Research article

Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1
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Abstract

Background: The Transforming Growth Factor-β (TGF-β) superfamily consists of a large group of structurally related polypeptides including various forms of TGF-β, bone morphogenetic proteins (BMPs), activins, growth and differentiation factors (GDFs) and the Anti-Mullerian Hormone (AMH, or MIS) [1,2]. Members of the TGF-β subfamily are molecular organizers for tissue and organ morphogenesis during embryonic development and play key roles in maintaining the homeostasis of various developed systems [3,4]. At the cellular level, diverse processes including cell proliferation, differentiation, adhesion and apoptosis are subjected to TGF-β regulation [5]. The intracellular signaling events are initiated upon TGF-β binding

Background

The Transforming Growth Factor-β (TGF-β) superfamily consists of a large group of structurally related polypeptides including various forms of TGF-β, bone morphogenetic proteins (BMPs), activins, growth and differentiation factors (GDFs) and the Anti-Mullerian Hormone (AMH, or MIS) [1,2]. Members of the TGF-β subfamily are molecular organizers for tissue and organ morphogenesis during embryonic development and play key roles in maintaining the homeostasis of various developed systems [3,4]. At the cellular level, diverse processes including cell proliferation, differentiation, adhesion and apoptosis are subjected to TGF-β regulation [5]. The intracellular signaling events are initiated upon TGF-β binding
to a pair of Ser/Thr kinase receptors known as the Type I receptor (TβR1) and the Type II receptor (TβRII), which are structurally similar but functionally distinct [6,7]. Upon binding to TGF-β, the Type II receptor recruits and activates the type I receptor by releasing the immunophilin FKBP12 from the type I receptor and also by mediating the trans-phosphorylation of the type I receptor at the GS domain, a highly conserved thirty-amino acid region containing a SGSGSG sequence [8-10]. The GS domain phosphorylation allows the Type I receptor to recruit and phosphorylate the cytoplasmic proteins belonging to the family of the Smad proteins [11,12].

The Smad proteins are the vertebrate homologues of the mothors against Dpp in Drosophila and the C. elegans Sma proteins [5,13-16]. Based upon their functional properties, Smad proteins are divided into three classes: 1) the receptor-regulated Smads, or R-Smads, which are phosphorylated respectively by TGF-β/activin receptors for Smad2 and Smad3 and by BMP receptors for Smad1, Smad5 and Smad8; 2) the co- mediator Smad (Co-Smads), namely Smad4 in mammals and Smad10 in Xenopus and 3) the inhibitor Smads (I-Smads) Smad6 and Smad7 which prevent the phosphorylation of the R-Smads and sometimes the formation of a complex between R-Smads and Co-Smads. Smad proteins have two conserved globular domains, namely N or Mad homology 1 (MH1) domain and C or MH2 domain, separated by a linker region of variable length and which can associate with one another in the inactive state. Upon phosphorylation by TGF-β type I receptor, Smad2 and Smad3 form a complex with Smad4 and translocate into the nucleus where they function as transcription factors. However, a novel activity of Smad3 in regulating the proteasomal degradation of the nuclear proto-oncoprotein SnoN and of the human enhancer of filamentation 1 (HEF1) has recently been reported. The data also suggest possible roles of proteasomal degradation of HEF1 and SnoN in a negative feedback mechanism of the TGF-β signaling pathway [17-19].

HEF1 was first isolated in a screen for human proteins capable of inducing pseudohyphal growth in Saccharomyces cerevisiae [20]. HEF1, also known as CasL, is a cytoplasmic docking protein belonging to the Cas family and structurally related to p130Cas and Efs. HEF1 is predominantly expressed in epithelial cells and lymphocytes. It contains multiple protein-protein interaction domains including a N-terminal SH3 domain that binds polