19-21bp double-stranded RNA with 2-4 nucleotide overhang at the 3' ends. One of the strands (the sense strand) is homologous to an area in the mRNA

of the target gene. To evaluate non-specific or toxic effects of the siRNA used, it is essential to include control siRNA unrelated to the target mRNA in all experiments. The transfection is achieved by the use of transfection reagents. HiPerFect was the transfection reagent used in this work. HiPerFect is a cationic lipid. si RNA complexed with the transfection reagent is efficiently released to the cells.

Prior to the day of transfection, 0,9 x 10^5 cells were seeded into each well of a 6 wells plate. The transfection mix, composed of siRNA in a final concentration of 5 nM mixed with 100 µl serum free medium and with 6 µl HiPerFect, was incubated for 10 min and added dropwise to the cells. The analysis of gene silencing was carried out 24-48 hrs after transfection by western blot.

10.6.2 Transient gene expression

Polyethylenimine (PEI) was the transfection reagent used for all transient transfections performed during this work. PEI has the capacity to condense DNA and allow its entry into cells by adsorptive endocytosis.

1 x 10^6 Cells were seeded one day before transfection. A transfection mixture composed of plasmids, 800 µl serum free and 20 µl PEI was prepared and mixed. 12 ug and 6 ug were the total amount of plasmids used for transfecting cells seeded in 10 cm^2 and in 6 cm^2 plate, respectively. The transfection mixture was incubated for 10 min at RT and added dropwise to the cells.

The pH of the PEI used was optimised for each cell line. The PEI used for HeLa was pH 7,5, whereas HEK293 was transfected with PEI pH 6,45.

48 hrs pre-transfection, the cells were harvested and analysed.

10.7 Flow cytometry

For flow cytometry analysis, cells were scraped and harvested by centrifugation at 800 rpm for 5 min. The resulting pellet was resuspended in cold PBS and pelleted again. After removal of PBS, the cells were fixed with 4 ml 70 % cold ethanol, which was added dropwise to the pellet under vortex. The cells can be fixed either for 1 h at -20℃ or overnight at 4℃. Cells can be stored at 4℃ until further analysis.

Before analysis, the fixed cells were washed with PBS and pelleted by centrifugation at 800 rpm for 5 min. The resulting pellet was resuspended in freshly prepared DNA staining solution containing 10 µg/ml propidium-iodide and 25 µg/ml RNase A in PBS. The cells were resuspended in a volume required to obtain $1x10^6$ cells/ml and incubated in dark at 37℃ for 30 min. The FACS machine from BD Bioscience was used to perform the analysis and FlowJo OSX was the software used for data analysis.

10.8 Quantification of apoptotic cells with annexin staining

Exposure of the membrane phospholipid phosphatidylserine (PS) to the outer leaflet of the plasma membrane is an early event of cells undergoing apoptosis.

Annexin V is a 35-36 kDa Ca2+dependent phospholipid-binding protein that has a high affinity for the PS and binds to cells with exposed PS. Conjugated to fluorochromes including FITC, annexin can serves as sensitive probe for flow cytometry analysis of cells undergoing apoptosis. In combination with a vital dye such propidium iodide, it is possible to distinguish between early and late stage of apoptosis. At late stage, the cells lose the membrane integrity and are positive for annexin and PI.

Apoptosis can be quantified either by fluorescence microscopy or by flow cytometry. For microscopy analysis, the cells were incubated for 15 min at 37℃ with 5 µl of annexin V alexa Fluor ® conjugates after inducing apoptosis. For flow cytometry, adherent and floating cells were collected and pelleted at 800 rpm for 5 min. The resulting pellet was resuspended in annexin binding buffer to get $1x10^6$ cells/ml. 100 µl of cell suspension were incubated in the dark with 5 µl annexin and 2 µl PI (50 ug/ml) for 15 min at RT. 400 µl of annexin binding buffer were added to the cells, which were incubated on ice for an additional 15 min. The analysis was carried out by flow cytometry within the next 1 hr.

11. Analytical cell culture techniques

11.1 Cell lysis

The cells were subcultured according to 4-1-2 in 10 cm^2 plate and harvested by centrifugation at 800 rpm for 5 min. The resulting pellet can be either stored at –

20℃ until use or directly lysed in RIPA-buffer containing protease and phosphatase inhibitors. The lysate was incubated on ice for 15 min, then sonicated twice for 20 s and centrifuged at 13000 rpm for 15 min at 4℃. The cell lysate was placed into a fresh tube and then used for immunoprecipitation therefore protein concentration was determined.

11.2 Protein quantification using DC protein assay

The concentration of a solubilised protein was determined using BCA protein assay kit following manufacturer's instruction. The adsorption was measured at 650-750 nm with a microplate reader. The protein concentration was calculated via a standard curve. For generating the standard curve, 4 concentrations of BSA were prepared in RIPA buffer ranging from 0,2 µg/ml to 1,2 µg/ml.

The lysates can be stored at –20℃ until use or can be diluted in 25 % 4XSB and boiled for 5 min at 95℃ for further protein analysis.

11.3 Immunoprecipitation

Selective immunoprecipitation of proteins is a useful tool for characterising proteins and protein-protein interaction.

Immunoprecipitations consist of 3 steps; lysing the cell, binding of the proteins to the antibody, and precipitation of the immunocomplexe through binding of the antibody to either A or G beads depending on the antibody used.

In this work, the cells were lysed in NP-40 buffer (3-6-4). After protein quantification (4-2-2), 0,5 mg lysates were incubated for 2 hrs at 4℃ with the 2 µg/ml specific antibody in a total volume of 500 µl with rotation. Controls antibodies were also included at the same concentration as the specific antibody used for immunoprecipitation. In order to precipitate the protein-antibody complex, the lysate was incubated with 30 µl G beads at 4℃ with rotation. The beads were prewashed once with water and twice with NP40-buffer. After 1 hr incubation, the beads were recovered by centrifugation at 4℃ and 800 rpm for 1 min, washed 5 times with NP40 (3-6-4) and boiled at 95℃ in 40 µl 2XSB (3-6-4) for 5 min. Input (1/30 of the resulting) and immunoprecipitated complexe were resolved by SDS-PAGE and detected by western blotting using specific antibodies.

11.4 *In Vivo* ubiquitination assays

This assay allows the detection of protein-His-ubiquitin adducts generated in cells after overexpression of protein of interest with His-Ubiquitin, which will be enriched on talon beads due to the histidine tag. A higher mobility forms of the protein of interest is detected by immunoblotting due to the addition of His-Ubiquitin.

HEK293 cells were seeded and transfected in the next day according to 4-1-5-2. For the entire experiment, HEK293 cells were transiently co-transfected with 3 µg His-Ubiquitin and expressing vectors encoding the tested interacting partners. 48 hrs post-transfection, cells were treated with 25 µM MG-132 for 3 hrs in order to inhibit the proteasome. Cells were then washed with PBS, lysed in urea buffer (3-6-4), and sonicated 2 times for 20 S. Cells debris were removed by centrifugation at 13000 rpm and 4°C for 15 min. The lysate was transferred to new eppendorf tube. 60 µl lysate from each sample served as input. The input samples were mixed with 20 µl 4xSB and boiled for 5 min at 95°C. The rest of the resulting lysates was incubated with 30 µl Ni^{2+} talon beads (prewashed once with ddH_2O and 2 times with urea buffer). The incubation was performed at RT for 2 hrs on a rotating wheel. Afterward the beads were washed 4 times with 1 ml urea buffer. After removing all traces of urea buffer, proteins were eluted from the talon beads by adding 40 µl 2XSB containing 200 mM imidazole and boiled for 5 min at 95°C. Imidazole displaces His-ubiquitin bound proteins by competing for binding to the nickel ions in the talon beads.

The ubiquitination of the desired proteins was analysed after SDS-PAGE separation and incubation with the appropriate antibodies.

11.5 Protein separation by SDS- Page

Discontinuous gel electrophoresis is a technique to separate proteins under denaturing conditions (in the presence of SDS) according to the molecular size. The proteins migrate through pores in the polyacrylamide gel matrix in response to an electrical field. The migration rate of the protein depends on the combination of pore size and protein charge, size and shape. The pore size increases with decreasing acrylamide concentration.

In this system two sequential gels are used; the separating gel topped by the stacking gel. The stacking gel is acid (pH 6,8) and has a low polyacrylamide

concentration (5,5 %). In this conditions the proteins are poorly separated but form a thin bands, which get separated during migration though the separating gel (more basic pH 8,8 with a higher polyacrylamide content).

The desired percentage of acrylamide in the separating gel depends on the size of the protein being separated. Generally 7,5 % gels is used for separation of SDS-denatured protein with a size ranging from 60-200 kDa, 10 % gels for SDS-denatured proteins up to 70 kDa and 15 % gel for small proteins (12-45 kDa) (3-6-4 table 2 and 3).

The glass-plate sandwich of the electrophoresis apparatus was assembled according to the manufacturer's instructions. The separating gel solution was poured immediately to the glass-plate sandwich. The top of the gel was covered with a layer of ethanol to allow polymerisation and guarantee the formation of a straight surface. The alcohol layer was removed after the gel formation. The stacking gel solution was poured to the glass-plate sandwich. The desired comb was used and removed from the stacking gel after polymerisation. The gel sandwich was attached to the upper chamber and placed to the lower buffer chamber. Both chambers were filled with the appropriate volume of running buffer.

Protein samples containing 1X SB at a final concentration were denatured by boiling at 95℃ for 5 min and applied to the gel wells. A protein-molecular-weight marker was used as a control during separation. The proteins migrate at 70 V thought the stacking gel. The voltage was increased to 100 V when the samples reach the separating gel.

The electrophoresis should be stopped as the molecular weight marker shows the desired separation. The gel can be stained with comassaie blue or transferred to a membrane for immunoblotting.

11.6 Western blot

Western blot or immunoblotting is the method used to transfer the proteins separated by SDS-PAGE from the gel to the membrane.

After finishing the electrophoresis, diassembling the gel sandwich and removing the stacking gel, the gel was equilibrated at RT in transfer buffer.

For the transfer, a nitrocellulose membrane was used. The membrane has to be first activated by a short incubation with 100 % methanol and rinsed with transfer buffer before proceeding to blotting.

The gel, topped by the membrane, was placed to the blotting cassettes according to the manufacturer. Transfers for 1 hr at 100 V and 0,35 mA was generally sufficient. For overnight transfer, a current of 30 V and 0,09 mA was required.

After the transfer, the membrane was removed from the transfer stack and the transfer efficiency was monitored with ponceau staining. After washing with TNT, the membrane was blocked in TNT containing 5 % non-fat milk for 15 min at RT with constant agitation. Blocking the blotted membrane reduces non-specific binding of the antibody. Following blocking, the blots were washed in TNT and incubated with the primary antibody overnight. In the next day unbound antibody was removed by washing 3 times for 10 min in TNT. The blots were incubated with HRP-conjugated secondary antibody for 1 hr at RT and washed 3 times for 10 min in TNT. The primary and secondary antibodies for the immunoblots used in this work were diluted to the desired working concentration in TNT and are listed in table 4 and 5 (3-7-1).

The protein detection was performed using ECL solution (3-6-4). This solution was prepared by mixing solution 1 and 2, followed by incubation for 5 min. The membrane was incubated for 1 min in ECl solution. In this system, HRP linked to secondary antibody catalyses in the presence of H_2O_2 the oxidation of luminol, which produces a chemiluminescence. The luminescence is enhanced by the addition of cumaric acid. The excess of the solution was removed and the blots were placed in a plastic bag. After smoothing out air bubbles, the wrapped membrane was placed in a film cassette. The film was exposed in dark until getting the appropriate signals and developed.

11.7 Determination of protein half-lifes

Half-life experiments allow the analysis of protein stability. In this work, the analysis was performed after inhibiting the protein synthesis by 25 µg/ml cycloheximide added to the cells in the day of the experiment. After addition of cycloheximide, cells were pelleted at different time points for protein extraction using RIPA buffer. Alternatively, the cells can also be scraped in 200 µl 2XSB, sonicated and boiled for 5 min at 95°C. The proteins were separated by SDS-PAGE.

11.8 Immunostaining

For immunofluoresence, cells were seeded in 10 cm^2 plates containing coverslips and placed in a humidified incubator at 37°C and 5 % CO_2. After 24-48 hrs, cells grown on coverslips were washed twice with PBS, fixed in 4 % PFA for 20 min, permeabilised for 2 min with 0,2 % triton X-100 in PBS and then blocked with PBS containing 2 % BSA for 1 hr at RT. After washing with PBS, cells were incubated in the dark with primary antibody either for 1 hr at 37°C or overnight at 4°C according to the manufacturer recommendations. After incubation with primary antibody, the cells were washed 3 times with PBS and then incubated in the dark with fluorescence-conjugated secondary antibody at 37°C for 1 hr. The antibody dilution was prepared in the blocking buffer. Table 4 summarizes the required dilutions for all the antibodies used in this work. To visualise the nuclei, cells were co-stained with DAPI for 1 min. After washing cells with H_2O, the cover-slips were mounted onto glass slides. ProLong gold antifade from invitrogen was the mounting medium used in this work. The analysis was performed directly under fluorescence microscopy. Alternatively, coated coverslips can be stored for several days in the dark at 4°C until analysis.

12. Molecular biological method

12.1 RNA analyses

12.1.1 RNA extraction

The RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. For the RNA extraction, cells were pelleted by centrifugation and resuspended in 500 µl solution D. The resuspended pellet can be either stored at -80°C or directly used for the RNA isolation.

50 µl of 2 M Na-acetat pH4 was added to the resuspended pellet. In order to dissociate proteins from DNA, 500 µl phenol was added. After vortexing, 100 µl chloroform was added. Chloroform denatures proteins and lipids and helps in separating nucleic acid from proteins. The resuspended pellet was incubated for 15 min on ice and subjected to a 5 min centrifugation at 4°C and 10000 rpm. The centrifugation allows the separation of two phases. The upper phase was transferred to a new eppendorf and extracted for a second time with 400 µl chloroform. After centrifugation for 5 min at 4°C and 10000 rpm, the supernatant containing RNA was

transferred to new eppendorf and precipitated with 1 volume of 100 % isopropanol for 90 min at -20°C. After spinning for 10 min at 4 °C and 10000 rpm the pellet was washed with 70 % ethanol. The pellet was shortly air-dried and solved in 10-30 µl DEPC H_2O. The RNA concentration was quantified using a photometer.

The RNA can be either stored at - 80°C or directly used for the synthesis of the complementary DNA (cDNA) for the RT-PCR.

12.1.2 cDNA synthesis

cDNA or complementary DNA is synthesised in vitro from RNA using a reverse transcriptase. The cDNA can be used for RT-PCR for expression analysis. For the synthesis, 1-2 µg of RNA were used depending on the RNA concentration obtained after extraction. The synthesis was carried out with a kit from BioRAD according to the manufacturer instructions.

12.2 DNA analyses

12.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction is a technique used in molecular biology to amplify specific DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification. PCR is based on the repetition of the cycle composed of 3 reactions; the first one is a denaturing step, which allows the separation of the double-stranded DNA. It is followed by an annealing reaction, in which the primers bind to complementary base on the single stranded DNA. In the third reaction the DNA is synthesised by the polymerase.

Parameters of each step (time and temperature) were optimised according to the size of target DNA fragment and annealing temperature used for amplification.

For the RT-PCR, the mastermix was prepared as follows:

17,5 µl MilliQ

2,5 µl 10x ThermoPol Reaction Buffer

1 µl Primer A

1 µl Primer B

1 µl dNTP's

1 µl TAQ polymerase

24 µl reaction

The 24 µl master mix were added to 2 µl of the synthesised cDNA in 4-3-1-2. After vortexing, the PCR reaction was started according to the next program:

Standard program:

Lid on 110°C			
94°C	3'	DNA Denaturing	
94 °C	30''	DNA Denaturing	25 - 30x
x °C	30''	Primer Annealing	
72 °C	30''	Strand Elongation	
72 °C	10'	Final Elongation	

The PCR product can be analysed using agarose gel electrophoresis.

12.2.2 Agarose electrophoresis

Gel electrophoresis is a technique used to separate nucleic acids according to the length of the molecules in an electrical field.

The agarose was dissolved to the desired concentration in 1X TBE, heated to boiling in a microwave and supplemented with 0,5 µg/ml ethidium. The concentration of the agarose gel varies according to the size of amplified DNA. Low agarose concentrations are used to separate large DNA fragment, whereas separation of small DNA molecules required less agarose concentration.

For the RT-PCR product, 1/10 volume of 10Xagarose loading buffer was added to the PCR master mix and pipetted into the well of the agarose gel. Inclusion of DNA marker on the gel allows the identification of the fragment depending on its size.

After completing the electrophoresis, the gel can be visualised on a UV transilluminator.

Agarose electrophoresis was also used to analyse the size of the DNA following restriction enzyme digest.

12.2.3 Restriction enzyme digestion

Restriction digest allows the cleavage of DNA at specific sites termed restriction sites using the respective restriction enzyme.

Analytical restriction digest was performed for 1-2 hrs at 37°C.

1-2 µg DNA Plasmid/PCR product

2 µl 10x restriction digest buffer (1/10th total volume)

1/100 of 100 x BSA if required for one of the enzymes

0.5-1 µl enzyme 1

0.5-1 µl enzyme 2

x µl ddH_2O (autoclaved)

20 µl reaction

12.3 Cloning

Full length and fragment of Gadd34 were cloned to the expression vector pCS+HA. Therefore appropriate primers were designed. 5' EcoRI and 3' Xho restriction sites were included to the designed primers. pCS+HA-ΔN1, pCS+HA-ΔN2 and pCS+HA-ΔN3 encode the part of Gadd34 located between 117-674 aa, 162-674 aa and 483-674 aa respectively.The full length Gadd34, ΔN1, ΔN2 and ΔN3 were amplified from Gadd34pCMV by PCR and cloned into 5' EcoRI and 3' Xho sites of pCS+HA vector.

Table 9 summarises the list of primers used for the cloning. The PCR amplification was performed using Pfu polymerase and was carried out for 25 cycles.

Following PCR, the genetrated products and the vector were digested with EcoRI and Xho. The restriction digest was performed as described in 4-3-2-3. The generated fragments were ligated to pCS+HA (4-3-3-2) after elution from the agarose gel (4-3-3-1).

12.3.1 DNA elution from agarose gel

A 0,8 % preparative agarose gel was used after restriction digest for the separation of the fragment required for cloning from other fragment. After completing the gel electrophoresis the DNA bands were visualised under UV and respective bands were cut from the gel and subjected to elution using an extraction Kit. In this work, ex-gel – OLS kit was used and the extraction was carried out according to the manufacture instruction.

The relative amount of the vector and insert was estimated after extraction by running a few µl on an analytical agarose gel (usually 0.8-1 %).

12.3.2 Ligation

This technique allows the covalent linking of two ends of DNA molecule using DNA ligase, which catalyses the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3' hydroxyl terminus of double stand DNA. The T4 DNA ligase was the ligase used in this work.

Ligation	Control reaction
100 ng Vector	100 ng Vector
x ng insert (see above)	-
2 µl 10x ligation buffer	2 µl 10x ligation buffer
0.5 µl ligase T4 (10U; NEB, Fermentas)	0.5 µl ligase (10U; NEB)

y µl ddH_2O (autoclaved)	z µl ddH_2O (autoclaved)
20 µl	20 µl

The reaction was carried out at RT for more than 1 hr.

12.3.3 DNA precipitation

Ethanol precipitation is the technique used for concentrating nucleic acid in aqueous solution. In this technique salt and ethanol are added to the aqueous solution, which forces the nucleic acid to precipitate out of solution.

PCR products or DNA after restriction digest were precipitated with this method. To this end, 1/10 volume of 3 M sodium acetate (pH 5,2) was added to the sample and mixed properly. For the precipitation 2,5 volume of 100 % ethanol was used and the precipitation was carried for 1 hr at –20°C. The precipitated nucleic acid was separated from the rest of the solution by centrifugation. The pellet was washed in cold 70 % ethanol and pelleted by centrifugation. The nucleic acid pellet was air dried and resuspended in the appropriate volume of TE or water.

12.4 Plasmid preparation

12.4.1 Transformation

Transformation of electrocompetent bacteria was achieved in this work using electroporation. This technique allows the entry of DNA apparently through hole in the cell membrane produced by a high-voltage electrical field applied briefly to the competent cells. Freshly prepared cells or frozen one can be used for the transformation. In the case of frozen cells it is required to allow the bacteria to cool down first on ice, 50-100 ul electrocompetent cells (prepared by sangita) were used for the electroporation. 1 µg of DNA (or 5 µl of ligation reaction) was added to the bacteria and mixed gently. The mixture DNA/bacteria was pippted into electroporation cuvettes, which were already pre-cooled on ice. A BioRAD-electroporator was used for the electroporation of bacteria, which were transferred back to the ice after the electrical shock. The bacteria were transferred carefully to 1

ml of 1XLB medium and incubated at 37°C under shaking either for 1h or for 30 min. To select for the transformed bacteria, 100 µl and 900 µl of the bacterial suspension were plated on LB with antibiotics and incubated at 37°C overnight.

Clones were picked and the DNA can be analysed after extraction.

12.4.2 Minipreps of plasmid DNA

The isolation of small amount of DNA from bacteria was performed using the alkaline lysis. The method consists of 4 steps; in the first one the bacteria are resuspended in a solution A (3-6-3). In the second step, the cells are lysed with solution B containing NaOH and SDS (3-6-3). SDS leads to lysis and release of the cell contents through solubilisation of the cell membrane. NaOH denatures proteins and chromosomal converting thereby the double-stranded DNA in the cell to single stranded DNA. The addition of potassium acetate in the third step allows the neutralization of the lysate, the re-naturation of the plasmid and the precipitation of SDS taking protein, lipids and chromosomal DNA. In the last step the plasmid DNA can be concentrated using the ethanol precipitation for example.

Prior the day of DNA extraction, 5 ml of 1XLB media (containing the required antibiotic) was inoculated with the tested clones. After 16 hrs (time to get sufficient growth), 1,5 ml of cells were pelleted by centrifugation at 6000g. The pellet was resuspended in 100 µl solution A (containing a tiny amount of lysozyme). 200 ul of solution B (3-6-3) was added to the resuspended pellet and mixed gently but thoroughly by inverting. 5 min incubation on ice allows maximum release of the plasmid DNA from the cells. For the neutralisation 150 µl of buffer C was added followed by 5-10 min incubation on ice. A 10 min centrifugation at 13000 rpm and RT enhances the precipitation of debris. For the precipitation of the plasmid DNA, the supernatant was transferred into new eppendorf containing 850 µl of 100 % ethanol, mixed and incubated for 15 min at –20°C. After a centrifugation at 4°C and 13000 rpm for 15 min, the DNA pellet was washed with 70 % ethanol and pelleted again by centrifugation. The pellet was dried for 5 min and dissolved in 20-50 µl TE containing 20 ug/ml of RNase.

12.4.3 Large-scale preparation of plasmid DNA: CsCl2

Isolation of DNA using cesium chloride gradient centrifugation allows the isolation of large-scale of clean plasmid DNA. In this method, DNA isolation by the alkaline lysis procedure is followed by equilibrium ultracentrifugation in cesium chloride-ethidium bromide gradients. The difference in the density enables the separation between the genomic DNA and plasmid DNA.

An overnight culture was prepared by inoculating 5 ml 1XLB medium with bacteria containing the desired plasmid. In the next day, 200 ml of 1XLB medium supplemented with the appropriate antibiotic was inoculated with 200 µl from the prepared overnight culture.

After harvesting the bacteria containing the desired plasmid by centrifugation for 20 min at 4°C and 4000 rpm, the pellet can be stored at -20°C or directly resuspended in 12 ml solution A (3-6-3) by pipetting up and down. Lysis buffer (prepared fresh) was added to the sample, mixed by inverting and incubated for 5 min at RT. 18 ml solution C (3-6-3) was added to the lysate, which was incubated for 20 min on ice and pelleted by centrifugation for 20 min at 4°C and 40000 rpm. The centrifugation allows the precipitation of detergent solubilised proteins and membranes with sodium acetate. The supernatant was then cleared by filtration.

The DNA-containing supernatant was transferred into a new tube, and the plasmid was precipitated by the addition of 40 ml of a 100 % isopropanol. After 20 min centrifugation at 4°C at 4000 rpm, the pellet was resuspended in a 10 ml TE buffer (3-6-3) pH 8,0. 2 ml of 10 M ammonium acetate was added to the resuspended pellet. After 20 min incubation on ice, a centrifugation for 20 min at 4°C and 40000 rpm allows the separation the rest of proteins and DNA.

The precipitation of the DNA-containing supernatant was achieved by addition of 32 ml of 100 % Ethanol followed by a centrifugation at 4°C and 4000 rpm for 20 min. The DNA pellet was air-dried shortly and resuspended in TE pH 8,0. 4,71 g of cesium chloride was dissolved in the DNA in suspension. 200 ul ethidium bromid and 51 ul triton 100X were added to the cesium chloride/DNA.

After a centrifugation for 10 min at RT and 4000 rpm, the samples were transferred into a Beckman Coulter ultra centrifuge tube and the tube were filled up to the neck

with TE buffer. Precautions to avoid air bubble were necessary. After sealing, the balanced tubes were placed in the rotor and the samples were subjected to ultracentrifugation overnight at 22℃ and 50000 rpm.

In the next day, two-ethidium bromide stained bands were visible. The lower Band (plasmid DNA) visualised under long wave UV light was carefully removed using a needle and a syringe and transferred to a 15 ml falcon.

Separation of the DNA from the ethidium bromide was achieved through extraction with an equal volume of 5 M NaCL saturated isopropanol. After inverting, the upper phase containing ethidium bromid was removed. The extraction was repeated several times until no trace of ethidium bromide was detectable in both phases.

In order to precipitate DNA, 2 volume of ddH_2O and 2,5 volume of 100 % ethanol was added to the solubilised DNA. The precipitation was performed at -20° for 1 hr. The precipitated DNA was pelleted by precipitation by centrifugation for 20 min at 4℃ and 4000 rpm. The DNA was washed with 70 % Ethanol, dried and resuspended in 300-500 µl TE buffer pH 8,0.

In the next day, the concentration and the purity of DNA were assessed using photometer by measuring the absorbance generated at 2 wavelengths (260-280 nm). The plasmid DNA was assayed by restriction digestion and detected on agarose gel electrophoresis. The DNA should be stored at 4℃.

IV. Results

1. Analysis of the interaction between overexpressed Gadd34 and Cdh1

1.1 Gadd34 interacts with Cdh1 in yeast and in mammalian cells

In order to define new interacting partner for the cell cycle regulator Cdh1, Dr. Kristine Bousset performed a yeast-two-hybrid screen with the full length human Cdh1. In this screen, she succeeded to identify Gadd34 interacting with Cdh1.

To examine whether Gadd34 and Cdh1 also interact in mammalian cells, co-immunoprecipitation assays were performed. HeLa cells were transfected with plasmids expressing Mt-Cdh1 and Gadd34 either separately or in combination. Cell lysates were subjected to immunoprecipitation either with Gadd34 or with 9E10 antibody. To confirm the specific interaction between the immunoprecipitated proteins and the used antibodies, lysates were also immunoprecipitated with control antibodies. An actin antibody and normal rabbit IgG were the controls for the 9E10 and the Gadd34 antibody respectively. 1/30 of the lysates served as input.

The immuno and co-immunoprecipitated proteins were collected with protein G beads and detected by immunoblotting using Gadd34 and Cdh1 antibodies.

Immunoprecipitation using the 9E10 antibody shows that it was possible to bring Gadd34 down only when MT-CDH1 and GADD34 were cotransfected. The interaction was specific as no immunoprecipitated proteins were detected after immunoprecipitation using an actin antibody (Fig 9A).

Mt-Cdh1 was also specifically co-immunoprecipitated with the Gadd34 antibody but not with rabbit IgG. The co-immunoprecipitation was only observed when Gadd34 and Mt-Cdh1 were coexpressed (Fig 9B).

In agreement with the first Co-immunoprecipitation assays performed by Dr. Bousset, these results demonstrate the specific interaction between Gadd34 and Cdh1 in mammalian cells after overexpression of these proteins.

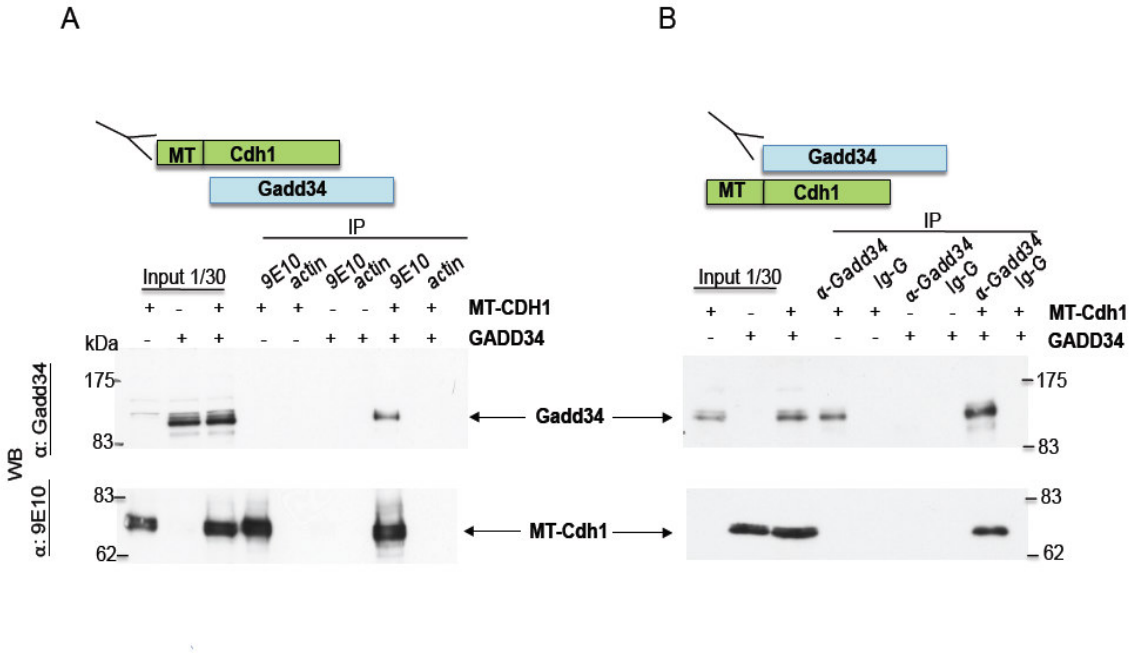

Fig. 9: Gadd34 interacts with Cdh1 in yeasts and mammals

A, B HeLa cells were transfected with 1 ug GADD34 and 1 µg MT-CDH1 separately or in combination. Lysates were immunoprecipitated with the anti-9E10 antibody (Fig 9A) and with anti-Gadd34 (Fig 9B). Control antibodies were used at the same concentration as the specific antibodies. Immunoprecipitation was followed by western blotting and detection with antibodies against 9E10 and Gadd34. 1/30 of cell lysates was used as input.

1.2 The C-terminal region of Gadd34 is sufficient for Cdh1 interaction

The APC/C^{Cdh1} substrates are characterised by the presence of D-Box (RXXL) and/or KEN-Box. Sequence analysis of human GADD34 revealed the absence of a KEN box and the existence of 5 RXXL motifs; one located at position 93-96 conserved between rat, mouse and human and 4 D-Boxes located at position 65-68, 139-142, 296-299 and 526-529 aa (Fig 10).

To determine the part of Gadd34 recognised by the APC/C^{Cdh1}, 3 deletion mutants were generated by PCR using the corresponding primers incorporating the appropriate restriction sites. The full length GADD34 and the 3 generated constructs (ΔN1 (117-674 aa), ΔN2 (162-674 aa), and ΔN3 (483-674 aa)) were subcloned into pCS2+ vector containing an HA-tag sequence (Fig 10).

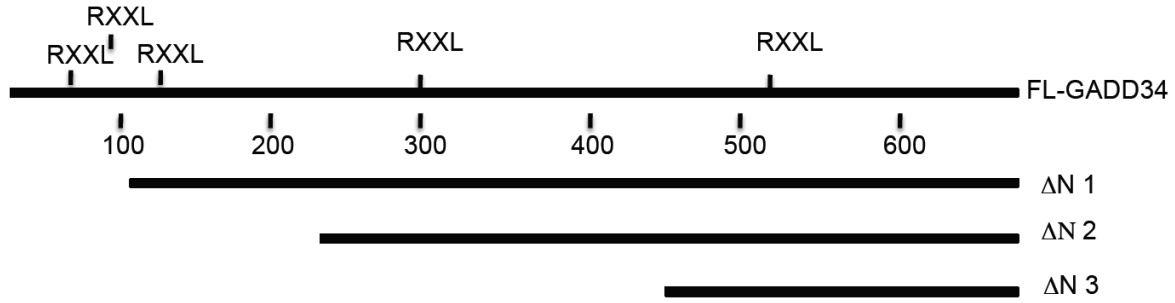

Fig. 10: Generation of deletion mutants

ΔN1 (117-674 aa), ΔN2 (162-674 aa) and ΔN3 (483-674 aa) constructs were generated by PCR using the appropriate primers and confirmed by sequencing. The different constructs and the full length GADD34 were cloned into the pCS2+HA tag plasmid. ΔN1 contains the D-Box located at position 139-142 aa, 296-299 aa and 526-529 aa. The 3 first D-Boxes, located at position 65-68, 93-96 and 139-142 aa, are absent in ΔN2 deletion mutant. Whereas ΔN3 contains only the D-Box located at position 526-529 aa.

Co-immunoprecipitation assays were performed in order to analyse the interaction of Cdh1 with the 3 generated deletion mutants and full length Gadd34. HEK293 cells were transiently transfected with the 4 constructs either separately or in combination with MT-CDH1. Cell lysates were subjected to immunoprecipitation using either anti-HA or anti-actin antibodies and Immunoprecipitates were analysed by immunoblotting using anti-HA and anti-9E10 antibodies (Fig 11).

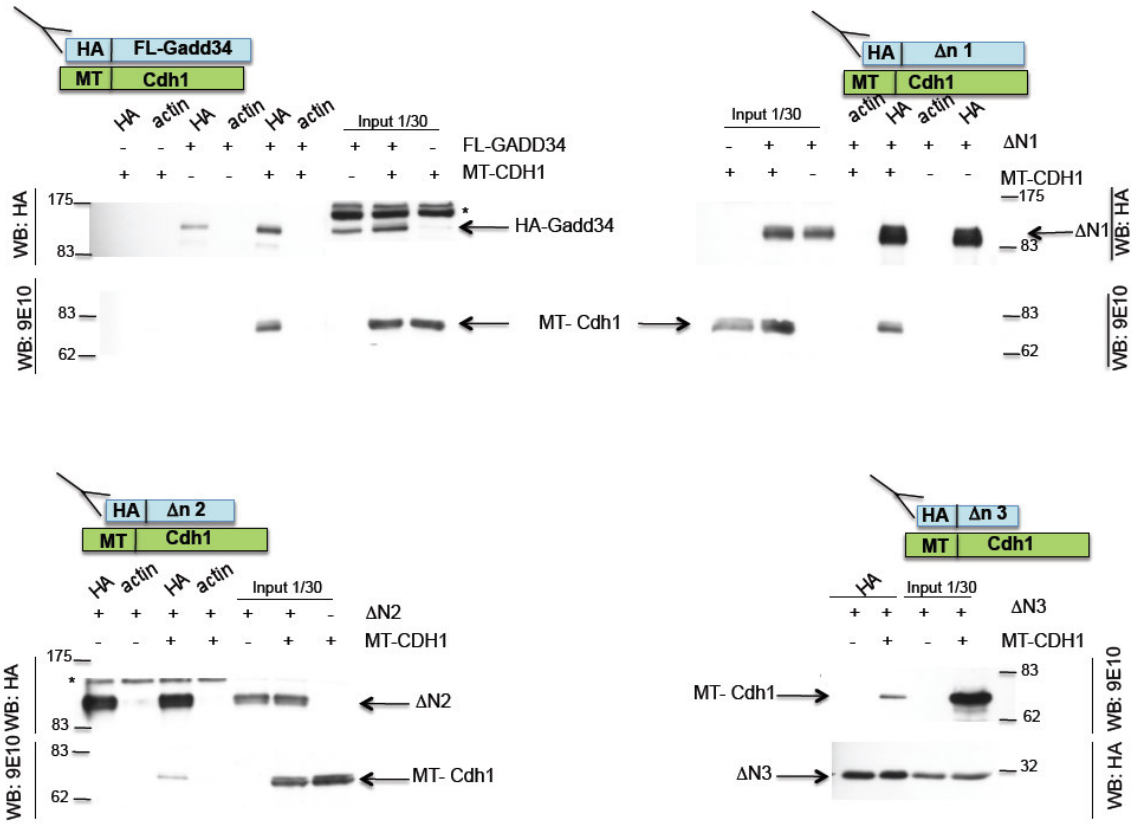

Fig. 11: Cdh1 interacts with C-terminal region of Gadd34

HeK293 cells seeded in 10 cm^2 plate were transfected with 1 µg plasmid expressing full length Gadd34 (3A), Δn1 (3B), Δn2 (3C) and Δn3 (4D) either separately or in combination with and 1 µg plasmid expressing Mt-Cdh1. Lysates were immunoprecipitated with anti-HA. An anti-actin antibody was used as a control. Immunoblotting was performed to detect immunoprecipitated HA- constructs and co-immunoprecipitated MT-Cdh1 using anti-HA and anti-9E10 antibodies, respectively. The asterics (*) point to unspecific Bands.

These Data show that Δn1, Δn2 and Δn3 were all able to interact with the overexpressed Cdh1. Cdh1 was only co-immunoprecipitated in the presence of either of these constructs. In addition, no immunoprecipitates were detected by using an actin antibody as unspecific control (Fig 11).

The results suggest that the C-terminal region (483-674 aa) is the part of Gadd34, which interacts with Cdh1. This in agreement with the result of the two-hybrid screen, as the C-terminus of Gadd34 was the part found in the screen.

1.3 Cdh1 can target Gadd34 for ubiquitination

APC/C^{Cdh1} is an E3 ligase in the ubiquitin pathway. To determine whether Cdh1 targets Gadd34 for ubiquitination, His-ubiquitination assays were performed.

Gadd34 was expressed in HEK293 cells in combination with His-ubiquitin and increasing levels of Mt-Cdh1. 48 hrs after transfection, the cells were treated for 3 hrs with MG-132 to accumulate polyubiquitinated proteins by inhibiting the proteasome and according to the scheme shown in figure 12A. 1/34 of the cell lysates served as input, whereas the rest of the cell lysates was incubated for 2 hrs with talon beads to bind the whole His-ubiquitin bound proteins. After SDS-PAGE, immunoblotting using anti-Gadd34 antibody was performed in order to detect the ubiquitinated Gadd34.

The polyubiquitinated proteins migrate on the SDS-PAGE as a smear caused by the addition of His-Ubiquitin to the target protein (about 18-20 kDa per His-ubiquitin).

Western blot analysis showed that overexpression of His-ubiquitin resulted in weak ubiquitination of Gadd34, which was strongly enhanced in after overexpressing CDH1 in a concentration dependent manner (Fig 12B).

To further confirm the direct involvement of Cdh1 in the Gadd34 ubiquitination, specific inhibitors of the APC/C^{Cdh1} activity were used. The Cdh1 inhibitor Emi 1 and

the N-terminal region of Cdh1 (N125), which acts as dominant negative, were cotransfected in combination with Cdh1 at two different levels (1 and 3 µg) (Fig 12C).

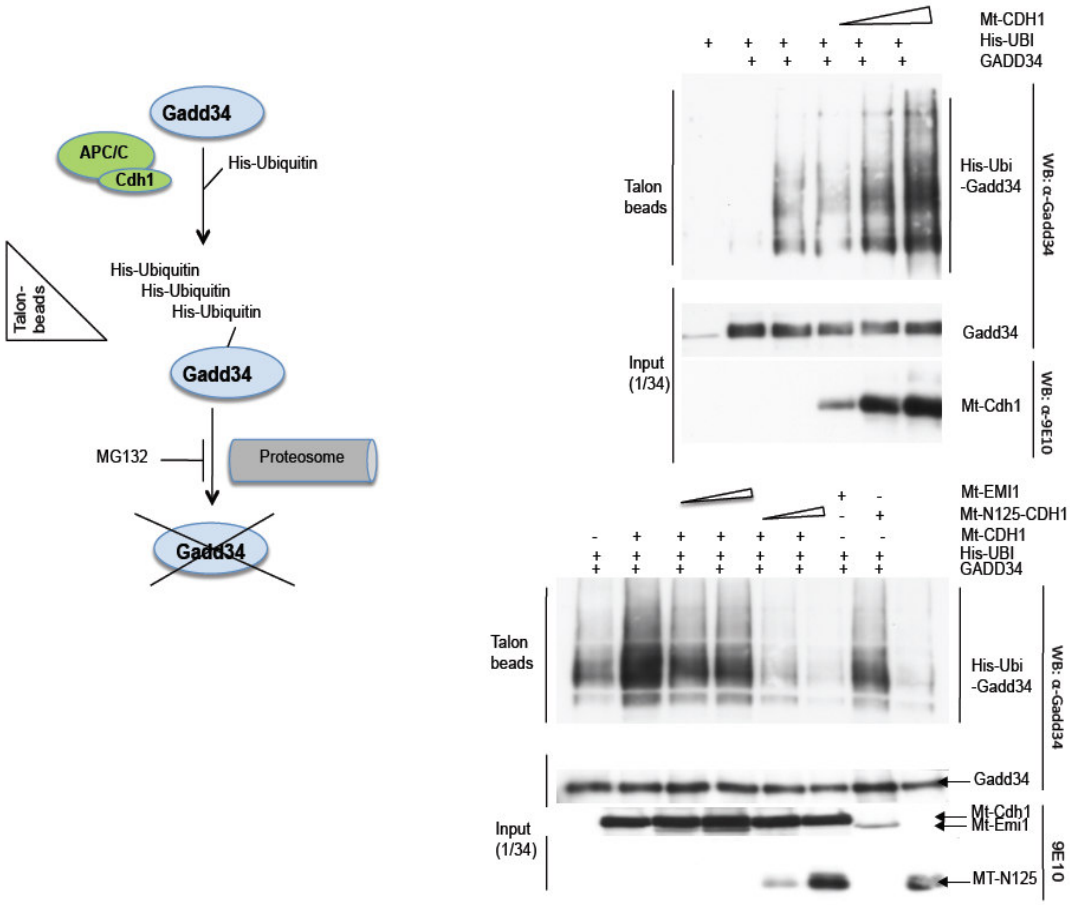

Fig. 12: Gadd34 is getting ubiquitinated in a Cdh1-dependent manner

A. Scheme describing the principle of the His-ubiquitination assay. B, 0,5 µg plasmid expressing Gadd34 was transfected into HEK293 cells, in combination with His-ubiquitin and increasing amounts of CDH1 plasmid (0,2, 1 and 2 µg). 2 days after transfection the cells were treated with MG132 for 3 hrs. The lysates were incubated for 2 hrs with talon beads, separated by SDS-PAGE and subjected to immunobloting to detect the level of ubiquitinated Gadd34. B, To inhibit Cdh1, 1- 3 µg of Emi-1 and Cdh1- N125 were cotransfected with Mt-Cdh1, and the level of Gadd34 ubiquitination was analysed as before. 1/34 cell lysates was used as input.

Western blot analysis confirmed that overexpression of Cdh1 resulted in an increase in Gadd34 ubiquitination, which was slightly decreased after overexpressing Emi-1. Gadd34 ubiquitination was also clearly decreased after overexpression N-terminal Cdh1 and almost completely inhibited after increasing the level of overexpressed Cdh1 (N125). In addition, Cdh1 (N125) caused a reduction of Gadd34 ubiquitintion in the absence of overexpressed full length Cdh1 (Fig 12C). Analysis of the level of overexpressed proteins revealed a difference in the expression level between Mt-Emi1 and Mt-Cdh1 (N125) (Fig 12C).

The first His-ubiquitination assay performed by Dr. Jörg Vervoorts showed the same results as the presented data.

Taken together, ovexpression of increasing levels of Cdh1 enhanced the ubiquitination of Gadd34. This ubiquitination decreased after overexpressing Cdh1 inhibitors. Thus, Cdh1 was directly involved in the ubiquitination of Gadd34.

2. Analysis of the interaction between Gadd34 and Cdh1 at the endogenous level

2.1 Gadd34 is induced during the ER stress

Physiological and pathological processes that affect protein folding can induce ER stress and thereby Gadd34. Tunicamycin, a specific inhibitor of N-glycolysation, and the proteaosome inhibitor MG-132 are well-known ER stress inducer and activator of the UPR. Treating HeLa cells with MG-132 (25 μM) for 3 hrs or incubation with 5 μg/ml tunicamycin for 7 to 16 hrs resulted in an accumulation of Gadd34 (Fig 13A).

Glucose deprivation was the first tool used to induce ER stress. The effect of glucose deprivation on HeLa cells concerning Gadd34 induction was examined after cultivating them for 24 hrs in medium lacking glucose. Western blot analysis revealed that depriving cells of glucose for 24 hrs resulted in the accumulation of Gadd34 (Fig 13B). The effect of glucose deprivation was examined for different cell lines including HCT116, HEK293 cells and U20S by cultivating them for 24 hrs in the absence of glucose. Analysis of the Gadd34 level indicated that the induction of Gadd34 by

glucose deprivation was not only related to HeLa cells, but was also observed in HEK293, HCT116 and U20S (Fig 13B).

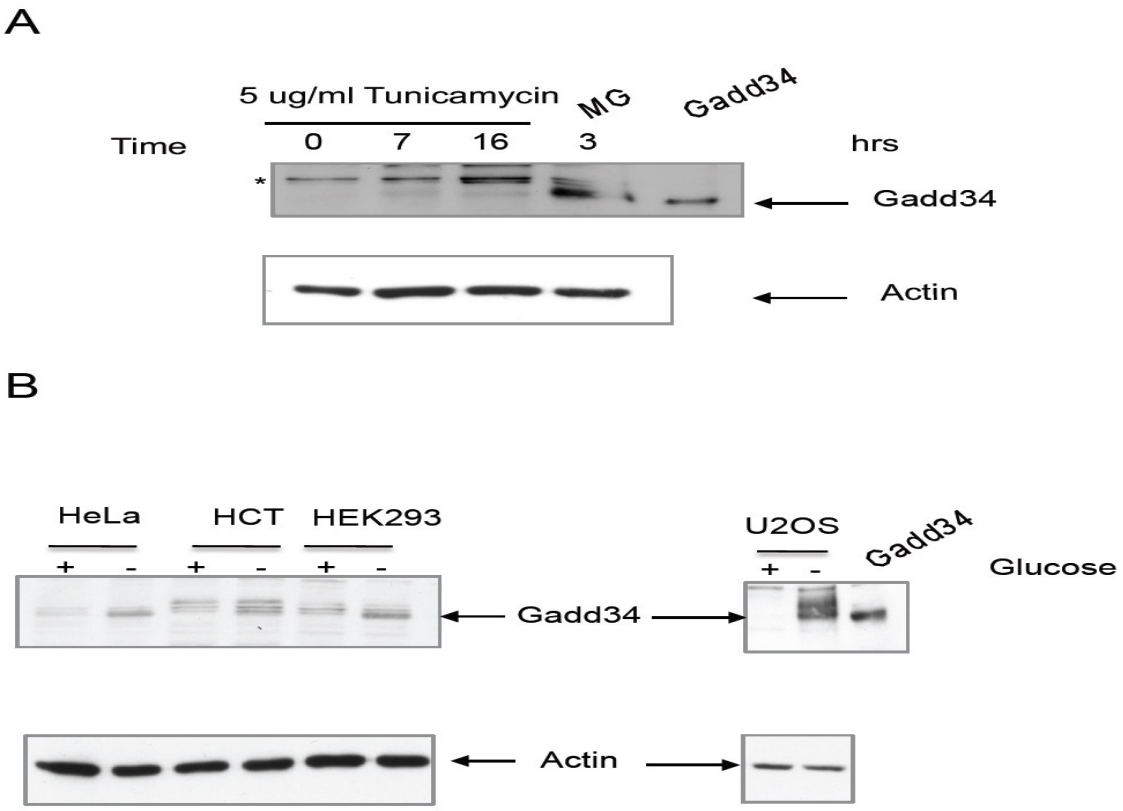

Fig. 13: Gadd34 is induced in different cell lines during ER stress

A. HeLa cells were treated either with MG-132 (25 µM) for 3 hrs or with Tunicamycin (5 µg/ml) for 7 and 16 hrs, the Gadd34 levels were analysed by western blot. Actin was used as loading control.

B. HCT116, HEK293 cells U2OS and HeLa cells were cultivated in medium lacking glucose for 24 hrs and the levels of Gadd34 were analysed by western blot. Actin served as control for equal loading.

To analyse the effect of glucose deprivation on HeLa cells in more detail time course experiments were carried out.

HeLa cells were deprived of glucose for 0, 4, 8, 12, 16 and 20 hrs and the GADD34 expression was examined at the transcriptional level by RT-PCR. The RNA was first extracted form samples taken at the indicated time points. cDNA synthesis was carried out. Two sets of primers were designed to detect a target sequence of Gadd34. The levels of Gadd34 mRNA were normalised to HPRT, which was used as

internal control in the RT-PCR. After semi semi-quantitative RT-PCR, GADD34 transcripts was first seen after 8 hrs glucose deprivation (Fig 14).

The Gadd34 protein levels and representative markers of the unfolded protein response such as eIF2α, P-eIF2α and Chop were analysed as well by immunoblotting at the indicated time points (Fig 14).

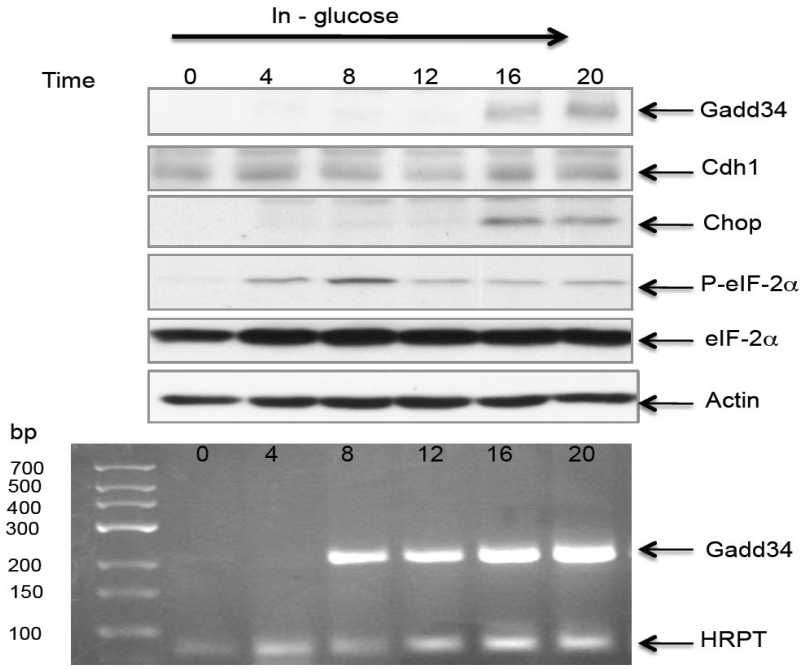

Fig. 14: Glucose deprivation induces the activation of the ER stress response and the accumulation of Gadd34

The figure shows a representative experiment performed in HeLa cells, which were cultivated in the absence of glucose to induce ER stress. GADD34 expression was analysed by RT-PCR and by western blot at the indicated time points. Cdh1 level and the level of important markers of ER stress were analysed by western blotting. HRTP was used as control for equal cDNA and actin for equal proteins loading.

Western blot analysis indicated that Gadd34 protein started accumulating after 16 hrs. In other experiment, Gadd34 was also detectable after 12 hrs glucose deprivation. The level of Chop (Gadd153) accumulated allways in parallel to Gadd34. eIF2α was highly phosphorylated after 8 hrs of glucose deprivation and became less phosphorylated later on (12 hrs after glucose deprivation). The level of Cdh1 was not affected during glucose deprivation (Fig 14).

2.2 Gadd34 levels decrease after releasing cells from stress

To analyse the level of Gadd34 after removal of stress, HeLa cells were deprived of glucose for 16 hrs, then released in medium containing glucose according to the scheme shown in figure 15A. The level of Gadd34 was examined by western blot at the indicated time points (Fig 15B).

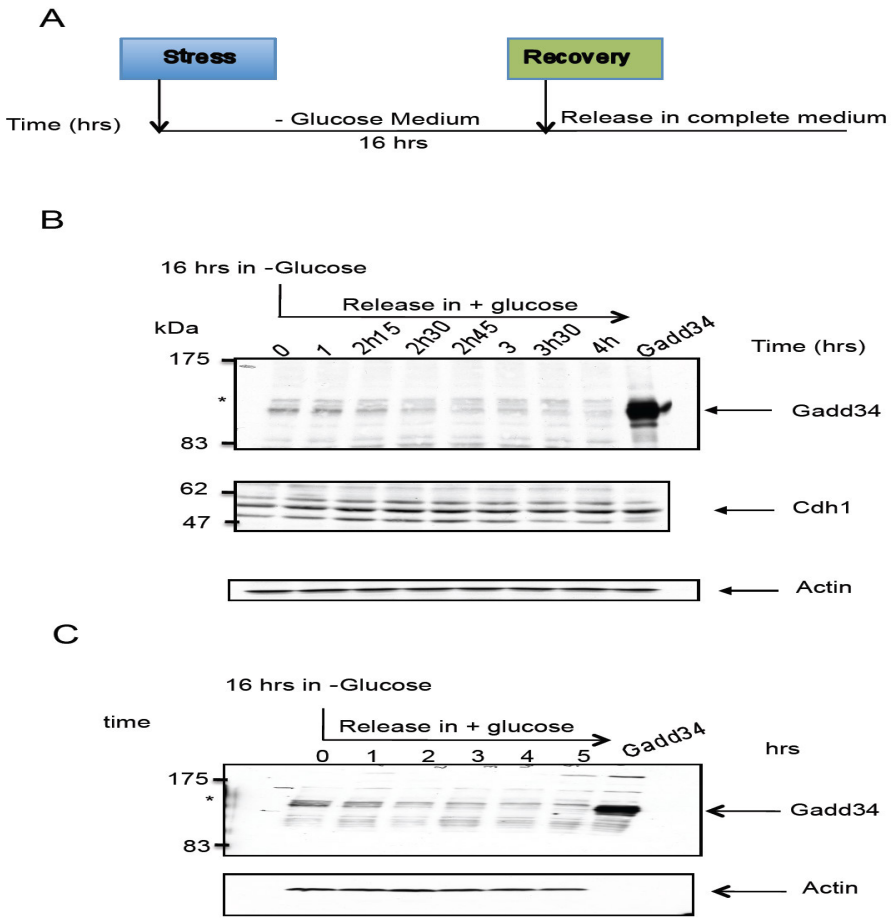

Fig. 15: Gadd34 level decreases after releasing cells from ER stress

A: Scheme describing the experimental design by which GD for 16 hrs was used to induce ER stress, followed by cultivation in complete medium for recovery. B, HeLa cells were cultivated in medium without glucose for 16 hrs and released in complete medium. The level of Gadd34 and Cdh1 were analysed after 16 hrs glucose deprivation and within the release at the indicated time points by western blot. C, HCT116 were subjected to glucose deprivation for 16 hrs and released in medium containing glucose. The level of Gadd34 was analysed by western blotting. Actin was used as control for equal loading. The asterisks (*) point to unspecific bands.

The result shows that the level of Gadd34 decreased after releasing the cell from the stress to reach a very low level after 4 hrs growth in complete medium (Fig 15B). This indicates that accumulation of Gadd34 is reversible after removal of the stress (Fig 15B).

This reversibility was also analysed in HCT116. After 16 hrs cultivation in medium lacking glucose, HCT116 cells were released into complete medium and the levels of Gadd34 were examined by western blotting for the indicated time points (Fig 15C). As in HeLa cells, the level of Gadd34 also decreased to a low level after releasing HCT116 from ER stress (Fig 15C).

2.3 Gadd34 is a protein with short half-life

To determine the stability of Gadd34, half-life experiments were carried out. HeLa cells were deprived of glucose for 16 hrs to induce the ER stress. The total protein synthesis was inhibited by adding cycloheximide in a final concentration of 25 µg/ml. Samples were taken at the indicated time points to analyse remaining proteins by immunoblotting (Fig 16A). The level of Gadd34 declined rapidly to less than 50 % within 30 min after cycloheximide addition. Quantification of 3 independent experiments confirmed that Gadd34 is a short-lived protein with a half-life of less than 30 min (Fig 16B).

To test whether the stability of Gadd34 is dependent on the type of the ER stress inducer, HeLa cells were treated with 5 µg/ml tunicamycin for 16 hrs. After inhibiting the total protein synthesis, Gadd34 stability was analysed by western blotting. The result indicated that if the ER stress was induced by tunicamycin, Gadd34 is an unstable protein, too (Fig 16C).

The turnover of Gadd34 was also examined in another cell line to analyse whether the instability of this protein is cell specific or not. Therefore, HCT116 cells were deprived of glucose for 16 hrs. The protein synthesis was inhibited by cycloheximide and the levels of Gadd34 were analysed for the indicated time points by western blotting (Fig 16D). The result confirmed that Gadd34 induced in HCT116 by glucose deprivation in is instable as shown in HeLa cells (Fig 16D).

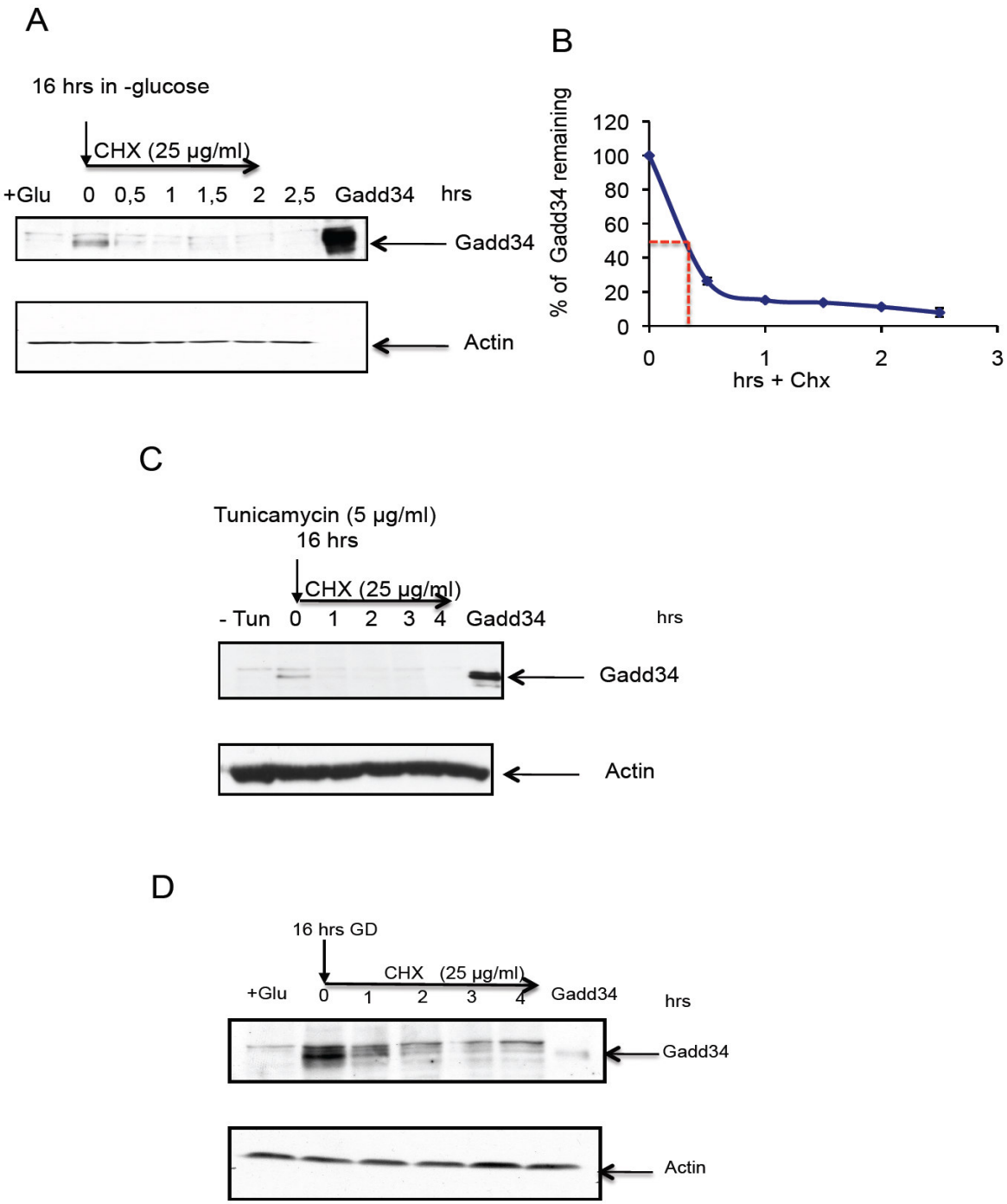

Fig. 16: Gadd34 is a protein with short half-life

A.Gadd34 stability was examined in HeLa cells subjected to 16 hrs glucose deprivation. The protein synthesis was inhibited by 25 µg/ml cycloheximide and the level of Gadd34 was analysed by immunoblotting. The graph in B represents the quantification of 3 independent experiments. Gadd34 levels were normalised to actin. All values are expressed relative to the zero time point. B. Gadd34 stability was analysed in tunicamycin-treated HeLa cells. D. Gadd34 stability was analysed in HCT116 as it was performed in HeLa cells. Actin was used as a control for equal loading.

Taken together, these data suggest that Gadd34 induced during the ER stress is a protein with a short half-life (Fig 16A). An effect, which was seen by different inducers of ER stress (Fig 16C) and seen in 2 different cell lines subjected to ER stress (Fig 16E).

2.4 The absence of Cdh1 results in accumulation and stabilisation of Gadd34

To understand the functional significance of the interaction between Gadd34 and Cdh1, it was important to know whether Cdh1 is involved in regulating Gadd34 turnover under physiological conditions. Therefore, the level and the stability of Gadd34 were analysed in the presence and after depleting Cdh1 using specific si RNA targeting Cdh1.

2.4.1 Gadd34 is slowly reduced in the absence of Cdh1 during recovery

The level of Gadd34 was first examined in the presence and absence of Cdh1. Therefore, HeLa cells were transfected either with Cdh1 si RNA or control si RNA in a final concentration of 5 nM. 24-30 hrs after transfection, cells were first starved of glucose for 16 hrs and then release in complete medium (Fig 17A). The level of Gadd34 was analysed by immunoblotting before, after glucose deprivation and within the release from stress, according to the scheme in figure 17A. The Cdh1 depletion was also analysed by western blot (Fig 17B).

Down regulation of Cdh1 resulted in a high accumulation of Gadd34 during stress and strongly inhibited the reduction of Gadd34 levels during the release (Fig 17B).

Quantification of Gadd34 levels of 3 independent experiments showed that in the presence of Cdh1 the level of Gadd34 declined to about more then 50 % after 3 hrs release, whereas in the absence of Cdh1 the Gadd34 remained up more then 80 % detectable after 3 hrs release (Fig 17C).

Down regulation of Cdh1 resulted in a high accumulation of Gadd34 during stress and slower reduction of Gadd34 levels during the release (Fig 17B).

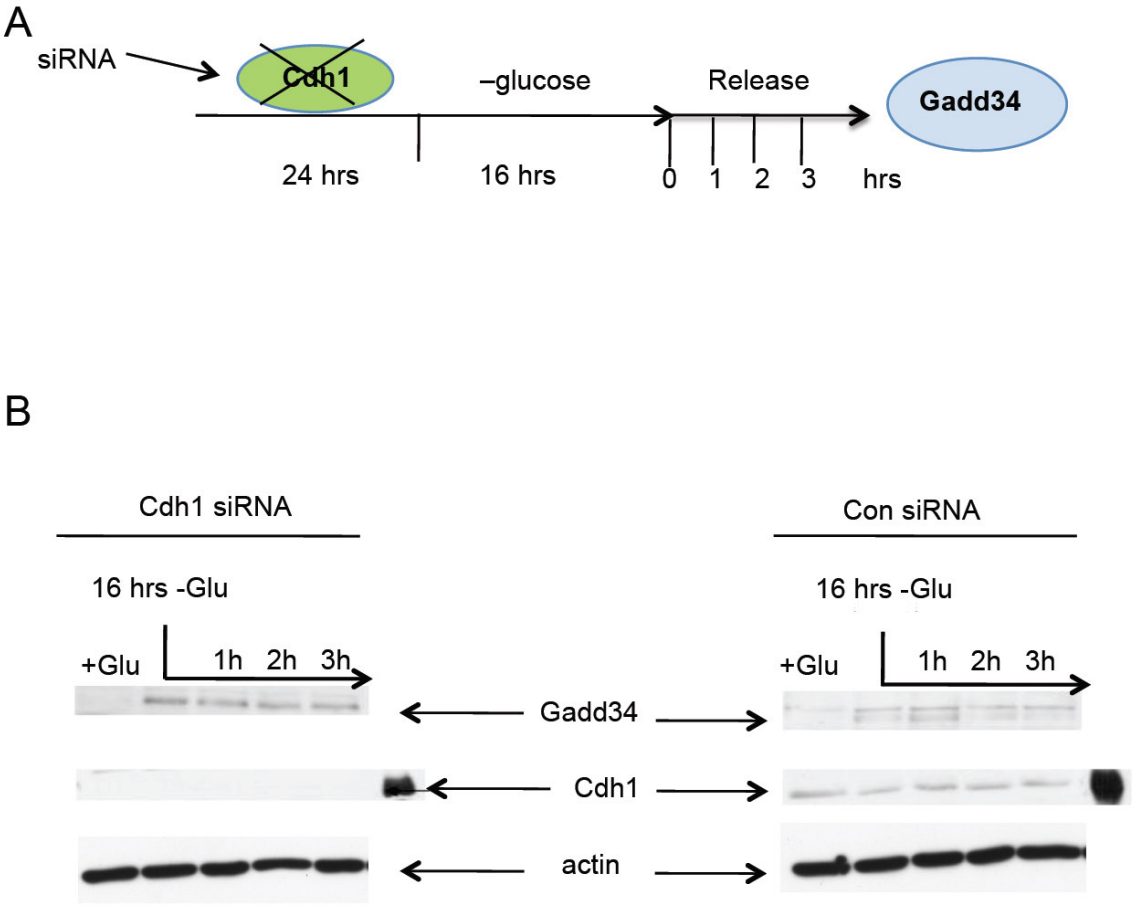

Fig. 17: Silencing of Cdh1 results in a higher accumulation of Gadd34 during stress and a slower reduction during the release

A. The scheme represents the experimental design by which the level of Gadd34 was analysed in the presence and absence of Cdh1. After transfecting HeLa cells either with Cdh1 si RNA or negative si RNA (5 nM), cells were subjected to glucose deprivation for 16 hrs and then released from stress in medium containing glucose. B. The level of Gadd34 and Cdh1 were analysed by immunoblotting after 16 hrs glucose deprivation and within the release at the indicated time points. Actin served as control for equal loading. C, The graph represents the quantification of 3 independent experiments showing a slower reduction of Gadd34 levels during the release in cells depleted of Cdh1 compared to cells transfected with control si RNA.

2.4.2 Gadd34 becomes more stable in the absence of Cdh1

In order to analyse whether the absence of Cdh1 might affect the stability of Gadd34, half-life experiments were carried out in the presence and after depleting Cdh1. HeLa cells were transfected either with Cdh1 si RNA or control si RNA and in the next day subjected to glucose deprivation for 16 hrs according to the scheme (Fig 18A). The levels of Gadd34 were assessed after inhibiting protein synthesis for the indicated time points by western blotting (Fig 18B). Quantification of Gadd34 level in three independent experiments show that the half-life of Gadd34 was extended from about 1 hr in con si RNA transfected cells to about 4 hrs in cells depleted of Cdh1 (Fig 18C).

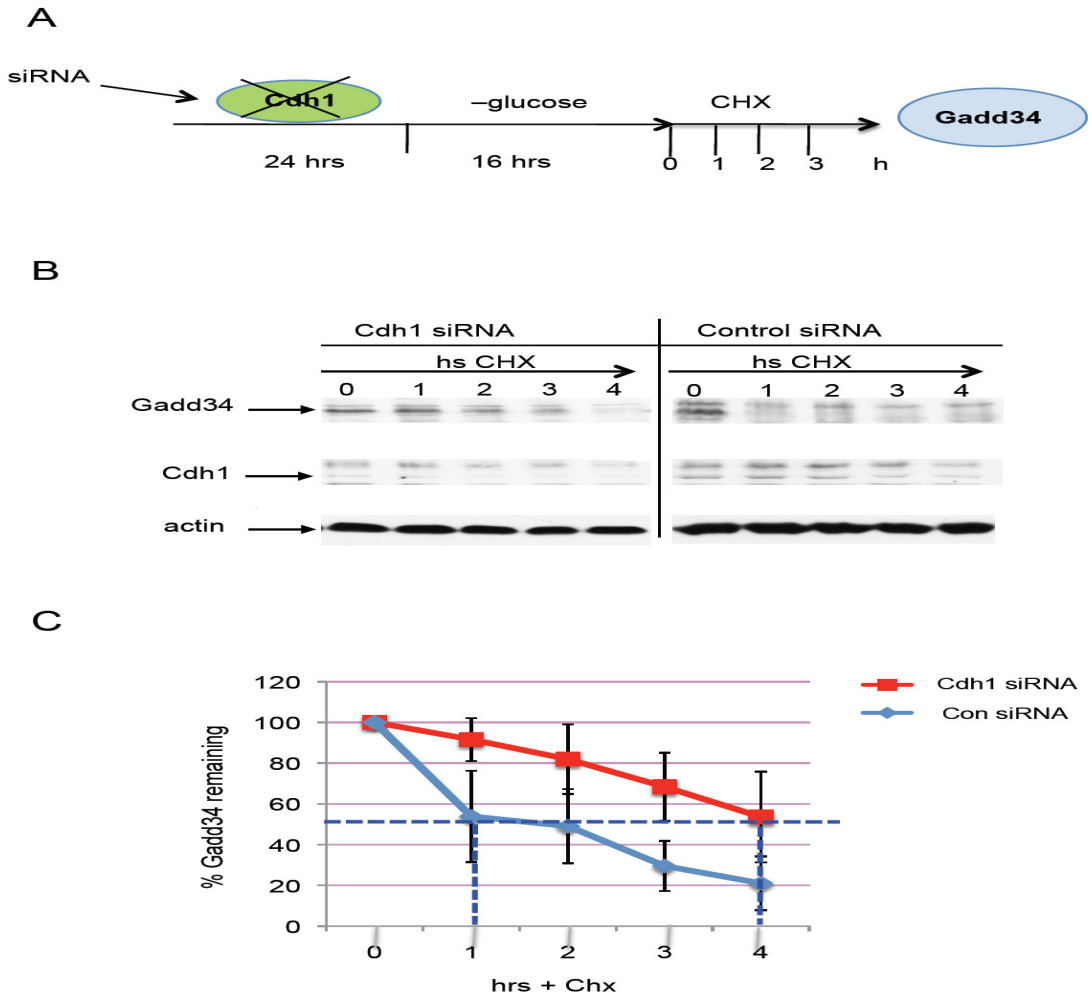

Fig. 18: Cdh1 regulates Gadd34 stability.

The scheme represents the design by which Gadd34 stability was analysed in the presence and absence of Cdh1. HeLa cells were transfected either with control si RNA or Cdh1 si RNA in a final concentration of 5 nM. After 30 hrs transfection, HeLa cells were subjected to glucose deprivation for

16 hrs. Gadd34 stability was examined within 4 hrs after adding cycloheximide by western blot. B: Cdh1 depletion and Gadd34 levels were examined using Cdh1 and Gadd34 antibodies, respectively. Actin was used as loading control to normalise Gadd34 levels. C. The levels of Gadd34 were quantified in the presence and absence of Cdh1. The graph shows the quantification of 3 independent experiments.

The data presented above indicated that depletion of Cdh1 results in stabilisation of Gadd34.

Taken together, the analyses of Gadd34 levels and turnover indicate that the APC/C^{Cdh1} is involved in targeting Gadd34 for degradation by the proteasome during and after releasing cells form ER stress (Fig 17 and 18).

3. Subcellular localisation of Gadd34 and Cdh1

The endogenous Gadd34 is principally associated with the ER, where Gadd34 recruits the protein phosphatases 1 (PP1) to dephosphorylate the initiation factor and to resume protein synthesis (Brush et al 2003). Cdh1 is predominantly located to the nucleus (Zhou el al., 2003). Immunofluoresence microscopy analyses were carried out in order to analyse the subcellular localisation of these two proteins.

3.1 Exogenous Cdh1 and Gadd34 are mainly located to two different compartments within the cell

The subcellular localisation was assessed by immunofluoresence for exogenously expressed Gadd34 and Cdh1. HeLa cells were transiently cotransfected with Mt-CDH1 and HA-GADD34. After 24 hrs, cells were fixed and co-stained with fluorescently labeled anti-Myc and anti-HA antibodies to determine the localisation of exogenous Cdh1 and Gadd34, respectively.

Microscopy analysis showed that exogenously expressed Gadd34 was located to the cytoplasm close to the nucleus. Cdh1 was mainly nuclear with a slight localisation to the cytoplasm (Fig 19).

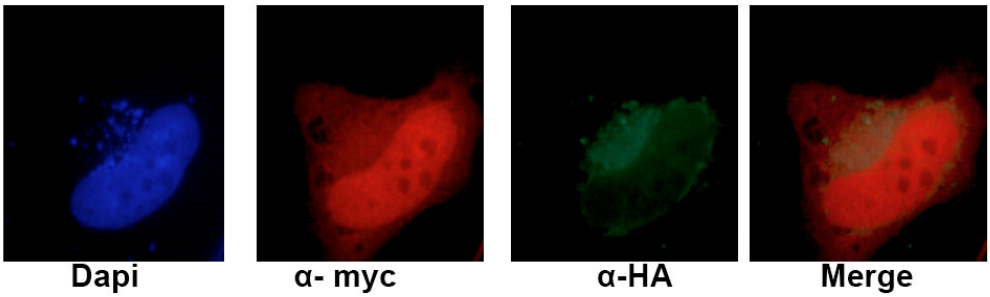

Fig. 19: Localisation of the exogenous Cdh1 and Gadd34

HeLa cells seeded in 6 cm^2 plate containing coverslips were co-transfected with 0,5 µg HA-Gadd34 and 0,5 µg MT-Cdh1. Transfected cells were fixed next day and stained with alexa Fluor® 555 labeled anti-Myc, and alexa Fluor® 488 conjugated anti-HA antibodies. Dapi was used to stain nuclei. The localisation of Cdh1 and Gadd34 was examined under the fluorescence microscope.

3.2 Exogenous Cdh1 localises to the cytoplasm under stress Conditions

To assess the localisation of exogenous Cdh1 under ER stress conditions, HeLa cells were transfected with Mt-CDH1 and subjected in the next day to a glucose deprivation time course. The cells were fixed after 0, 8, 12 and 16 hrs glucose starvation and stained with alexa Fluor® 555-conjugated anti-Myc antibody in order to examine the localisation of the overexpressed Cdh1 under these conditions. To determine the integrity of the nucleus, cells were costained with anti-Eset antibody. Eset is a histone methyltransferases, which binds to DNA, mediates histone methylation and thereby regulates gene expression (Yang t al., 2002, Ryu et al., 2006).

The localisation of Cdh1 was examined under a fluorescence microscope before and after depriving cells of glucose. Notably, a mainly nuclear localisation pattern of Cdh1 was seen with alexa Fluor® 555 labelled anti-Myc antibody in unstressed cells. The localisation of exogenous Cdh1 changed during glucose deprivation to become distributed both in the cytoplasm and the nucleus (Fig 20A).

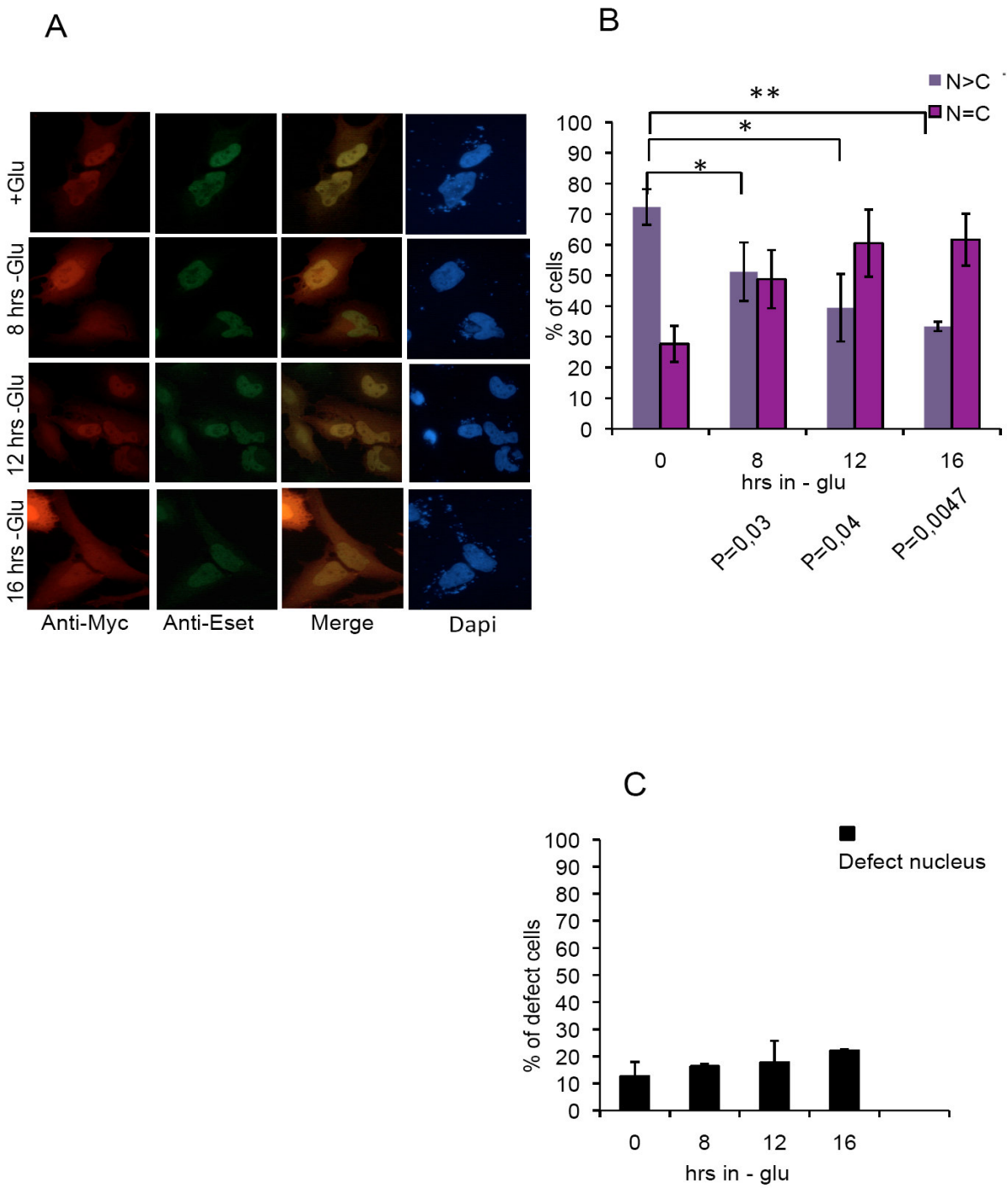

Fig. 20: Exogenous Cdh1 shifts to the cytoplasm under stress conditions

HeLa cells were transfected with 0,5 µg Mt-Cdh1and subjected to glucose deprivation in the next day. The localisation of Cdh1 was analysed for the indicated time points by immunofluoresence using Alexa Fluor® 555 labeled anti-Myc antibody. Cells were costained with Dapi to show the nuclei. The nuclear integrity was analysed using anti-Eset, which was visualised by alexa Fluor® 488 anti-rabbit secondary antibody. A. Shown are representative pictures taken at the indicated time points. B, Quantifications were performed to determine the percentage of Cdh1 transfected cells showing more

nuclear localisation of Cdh1 (N>C) and Cdh1 transfected cells showing about equal distribution to the nucleus and to the cytoplasm (N=C). The graph in B represents the quantification of 3 independents experiments. The graph in C represents the quantification of transfected cells with a defective nucleus in the same 3 independents experiments for the indicated time points. Statistical significance (P value) was determined using an unpaired t test (P<0,05) was considered as statistically significant).

The localisation of exogenously expressed Cdh1 was quantified during glucose deprivation by counting cells showing a mainly nuclear localisation and cells showing a cytoplasmic and nuclear distribution of Cdh1. To exclude that the shuttling of exogenous Cdh1 to the cytoplasm can be caused by a loss of nuclear integrity, transfected cells with defect nucleus manifested by a cytoplasmic distribution of Eset were excluded from the quantifications (Fig 20B). In addition, cells with defect nucleus were counted (Fig 20C).

The results of three independent experiments are illustrated in the graph 20B. Almost 72 % of counted cells were showing a mainly nuclear localisation of exogenous Cdh1, when cells where cultivated in the presence of glucose. This percentage decreased significantly to reach 51 % after 8 hrs (P=0,04), 39 % after 12 hrs (P=0,01) and 33 % after 16 hrs glucose deprivation (P=0,0047) (Fig 12 B).

The results indicated that stress conditions caused by glucose deprivation induced a partial cytoplasmic accumulation of Cdh1 (Fig 20).

3.3 Cdh1 becomes mainly located to the nucleus during recovery from Stress

The localisation of Cdh1 was as well analysed during recovery from stress. To this end, HeLa cells were transfected with MT-Cdh1. 24 hrs post transfection, cells were deprived of glucose for 16 hrs and released from stress in medium containing glucose. The localisation of the exogenous Cdh1 was analysed by immunostaining during the course of the experiment at the indicated time points in three independent experiments (Fig 21).

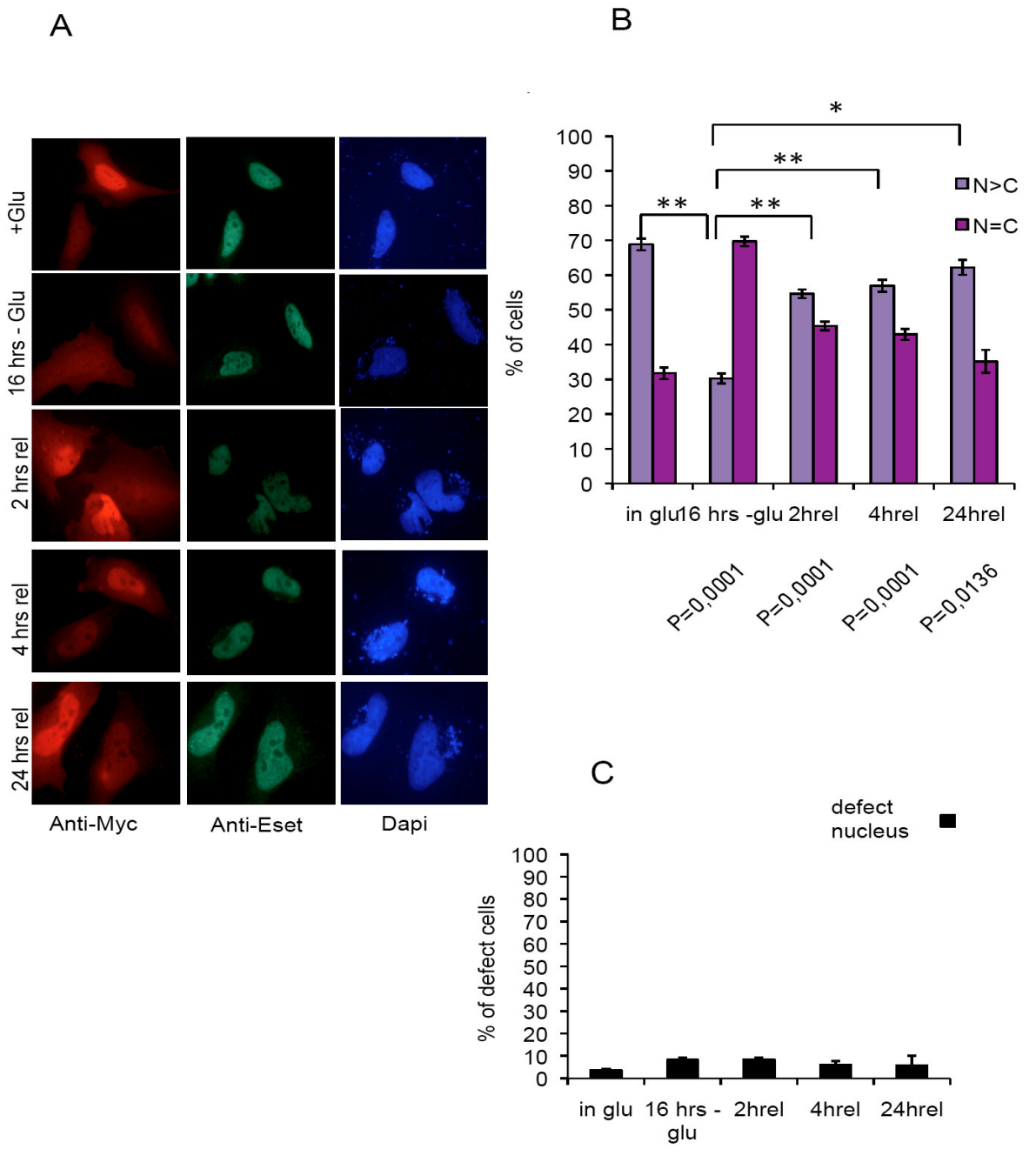

Fig. 21: Recovery from stress results in a nuclear relocalisation of overexpressed cdh1.

HeLa cells were transfected with 0,5 µg MT-Cdh1. The localisation of Cdh1 was analysed by immunofluoresence. A. Representative pictures of cells analysed at the indicated time points, showing Cdh1 (stained with alexa® 555 labeled anti-Myc antibody) and the nucleus (stained with Dapi). Nuclear integrity was examined with anti-Eset, which was visualised by alexa Fluor® 488 anti-rabbit secondary antibody. B. The graph shows the localisation of exogenous cdh1 quantified in 3 independents experiments. The graph in C represents the percentage of Cdh1 transfected cells with defect nucleus, which were not taken into consideration when Cdh1 localisation was examined. P value was determined using an unpaired t test. $P<0,05$ was considered as statistically significant.

Prior to glucose deprivation, exogenous Cdh1 was localised in 70 % of transfected cells primarily in the nucleus. As seen before after 16 hrs glucose deprivation, Cdh1 became in about 30 % of transfected equally distributed between the cytoplasm and the nucleus. The observed distribution of exogenous Cdh1 changed significantly during recovery: 54 % of transfected cells were showing a nuclear localisation of Cdh1 after 2 hrs release (P= 0,0001), 57 % (P=0,0001) after 4 hrs and about 62 % after 24 hrs release (P=0,0136) (Fig 21).

The results indicated that during recovery from stress Cdh1 become gradually more located to the nucleus again.

3.4 Under ER stress, exogenous Cdh1 can still interact with the APC/C

To analyse whether Cdh1 interacts with APC/C under ER stress conditions, immunoprecipitation assays were performed. For this purpose, HeLa cells were transfected with Mt-Cdh1. The next day, the cells were further cultivated for 16 hrs in medium either containing or lacking glucose. Cell extracts were subjected to immunoprecipitations using anti-Myc antibody and an anti-actin antibody was used as control. Immuno-complexes were detected by immunoblotting using anti-Myc and anti-Cdc27 antibodies (Fig 22).

Immunoprecipitation using anti-Myc antibody shows that it was possible to bring Cdc27 down in lysates extracted from cell growing either in the presence of glucose or for 16 hrs in the absence of glucose. The interaction between overexpressed Cdh1 and endogenous Cdc27 was specific, as no immunoprecipitates were detected after immunoprecipitation using actin antibody (Fig 22).

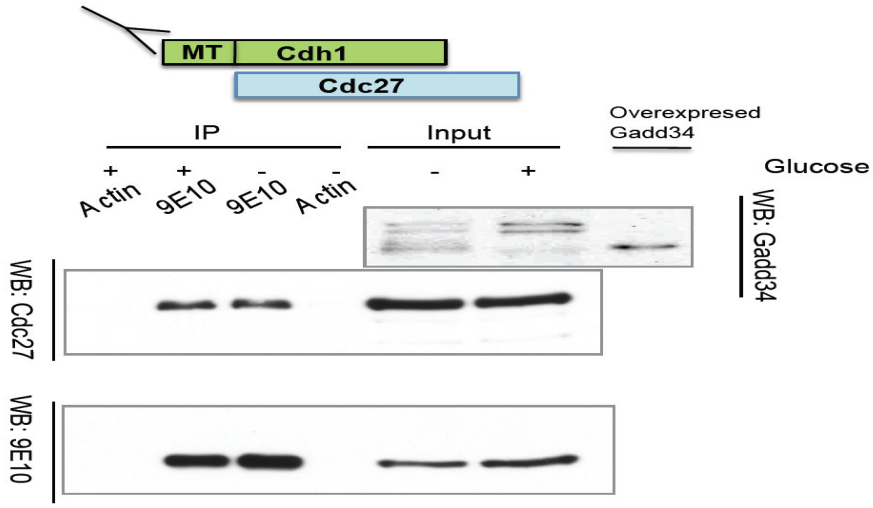

Fig. 22: Cdh1 interacts with the APC/C subunit Cdc27 in stressed and unstressed cells.

HeLa cells were transfected with 1 µg Mt-Cdh1 and cultivated in the next day in medium either containing or lacking glucose for 16 hrs. The interaction between the exogenous Cdh1 and endogenous APC/C subunit Cdc27 was examined by immunoprecipitation using anti-Myc antibody. Immunoprecipitates were detected by western blot using specific antibodies.

The result of the immunoprecipitation assay indicated that overexpressed Cdh1 was able to interact with endogenous Cdc27 in stressed and unstressed cells.

4. Functional significance of the interaction between Gadd34 and Cdh1

The data presented above show that Cdh1 controls the Gadd34 turnover under ER stress conditions. Does Gadd34 interfere with the cell cycle progression and is therefore a target for degradation by APC/C^{Cdh1}? Does Cdh1 control the ER stress response by modulating the Gadd34 turnover? Or both?

4.1 Accumulation of Gadd34 does not impede the cell cycle progression in HeLa cells

Cell cycle analyses were performed under glucose deprivation in order to test the impact of the ER stress on the cell cycle progression. Therefore, HeLa cells were subjected to glucose deprivation and the cell cycle profiles were analysed by flow cytometry (Fig 23A). Protein levels of important regulators of the cell cycle were also assessed by western for the indicated time points (Fig 23B).

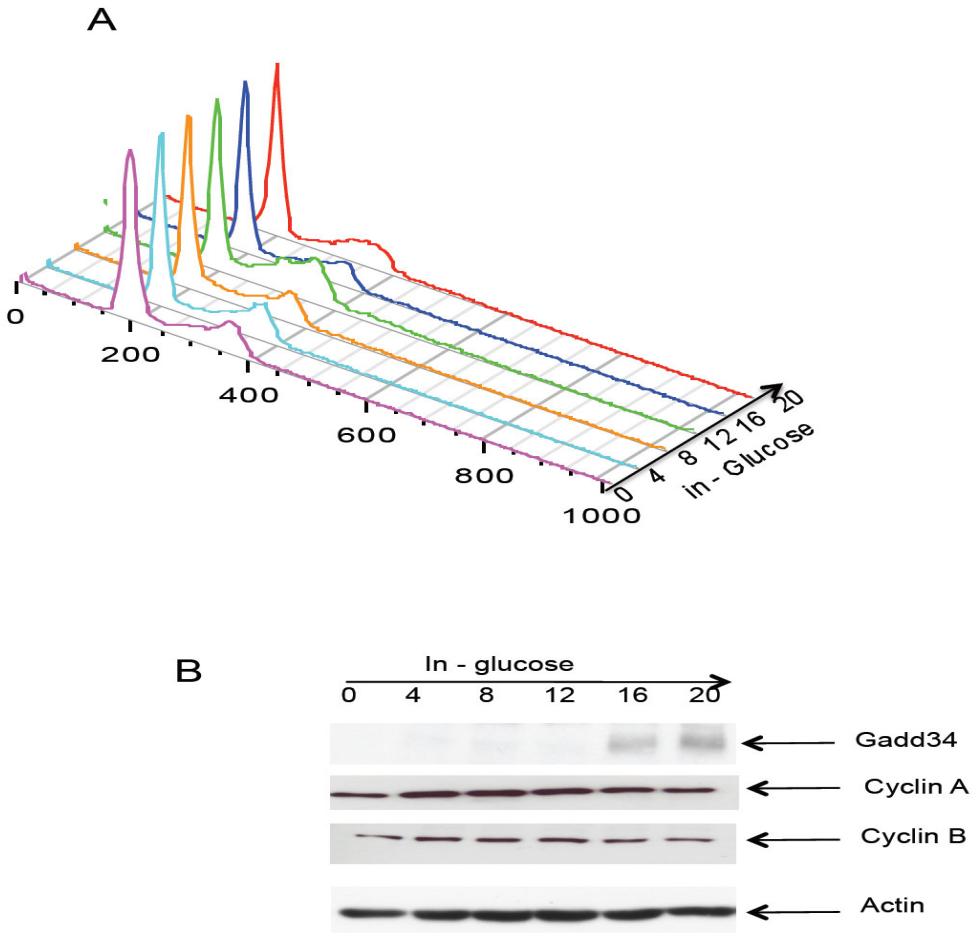

Fig. 23: Glucose deprivation is not interfering with the cell cycle progression in HeLa cells.

A. HeLa cells were subjected to glucose deprivation for the indicated time points and the cell cycle distribution was analysed by propidium iodide-based flow cytometry. B Western blot analyses were carried out using the appropriate antibodies to detect the level of cell cycle regulators during glucose deprivation and actin was used as control for equal loading.

The results of the flow cytometry analyses indicated that no cell cycle effect could be observed during glucose deprivation. Cell cycle arrest was not detected in HeLa cells even after depriving them of glucose for 20 hrs (Fig 23). In addition, the level of cyclin A and Cyclin B remained the same during glucose deprivation as analysed by immunobloting. Therefore, the accumulation of Gadd34 observed by cultivating HeLa cells in medium lacking glucose was not interfering with cell cycle progression (Fig 23B).

4.2 Glucose deprivation induces cell death in HeLa cells

Microscopic analysis of HeLa cells cultivated for 16 to 20 hrs in the absence of glucose revealed that a percentage of the cells were dying. As a persistence of ER stress can contribute to cell death, it was interesting to determine the mechanism behind the death observed after glucose deprivation.

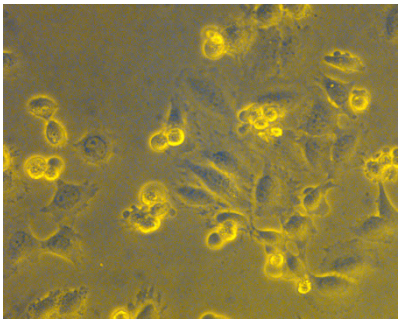

Fig. 24: HeLa observed under light microscope after 16 hrs growths in medium not-containing glucose.

The image represents the status of HeLa cells observed under light microscope after 16 hrs growth in medium lacking glucose.

4.3 Glucose deprivation induces apoptosis in HeLa cells

To examine whether the dying cells were undergoing apoptosis, annexin incorporation was tested in HeLa cells deprived of glucose. Therefore, HeLa cells were subjected to glucose deprivation for the indicated time points and then

incubated with annexin in combination with PI. Cells stained only with annexin are early apoptotic cells, whereas the uptake of PI indicates the loss of membrane integrity and pointed to terminal events of apoptosis (Fig 25). Detection of apoptotic cells was carried after 0, 12, 16 and 22 hrs glucose deprivation by flow cytometry (Fig 25).

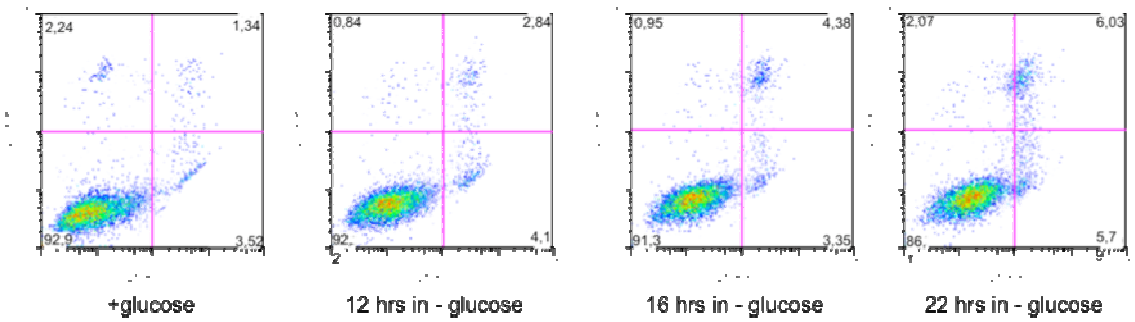

Fig. 25: HeLa cells undergo apoptosis in the absence of glucose.

HeLa cells were subjected to glucose deprivation. Quantification of apoptotic was carried out by flow cytometry after incubating cells with annexin and PI for the indicated time points. PI was used as indicator for late apoptosis (cells stained with PI and annexin). The lower left square indicates the percentage of living cells. The lower right and upper right show the percentage of early (positiv for annexin) and late (stained with annexin and PI) apoptotic cells, respectively.

The analyses showed that 5 % of cells were stained with annexin and PI at 0 h time points. The percentage of early and late apoptotic cells (stained with PI and annexin) increased during glucose deprivation from 7 % after 12 hrs to reach 12 % after 22 hrs growth in medium lacking glucose (Fig 25 B).

The results indicated that apoptosis is a cause of death observed during glucose deprivation.

4.4 Autophagy is activated in HeLa cells subjected to glucose Deprivation

Activation of autophagy could be another mechanism induced by glucose deprivation and finally resulting in death of HeLa cells. Therefore, analysis of of LC3 as an

autophagic marker was carried out in the course of the experiment. LC3 (microtubule-associated protein1 light chain 3) is a protein, which is located diffusely in the cytoplasm under normal conditions. Activation of autophagy results in the translocation of the cytosolic form of LC3 (LC3-I) to the surface of autophagosomes, its binding to phosphatidylethanolamine and its cleavage to LC3-II. Investigation of the autophagic response under glucose starvation by western blot confirmed an early cleavage of LC3-I. 4 hrs after cultivating HeLa in medium lacking glucose, the conversion to LC3-II was observed. LCII could be seen also during the whole glucose deprivation time course (Fig 26).

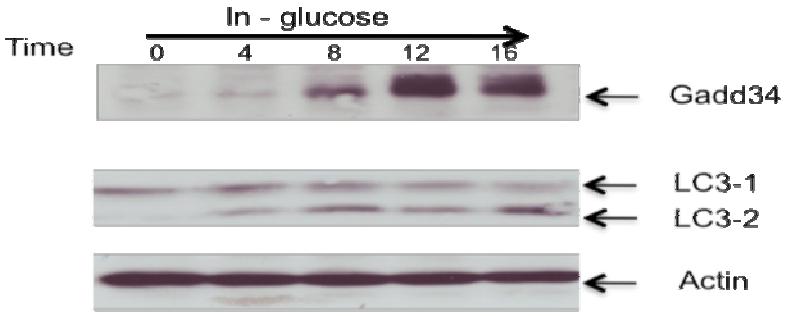

Fig. 26: Autophagy is activated in HeLa cells subjected to glucose deprivation.

HeLa cells were subjected to glucose deprivation. The level of Gadd34 was analysed by western blotting using Gadd34 antibody. Cleavage of LC3 was examined by western blotting at the indicated time points. Actin served as loading control.

Activation of autophagy in starved cells suggested that autophagy can protect starved cells from death and can also be another trigger for cell death observed in HeLa cells during glucose deprivation.

5. The absence of Cdh1 sensitises cells to glucose deprivation

Microscopic observations revealed that cells depleted of Cdh1 were showing more cell death when they were cultivated in the absence of glucose. These observations

suggested that Cdh1 might be involved in modulating the survival of cells undergoing ER stress. To verify this hypothesis, the sensitivity of HeLa cells to ER stress caused by glucose deprivation was analysed in the presence and absence of Cdh1 in more details. Therefore, HeLa cells were transfected either with Cdh1 si RNA or control si RNA, then in the next day subjected to glucose deprivation. The cell viability was assessed by trypan blue staining, for the indicated time points according to the scheme in figure 27A. Adherent and floating cells were collected for the quantification. Trypan blue was used to quantify all kind of death, which can be activated during glucose deprivation. Immunoblotting was performed to determine the levels of Gadd34 and to examine Cdh1 depletion (Fig 27B).

Western blot analyses confirmed the depletion of Cdh1 in cells transfected with Cdh1 si RNA. Gadd34 was highly induced during glucose deprivation in cells transfected with Cdh1 si RNA compared to cells transfected with control si RNA. In addition, Gadd34 started accumulating earlier in the absence of Cdh1. A high induction was detected after 12 hrs in cells transfected with Cdh1 si RNA, whereas control si RNA transfected cells showed only a slight accumulation of Gadd34 at the same time point (Fig 27B).

The percentage of living cells was quantified after 0, 12, 16 and 22 hrs glucose deprivation. In complete medium (0 hour time point), no significant difference in survival ($P>0,5$) was observed between cells transfected with control si RNA and Cdh1 si RNA (Fig 27C). Therefore, the percentage of viability was considered to be 100 % in cells containing Cdh1 or depleted of it at the 0 hour time point (Fig 27C). The percentage of living cells was calculated by dividing the percentage of viability in each time point by the percentage of viability at the 0 h time point for both control and Cdh1 si RNA transfected cells. The graph in 25D illustrates the percentage of survival averaged from 3 independent experiments.

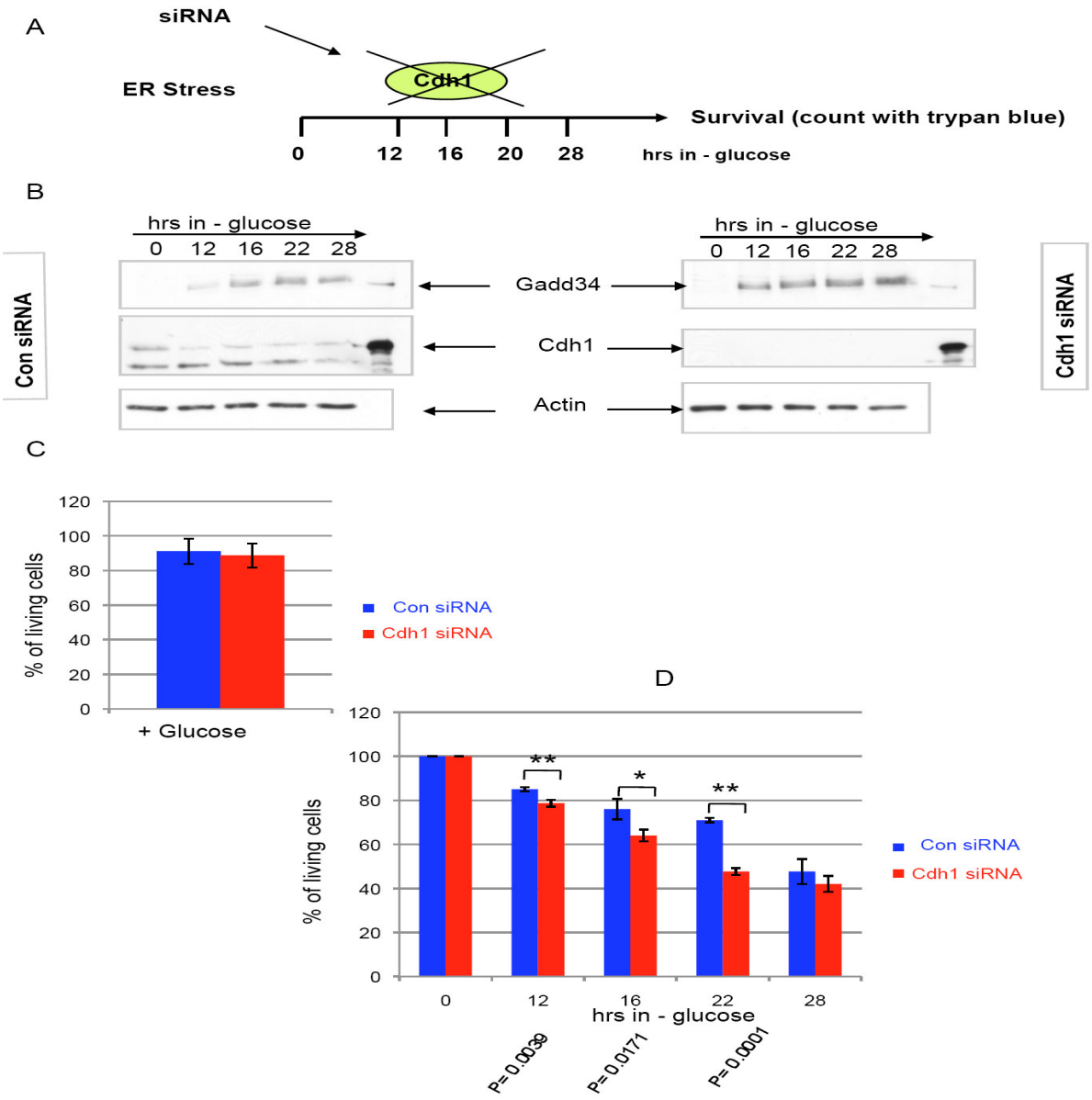

Fig. 27: Cdh1-depleted HeLa cells were more sensitive to glucose deprivation.

A. The scheme represents the design by which the viability was determined after glucose deprivation in cells expressing and depleted of Cdh1. HeLa cells were transfected either with control si RNA or Cdh1 si RNA. 24 hrs after transfection, the cells were subjected to glucose deprivation for the indicated time points. B. The graph represents the percentage of living cells quantified by trypan blue. C. The levels of Gadd34 and Cdh1 were examined in lysates by western blotting at the indicated time points and actin served as control for equal loading. D: The graph shows the % of survival quantified with respect to non deprived cells in the presence and absence of Cdh1 in 3 independents experiments. Statistical significance (P value) was determined using an unpaired t test (P<0,05) was considered as statistically significant).

Quantification of 3 independent experiments was performed. In con si RNA transfected HeLa cells, 85 % of cells were viable after 12 hrs. The survival decreased to 76 % after 16 hrs and to 71 % after 22 hrs to reach about 48 % after 28 hrs glucose deprivation. In the absence of Cdh1, the sensitivity to glucose deprivation was more enhanced compared to con si RNA transfected HeLa cells. Quantification showed that about 79 % of cells were viable after 12 hrs glucose deprivation in Cdh1 si RNA transfected cells, 64 % after 16 hrs, about 48 % after 22 hrs and reached 42 % after 28 hrs growth in the absence of glucose (Fig 27D).

The higher sensitivity to glucose deprivation in Cdh1 depleted cells was highly significant after 12 (P=0,0039), 22 hrs (P=0,001) glucose deprivation and significant after 16 hrs glucose deprivation (P=0,0171) (Fig 27D).

Taken together, quantification of survival in cell depleted of Cdh1 subjected to glucose deprivation suggested the implication of Cdh1 in controlling the survival under ER stress conditions.

6. Co-depletion of Gadd34 partially rescues from glucose deprivation induced cell death

Gadd34 induction and even overexpression correlates with apoptosis (Hollander et al 2003). It has been also reported that Gadd34 enhances autophagy during starvation (Nizam Uddin et al 2011). The data presented above strongly showed that Cdh1 controls Gadd34 turnover and controls survival under ER stress conditions. The reports and the finding suggested that Cdh1 could be involved in controlling the survival under ER stress conditions through regulating Gadd34 turnover. Therefore and to verify this hypothesis, HeLa cells were transfected either with control si RNA, Cdh1 si RNA, Gadd34 si RNA or the combination Gadd34/ Cdh1 si RNA in 5 nM final concentration. The cells were subjected in the next day to glucose deprivation. The cell viability was assessed by trypan blue staining as it is described in the sheme Fig 28A and the protein levels were detected by immunoblotting at the indicated time points (Fig 28B).

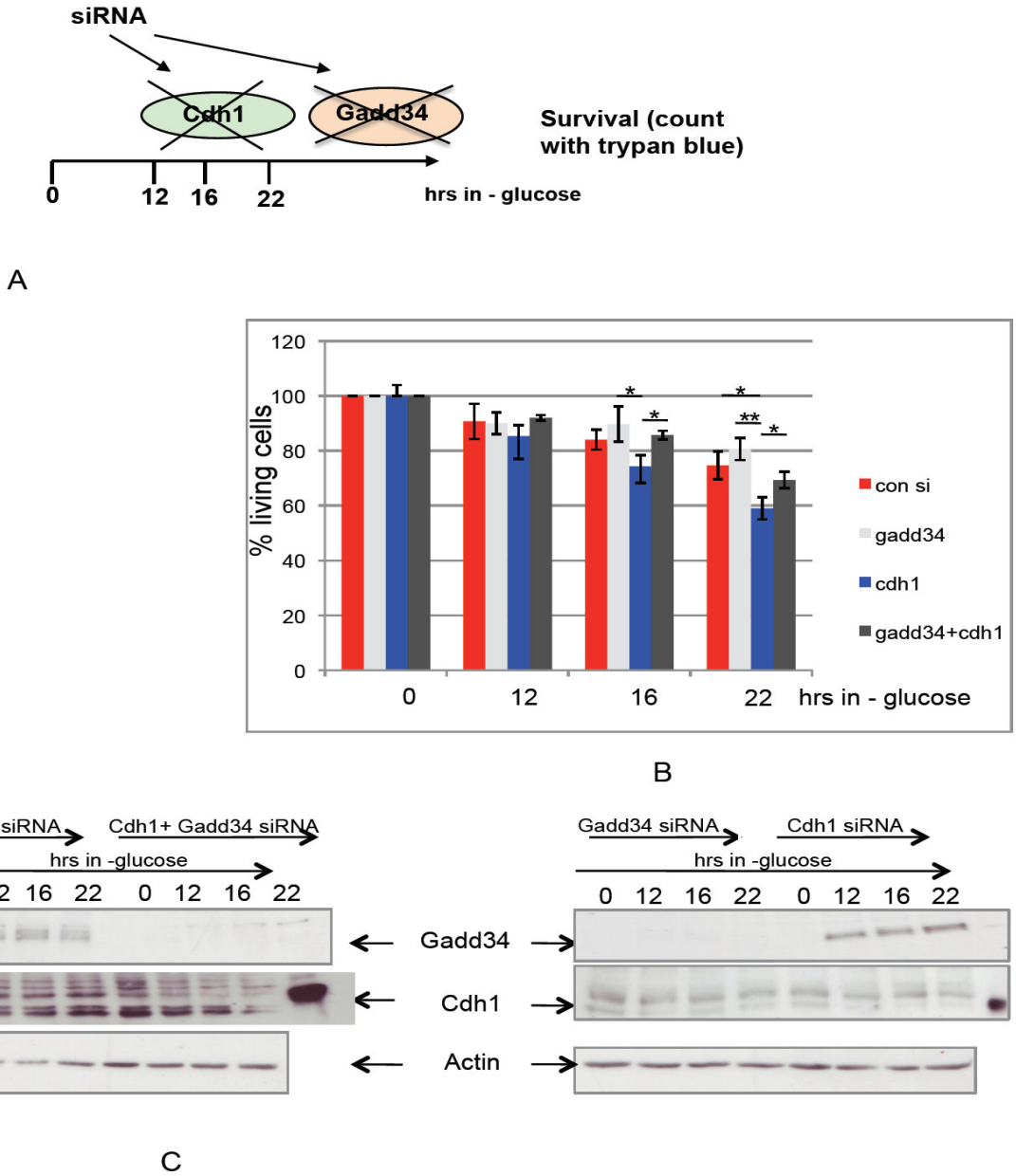

Fig. 28: Co-depletion of Gadd34 rescues HeLa cells partially from the death induced by glucose deprivation.

A. HeLa cells were transfected either with control si RNA, Cdh1 si RNA, Gadd34 si RNA separately or with the combination of Cdh1 and Gadd34 si RNA in a 5 nM final concetration. 24 hrs after transfection, the cells were subjected to glucose deprivation and the cell viability was assessed by trypan blue staining at the indicated time points. B. Western blot analyses were pereformed to detect Cdh1 and Gadd34 levels. Actin was used as control for equal loading C. The graph represents the percentage of living cells quantified in 3 independent experiments by trypan blue in cells transfected with the indicated si RNAs and culivated in the presence of glucose. D. The graph represents quantification of survival in cells depleted either of Cdh1, or Gadd34 or both or cells transfected with

control si RNA avereged from 3 independent experiments. The statistics in Fig D were kindely done by Dr. Bousset.

Quantification of cell viability was performed as described in the experiment before (3-3). The cell viability at the 0 h time point was considered as 100 % in all si RNA transfected cells as it was no significant difference to cell transfected with control si RNA (Fig 28C).

The cell viability was quantified by trypan blue staining in each time point of glucose deprivation. The percentage of survival was calculated as done before. The graph in 26B represents the average of 3 independent experiments (Fig 28D).

Depletion of Gadd34 showed 90 % of viability after 12 hrs glucose deprivation. Almost no difference in the viability if compared to cells transfecetd with control si RNA and subjected to glucose deprivation for 12 hrs (90,67 %). A slight but not significant increase was observed after depleting Gadd34 in cells deprived of glucose for 16 and 22 hrs. The viabilty increased from 84 % in control si RNA transfected cells to 89,67 % in Gadd34 depleted cells after 16 hrs glucose deprivation and from 74,67 % in the presence of Gadd34 to 80,67 % in Gadd34 si RNA transfected cells deprived of glucose for 22hrs (Fig 28D).

In Cdh1 depleted cells, 85 % of cells were viable after 12 hrs growth in the absence of glucose, 74 % after 16 hrs and 59 % after 22 hrs. In this set of experiments, statistical analysis showed that the higher sensitivity to glucose deprivation observed here was significant at the 22 hrs time point (P=0,01) (Fig 28D).

When Gadd34 and Cdh1 were co-depleted, the viability quantified after 12 hrs glucose deprivation increased to 92 % compared to Cdh1 si RNA transfected cells (85,33 %). This change was also observed after 16 and 22 hrs glucose deprivation. Statistical analyses revealead that co-depletion of Gadd34 and Cdh1 increased the viability of cells significantly from 74,33 % to 85,67 % (P=0,03), and from 59 % to 69,33 % (P=0,02) when deprived of glucose for 16 and 22 hrs, respectively (Fig 28D). At the 22 hrs time point a significante loss of viability was observed after depleting Cdh1, which could be nearly rescued by co-depleting Gadd34.

The results indicated that co-depletion of Gadd34 rescued partially from the cell death observed during glucose deprivation (Fig 28D).

In Parallel, flow cytometry analyses were carried out in order to determine the effect of the depletion of Gadd34, Cdh1 and both on the cell cycle progression in cells subjected to glucose deprivation for the indicated times (Fig 29). The results indicated that depletion of Gadd34, Cdh1 and both was not interfering with cell cycle progression upon stress caused by glucose deprivation (Fig 29).

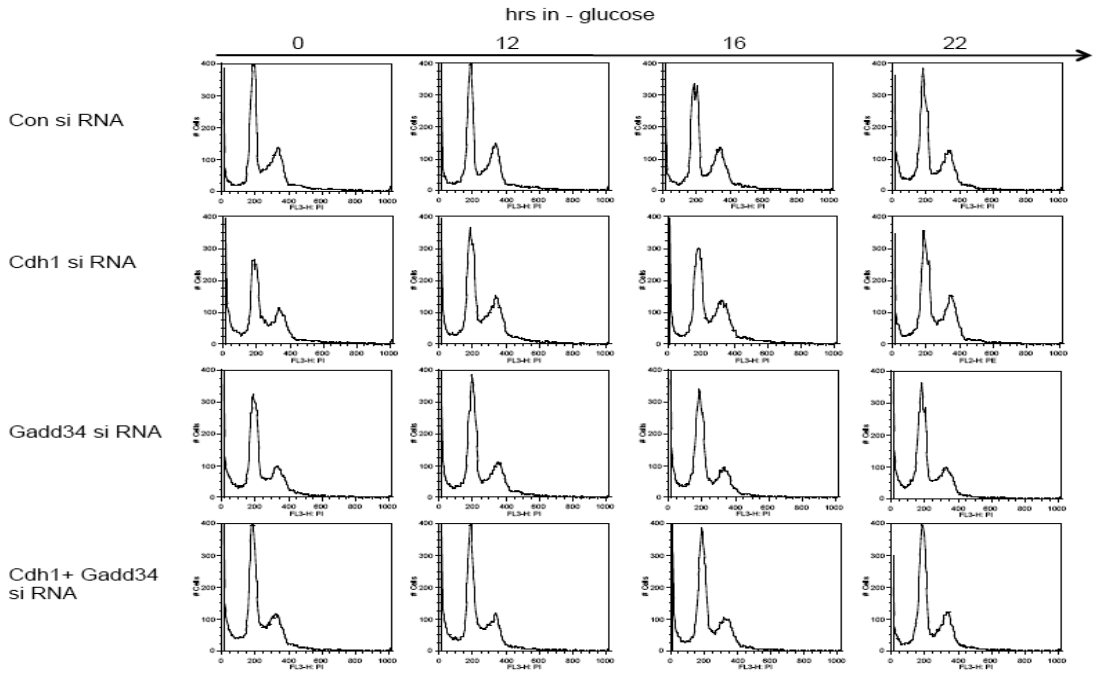

Fig. 29: Cell cycle progression is not affected during glucose deprivation in cells depleted of either Cdh1 or Gadd34 or both.

Flow cytometry analyses were carried out to examine the effect of the depletion of Cdh1, Gadd34 and both on cell cycle progression during glucose starvation.

Taken together, the data presented above indicate that accumulation or depletion of Gadd34 under ER stress conditions is not interfering with the cell cycle progression. Furthermore, they suggest that APC/CCdh1 is involved in controlling the cell survival under ER stress conditions through targeting Gadd34 for degradation by the proteasome.

7. Depletion of Cdh1 is not interfering with the recover of cells subjected to glucose deprivation

HeLa cells subjected to glucose deprivation showed a decrease in cell viability. This reduction was more enhanced in the absence of Cdh1. Do stressed cells reach of point of no return after this they are not able to recover? And if this point exists, is it dependent on the presence or absence of Cdh1?

To answer these questions, HeLa cells were transfected either with control si RNA or with Cdh1 si RNA. Next day, the cells were deprived of glucose for 0, 12, 16, 22 and 28 hrs. To monitor the number of living cells, cells were counted after trypan blue staining during glucose deprivation for the indicated time points and after releasing these cells for 24 hrs in complete medium. Western blot analyses were performed to detect Gadd34 induction and to examine Cdh1 depletion during glucose deprivation and after release (Fig 30A).

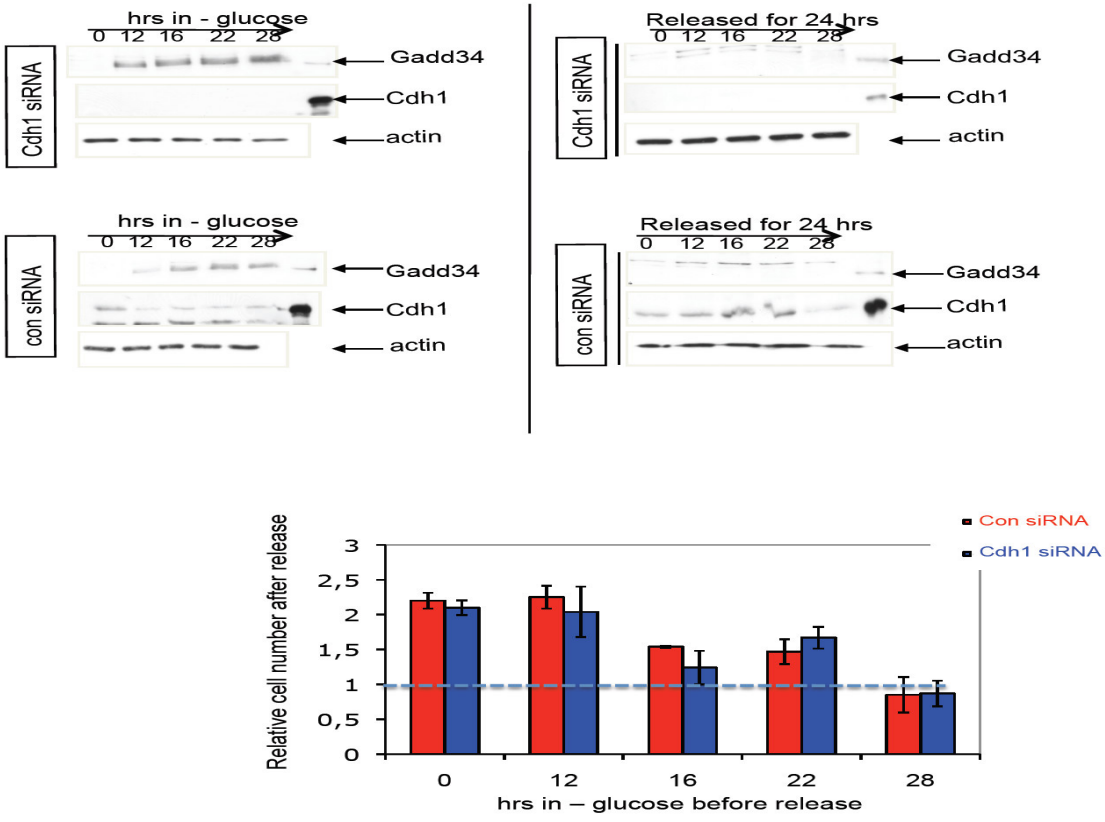

Fig. 30: HeLa cells were able to proliferate in the presence and absence of Cdh1 after releasing them from stress caused by glucose deprivation.

HeLa cells were transfected either with Cdh1 si RNA or with control si RNA. 24 hrs later, the cells were deprived of glucose for the indicated time points. At every time point cells were released for 24 hrs in complete medium for recovery. The cell viability was assessed before and after recover by trypan blue staining for the indicated time points. A. The levels of Gadd34 and Cdh1 were examined by western blotting using specific antibodies. Actin was used as a loading control. B. The graph shows the percentage of cells, depleted or containing Cdh1, which were able to recover after 24 hrs release from stress. The graph represents the mean of two independent experiments.

Western blot analyses confirmed the depletion of Cdh1 in cells transfected with Cdh1 si RNA and showed the Gadd34 induction during glucose deprivation in the presence and absence of Cdh1. After 24 hrs release, Gadd34 was completeley removed. This was observed both in control and Cdh1 si RNA transfected cells. A slight level of Gadd34 was detected in the absence of Cdh1 in cells releasd from 28 hrs glucose deprivation (Fig 30A).

The relative cell number was calculated for each time point by dividing the number of living cells after 24 hrs release by the number of living cells before release. The graph in 30B represents this value averaged from 2 independent experiments in control and Cdh1 si RNA transfected cells for the indicated time points.

The quantification showed that the cell number doubled within 24 hrs in cells growing in complete medium. The same was observed, when they were deprived of glucose for 12 hrs. After been subjected to glucose deprivation for 16 to 22 hrs, the cells were still able to recover, but the relative cell number counted after 24 hrs release decreased from 2 (observed in earlier time points) to almost 1,5. This effect was observed both in the presence and absence of Cdh1. This decrease can be explained by the fact that some damaged cells were not able to recover and were eliminated within the 24 hrs release. After 28 hrs glucose deprivation, the cell number didn't increase after addition of glucose. This suggests that the cells were not able to recover and to proliferate both in control and Cdh1 si RNA transfected cells (Fig 30B).

The results indicated that HeLa cells were able to recover up to 22 hrs glucose deprivation both in the presence and absence of Cdh1. Furthermore, the cells had reached a point of no-return after been deprived of glucose for longer than 22 hrs as

seen at the 28 hrs time point. At this point, HeLa cells didn't recover and showed a decrease in the the relative cell number counted after 24 hrs release in complete medium. This effect was not dependent on Cdh1, as it was also observed in control si RNA transfected cells.

V. Discussion

Proteasomal dependent degradation of Gadd34 has been previously documented (Brusch & Shenolikar 2008). However, the ubiquitination system involved in this degradation is up to now unknown. The results presented in this work give the first evidence for the specific interaction between Gadd34 and Cdh1 and the direct involvement of Cdh1 in the Gadd34 turnover. In this function Cdh1 controls the survival of cells subjected to ER stress. By defining the ER stress protein Gadd34 as a new interacting partner of the cell cycle regulator, it was also possible to characterise a new function played by APC/C^{Cdh1} under ER stress conditions.

1. Gadd34 is a new target of the APC/C^{Cdh1}

In the first part of this work, the interaction previously discovered in a yeast-two-hybrid screen between Cdh1 and Gadd34 was analysed in human cells. Co-immunoprecipitation assays demonstrated the specific interaction between Gadd34 and Cdh1 in mammalian cells after overexpression (Fig 1). Furthermore, and in agreement with the results of the yeast two-hybrid screens, the C-terminals of Gadd34 was sufficient for the interaction with Cdh1. This was concluded after co-immunoprecipitating exogenous Cdh1 with the 3 deletion mutants (Fig 2-3). To exclude a contribution of the N-terminus in the interaction with Cdh1, a construct lacking the C-terminus (Gadd34 1-483 aa) is already cloned by Dr. Bousset in HA-Tag expressing vector and will be used in co-immunoprecipitation assays. The C-terminal region of Gadd34 is also interacting with most important regulators of its activities (Hollander et al., 2003). The observation that the D-Box located at the C-terminal was not found to be conserved between human, rat and mouse suggests that Cdh1 may need a motif other than D-Box for Gadd34 recognition. A-box (RxLxPSN), O-box, and CRY box (CRYxPS) are additional destruction motifs recognised by Cdh1 (Leuken et al., 2006). There is also evidence suggesting that Cdh1 in some cases needs more than one motif for recognising its targets. The Aurora B kinase is an example of substrates recognised by APC/C^{Cdh1} via KEN and A-box (RxLxPSN) (Nguyen, et al., 2005). Therefore, mutating this motif will help to

further test the importance of the D-Box at position (526-529) in the recognition of Gadd34 by Cdh1.

The function of APC/C^{Cdh1} as an E3 ligase in the ubiquitin pathway suggested also that this interaction could result in Gadd34 ubiquitination. This hypothesis was tested using a His-ubiquitination assay. This assay is an efficient system to detect E3 ligase function toward specific substrates (Bloom and Pagano 2005). The detection of protein ubiquitination occurs after transient expression of the E3 ligase, the target protein and His-Ubiquitin. Increasing the level of the E3 ligase should result in increasing the ubiquitination of the substrate. Furthermore, inactivation of the E3 ligase can strengthen the argument for a direct ubiquitination of the substrate by the tested E3 ligase. The performed His-ubiquitination assays showed that Gadd34 ubiquitination was enhanced by Cdh1 in a dose dependent manner. An effect, which was reversed after overexpressing Cdh1 inhibitors Emi1 and the N-terminal of Cdh1 (N125) thereby giving evidence for the direct involvement of Cdh1 in Gadd34 ubiquitination. The ubiquitination of Gadd34 was more inhibited by the dominant negative Cdh1 (N125) than Emi1. The difference in the level of Gadd34 ubiquitination could be a result of the difference in the expression level between Emi1 and Cdh1 as it was seen in the western blot (Fig 4). It has been already reported that the C-terminal region was the part of Gadd34, which is subjected to ubiquitination (Watanabe et al., 2007). The data presented above support this result as the C-terminal was the part of Gadd34 interacting with Cdh1. Overexpression of the C-terminal region of Gadd34 in combination with Cdh1 and His-ubiquitin could be an additional experiment to further show that the C-terminus is the part of Gadd34, which is getting ubiquitinated by Cdh1. It was suggested that a construct lacking the first 60 amino acids at the N-terminal (Gadd34 (Δ-60)) was seen to be more stable compared to the full length Gadd34 due to different localisation (Brush & Shenolikar 2008). This observation should be taken into consideration, when analysing the ubiquitination of Gadd34 deletion constructs.

2. Cdh1 regulates Gadd34 turnover under physiological Conditions

The functional significance of the interaction between the cell cycle protein Cdh1 and the ER stress protein Gadd34 was analysed under conditions causing ER stress.

Physiological and pharmaceutical stress inducers were tested during this work in order to induce ER stress and thereby to accumulate Gadd34 (Fig 5). In line with previously published data glucose deprivation was effective in inducing Gadd34 in HeLa and in other human cell lines (Fig 5) (Diehl 2006, Brush & Shenolikhar 2008). The results also indicated that Gadd34 was not expressed in unstressed HeLa and the other tested cells (Fig 6). Its transcription as analysed by RT-PCR started between 4 and 8 hrs glucose deprivation when eIF2-α was higher phosphorylated (Fig 6). It has been reported that the expression of Gadd34 is controlled by the transcription factor ATF4. Both ATF4 and Gadd34 are selectively translated when eIF2-α is phosphorylated in a 5'UTR dependent mechanism (Ma & Hendershost 2003, Ron 2002, Lee et al., 2009). The decrease in eIF2-α phosphorylation observed later during glucose deprivation could be attributed to the feedback loop played by Gadd34 by assembling the phosphatase type I (Fig 6). In this function Gadd34 allows partial translational recovery and strengthens the ER stress response. This leads to more expression of many ER stress inducible-genes including Bip, ATF4 and Chop (Novoa et al. 2003). This could explain the increased levels of Chop observed later during glucose deprivation (Fig 6). Other studies showed that ATF4 induces the expression of Chop, which leads in turn to the accumulation of Gadd34. Tunicamycin treated Chop-/- cells showed a reduced expression of Gadd34 (Marciniak et al., 2004). Thus, Chop was placed upstream of Gadd34, which can also be an argument for the high accumulation of Gadd34 observed late during ER stress (Fig 6) (Harding et al., 2000, Kojima et al., 2003. Marciniak et al., 2004, Zhang & Kaufman 2008).

Consistent with a previous study performed by Brush and Shenolikar (Brush & Shenolikar 2008), the accumulation of Gadd34 was reversible after removal of stress (Fig 7) and Gadd34 was found to be a short lived protein (Half-life< 30 min) (Fig 8A). Together, these data suggest the existence of transcriptional and translational regulation, which allows Gadd34 as an ER stress inducible gene to be exclusively and selectively expressed and translated under ER stress conditions. Inactivating Cdh1 activity resulted in a higher accumulation of Gadd34 in ER stressed cells, slower reduction of Gadd34 during the release (Fig 9) and extended Gadd34 half-life (Half-life> 4hrs) (Fig 10). These data implicate strongly Cdh1 in the regulation of Gadd34 turnover in stressed cells and after releasing cells from stress thereby giving the first evidence that APC/C^{Cdh1} is the E3 ligase involved in the proteasomal degradation of Gadd34 (Fig 9-10).

Gadd34 was not completely stable in cells depleted of Cdh1 (Fig 9-10). It is possible that low levels of Cdh1 remaining after depletion, which were not detected by western blotting, can degrade Gadd34. It can also be explained by the existence of another E3 ligase, which can be activated to regulate the turnover of Gadd34 in the absence of Cdh1. Proteins directed for proteasomal degradation by two different ligases have been already documented. For example, SAS6 proteasomal dependent degradation is controlled by APC/C^{Cdh1} during G1 (Tang et al., 2009) and by SCF^{Fbxw5}, recently discovered in our group, during S phase (Puklowski et al., 2011). Preliminary data showed that overexpressed Cdc20 can interact with Gadd34 and resulted also in its ubiquitination (data not shown in this work). These observations suggest that Cdc20 could possibly compensate for the loss of Cdh1 and require further investigations.

3. Gadd34 and Cdh1 localise to the cytoplasm under ER stress Conditions

In the absence of specific antibodies suitable for immunofluoresence to recognise endogenous Gadd34 and Cdh1, analysis of the subcellular localisation of Cdh1 and Gadd34 was carried out for exogenously tagged expressed proteins. Consistent with previous data, the result of the immunostaining revealed that Cdh1 was mainly nuclear with a slight staining in the cytoplasm in unstressed cells (Fig 11). Gadd34 was located in the cytoplasm close to nucleus (Fig 11). The use of specific fluorescent dyes like DioC6 staining the ER or specific antibodies that recognise ER-associated proteins like anti-Calnexin is required to further indicate that Gadd34 is localised to the ER. The localisation of Gadd34 was analysed in detail in a recent study performed by Zhou and colleagues. In this work, they described a hydrophobic region located between the 16 and 65 amino acids in targeting Gadd34 to the ER. This sequence is highly conserved in mammalians species. Deletion of this sequence or substitution of valine located at position 25 to arginine resulted in cytosolic distribution of Gadd34. The localisation of Gadd34 to the ER was seen to be important for its proteasomal degradation, as the cytsolic form of Gadd34 was more stable than the wild type (Zhou at al., 2011).

Analyses of the localisation of exogenous Cdh1 revealed that Cdh1 was mainly located to the nucleus in unstressed cells. Depriving cells of glucose resulted in an equal distribution of Cdh1 between the nucleus and the cytoplasm. This change in the localisation was significant (Fig 12). After releasing cells from the stress caused by glucose deprivation, this localisation changed significantly to become again mainly nuclear (Fig 13).

Eset was used as a control to examine nuclear integrity under glucose deprivation. The results indicated that only the localisation of Cdh1 was changed during glucose deprivation. Further investigations are required to test whether the effect observed is due to overexpression of CDH1 or can be seen in endogenous protein.

The subcellular localisation of Cdh1 is cell cycle dependent. Cdh1 is phosphorylated and located to the cytoplasm during S, G2 and early M, whereas unphosphorylated Cdh1 is mainly located to the nucleus at the mitotic exit and during G1 (Kramer et al., 2000). The mechanism explaining the cytoplasmic distribution of Cdh1 under stress conditions might be different from the cell cycle regulation. Changes in protein localisation following stress have already been reported in the literature. Stress caused by DNA damage was seen to promote the nuclear import of p53, which is predominantly localised and degraded in the cytoplam after translation in unstressed cells (Marchenko, et al., 2010). Genotoxic stress promotes also dephosphorylation of Cdh1, which becomes nuclear in order to reactivate the APC/C and thereby to control the G2 arrest in DNA damaged cells (Basserman et al., 2008). But so far there are no reports on cytoplasmic distribution of Cdh1 under any from of stress. An active cytoplasmic isoform of Cdh1 lacking the nuclear localisation side was already described but the conditions allowing the translation of this isoform are not known (Zhou et al., 2003). It could also be possible that the cytoplasmic Cdh1 observed under ER stress is an isoform of Cdh1, which is selectively translated under ER stress. Indeed, examples of protein isoforms translated under ER stress are already documented. p53/47 for example is an isoform of p53 translated selectively under ER stress (Bourougaa et al., 2010).

4. Modulation of Gadd34 turnover highlights a new function of Cdh1 during ER stress

4.1 ER stress caused by glucose deprivation is not affecting the cell cycle progression

Flow cytometry analyses revealed that depriving HeLa cells of glucose cells up to 22 hrs was not resulting in any obvious cell cycle effect (Fig 20 - 27). A reduced proliferation due to a G1 arrest was already reported in ER stressed cells (Brewer et al., 1999, 2000). Glucose limitation was also suggested to activate a p53-dependent restriction checkpoint at the G1/S boundary and cell cycle arrest (Jones et al., 2005. See introduction: 5- UPR and cell cycle). The dependency of the arrest observed on p53 can be an explanation for the absence of G1 arrest during glucose deprivation. In fact, p53 is instable due to an enhanced degradation by the of p53 protein by the E6 viral oncoprotein expressed in HeLa cells used in this work (Wesierska-Gadek et al., 2000).

4.2 Cdh1 modulates the survival under ER stress through targeting Gadd34

Quantification of survival revealed that depletion of Cdh1 significantly sensitised HeLa cells to cell death during the course of glucose deprivation. Gadd34 was also detected earlier and to higher level in cell depleted of Cdh1 compared to control si RNA transfected cells when they were subjected to glucose deprivation (Fig 25). These results suggested that the absence of Cdh1 as the E3 ligase controlling Gadd34 turnover could be a reason for the increased cells death observed during glucose deprivation. This hypothesis was tested by analysing the effect of depleting Cdh1, Gadd34 and the co-depleting both Cdh1 and Gadd34 on the survival of stressed cells. In this set of experiments Cdh1 depleted cells were seen to be sensitive to glucose deprivation compared to control si RNA transfected cells (Fig 26). This sensitivity was only significant at the 22 hrs time point which was not the case for the previous experiments (Fig 25). A variation in the cell culture conditions could be an explanation for the difference observed between the results illustrated in Figure 25 and 26. Depletion of Gadd34 increased slightly but not significantly the

survival in cells subjected to glucose deprivation (Fig 26). The sensitivity of Cdh1 depleted cells to glucose deprivation was significantly reduced after co-depleting Gadd34 and Cdh1 in cells subjected to glucose deprivation for 16 and 22 hrs. Therefore it is very likely that Cdh1 is involved in the modulation of cell survival under ER stress conditions by controlling the level of Gadd34 (Fig 26).

An involvement of Gadd34 in cell death was already documented. It has been reported that overexpression of Gadd34 correlates with and even enhances apoptosis (Adler et al., 1999, Hollander et al., 1997, 2001, 2003). This function can be negatively modulated through binding of HRX (expressed as a result of chromosomal translocation in Leukemia) to the C-terminus of Gadd34 (Adler et al., 1999) and through phosphorylation by Lyn (Scr-related protein tyrosine kinase activated during DNA damage) (Grishin et al., 2001). The apoptotic function played by Gadd34 was also described in prolonged ER stress. Gadd34$^{\Delta C/\Delta C}$ mutant mice (homozygous for a mutation that eliminates the C-terminal region of Gadd34 required for the binding to the phosphatase type1) were resistant to apoptosis induced in kidney by tunicamycin treatment. This could be most likely due to a prolonged shut off of protein synthesis (Marciniak et al., 2004). Prolonged inhibition of protein synthesis might relieve in some context the stressed cells from an overload of additional newly synthesised proteins and even the expression of other pro-apoptotic proteins like Chop. But it can also block the expression of pro-survival proteins and promote thereby cell death. Gadd34$^{\Delta C/\Delta C}$ mousse fibroblasts were more sensitive to thapsigargin induced cell death. An effect, which correlated with impaired expression of ER stress-inducible chaperones due to a persistent inhibition of protein synthesis (Novoa et al., 2003).

The finding presented in my thesis suggested a new function of Cdh1 under ER stress. In this function Cdh1 protects cells from cell death activated during ER stress. A recent study supports also this notion, as Cdh1 was shown to control the degradation of TRB3 another ER stress protein (Ohoka et al., 2010). TRB3 is also induced via the ATF4 - Chop pathway and promotes cell death under prolonged ER stress conditions (Ohoka et al., 2005). Therefore, Cdh1 might play a more general role in survival after ER stress response.

Evidences suggest that the balance between survival and death during ER stress is maintained by intrinsic instability of mediators of cell death during ER stress response

such as Chop and Gadd34. Prolonged ER stress increases the level of these proteins and promotes death of ER-stressed cells (Rutkowski et al., 2006, Ron & Walter 2007). The finding presented in this work and the above mentioned reports strongly suggest that Cdh1 can be an inhibitor of the switch from the pro-survival to pro-death response of the ER stress response. This function can be achieved through reducing the level of pro-apoptotic mediators through targeting them for degradation by the proteasome.

The rescue observed after depletion of Gadd34 was only partial during the course of the experiments (Fig 26). This could be explained by the existence of other pro-death proteins activated by the other axes of the UPR. JNK activated by the IRE1 signalling pathway for example can activate apoptosis though phosphorylation of Bcl2 family proteins and subsequent release of Bax and Bid (Lai et al 2007, Lamara & Scorrano 2009). Phosphorylation of Bcl2 can also promote autophagic cells death (Verfaillie el al 2010). Furthermore, Chop can also be induced by pathways other than PERK (see introduction Fig 7) and promote apoptosis. This is in line with the observation that cells were not completely protected from the cell death observed when Gadd34 was depleted (Fig 26).

Both autophagy and apoptosis were activated during glucose deprivation (Fig 22-23). However, it was not clear whether autophagy is activated as cytoprotective or cytotoxic mechanisms. Therefore, inactivation of autophagy using specific inhibitors like 3-methyladenine or by targeting the expression of autophagic genes using specific si RNA can be useful to analyse whether autophagy promotes survival or eliminates damaged cells during glucose deprivation and thereby to characterise the mechanism of the cell death activated under these conditions.

The absence of Cdh1 sensitised cells significantly to glucose deprivation without affecting their ability to recover and to proliferate after removal of stress (Fig 28). Cdh1 depleted cells deprived of glucose for 16 hrs reduced Gadd34 slowly in the first 3 hrs of release (Fig 9). In these cells, the high accumulation of Gadd34 observed within the first hours of release was not interfering with the recovery. This can be explained by the fact that as long as a cell didn't reach the point of no-return they still have the opportunity to recover and to divide. Furthermore, the cells might not accumulate Gadd34 to the same levels. Cells with a high level of Gadd34 will die within the release and cells with low levels can most likely activate other mechanism

to degrade Gadd34 and thereby to survive and proliferate. In fact, Gadd34 was seen to be completely removed in these cells after 24 hrs release (Fig 28). This effect was also observed when cells were stressed up to 22 hrs (Fig 28).

After 12 hrs glucose deprivation, the relative cell number doubled in a similar way as cell growing in the presence of glucose. A decrease in the relative cell number was observed after releasing cells from 16 and 22 hrs glucose deprivation (Fig 28). This can be explained by the fact that some cells which were determined to die before the release and were not detected with trypan blue staining (early apoptotic for example) were eliminated within the 24 hrs of release. Quantification of apoptotic cells with annexin before and after the release could be used to quantify these cells.

The results presented by this work revealed the importance of Cdh1 in controlling the ER stress response through targeting Gadd34 for degradation, whereas the release from stress was not seen to be dependent on this function.

5. Targeting Cdh1 can be a promising novel strategy for cancer Treatment

The results discussed above could be exploited for inhibiting tumour growth. For their energy supply, tumour cells are more dependent on glycolysis and therefore they are characterised by an increased glucose uptake compared to normal cells (Garber 2006, Simons et al., 2009). In tumour cells a decrease in ATP can leads to apoptosis (Heiden et al., 2009). An increased activation of autophagy in the absence of glucose can also result in cell death (Dang et al., 2011). Data pointed out that manipulation of glucose metabolism can be effective in eliminating cancer cells (Garber 2006). In addition, that some drugs were more effective in eradicating tumours, when cells were deprived of glucose. This was for example observed with Versipelostatin and biguanides, inhibitors of GRP78, ATF4 and XBP1, when they were combined with glucose deprivation (Saito et al., 2009).

The effect of Cdh1 depletion in increasing cell death of HeLa cells subjected to glucose deprivation was an important result presented in this work. This result suggests that strategies to either downregulate or inhibit Cdh1 can represent a promising therapeutic approach in the context of tumours therapy. This idea is

supported by other growing evidences, which point out to the importance of inhibiting proteolysis as target for cancer treatment. Interfering with the activity of SCF by down regulating Skp2 either by si RNA or using specific inhibitors can induce either apoptosis or growth arrest in tumour cells (Wäsch & Engelbert 2005). Other studies revealed that inhibition of Cdc20 can be a new tool in targeting cancer cells. Depletion of Cdc20 was observed to induce cell cycle arrest at G2/M and to inhibit cell growth (Kidokoro et al., 2008). In addition, our group and others demonstrated the importance of targeting the proteasome for cancer therapy. Argyrin discovered by our group, because of its specificity towards p27, is a very promising future drug, showing an *in vivo* antitumour activity by inhibiting the proteasome (Nickeleit et al., 2008).

ER stress is activated in solid tumours due to hypoxia and nutrients starvation. Depletion of Cdh1 using small drugs could be used to increase the ER stress response. This effect can be more enhanced if combined with glucose deprivation. Therefore combining Cdh1 inhibitors with glucose deprivation or inhibitors of glucose metabolism can be a promising strategy to further stress and to eliminate cancer cells. The use of inhibitors of glucose metabolism is suggested as it is to some extend not possible to deprive cell completely of glucose *in vivo.* Treatment with 2-deoxy-D-glucose (2DG) was seen to be effective in inhibiting glucose metabolism by competing with glucose for uptake and phosphorylation at the entry of glycolysis (Simons et al., 2009).

Inhibition of Cdh1 using small drugs can induce senescence in non-tumour cells and can affect many aspects of APC/C^{Cdh1} functions in terminally differentiated cells (see introduction: 6-Life without Cdh1). With respect to the haplo-insufficient tumour suppressor function played by Cdh1, the use of inhibitors that partially inactivate APC/C^{Cdh1} can result in developing other tumours (Garcia-Higuera et al., 2008). Therefore, targeting tumours cells exclusively and selectively can be effective in reducing any side effects caused by inhibiting Cdh1 during cancer therapy. This could be achieved for example by combining Cdh1 inhibitor with specific monoclonal antibodies recognising markers specifically expressed on the surface of tumours (like Bip). Thus, healthy cells would not be affected (Verfaillie et al., 2010).

An *in vivo* tumour model will help to explore and to exploit the finding presented in this work. Multiple myeloma cells are characterised by increased activation of the ER

stress response to induce XBP-1, which is necessary for plasma cell differentiation and immunoglobulin secretion (Lee et al., 2003). Myeloma xenograph models are already documented and were used in several *in vivo* studies (Pan et al., 2011, Papandreou et al., 2011). This system can be useful for exploring the effect of Cdh1 depletion on tumours growth and survival. This can be achieved for example through generating multiple myeloma stably expressing inducible Cdh1 si RNA, which can be xenographed to nude mice. If the *in vivo* analyses confirm the importance of depleting Cdh1 in modulating survival of tumour cells, this system can be exploited for screening for small molecules inhibiting Cdh1 or stabilising Gadd34, which could be in combination with inhibitors of glucose metabolism an effective tool for treating cancer.

VI. List of abbreviation

A	Ampere
APS	Ammonium persulfate
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
℃	Degree celsius
CsCL	Cesium chloride
Da	Dalton
dd	Double distillate
DAPI	4´,6-diamidino-2-phenylindole
DEPC	Diethyl pyrcarbonat
DMEM	Dulbecoo's modified egal medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DNase	Deoxyribonuclease
DOC	Deoxycholic acid
dNTPs	2-deoxyribonucleotides
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetraacetic acid
et al	and others

FACS	Fluoresence activated cell sorting
FCS	Fetal calf serum
Fig	Figure
G	Gram
GD	Glucose deprivation
Glu	Glucose
HEPS	4-(2-Hydroxyethyl)-1-piperzine-N-ethanesulfonic acid
Hrs	Hours
H_2O_2	Hydrogen peroxide
HPRT	Hypoxantin-phosphoribosyl-transferase
HRP	Horseradish peroxidase
mRNA	Messenger RNA
kb	Kilobase
kDa	Kilodalton
M	Molar
mA	Milli-ampere
MG132	z-leu-leu-leu-al
min	Minute
mRNA	Messenger RNA
ml	Milliliter
n	Nano (10^{-9})
NaF	Sodium fluoride
NP-40	Nonidet P-40
Nr	Nummer

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SB	Sample buffer
siRNA	Small interfering RNA
Rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris-borate-EDTA
TE	Tris EDTA
TEMED	(N, N, N', N'- tetramethyethylenediamine
TNT	Tris-NaCL-tween
Tris	Tris-(hydroxymethyl) aminomethane
µg	Micro (10^{-6})
µg	Microgram
µM	Micromolar
PFA	Paraformaldehyde
PAA	Polyacrylamid
V	Volt

VII. References

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“Where there is a will there is a way„

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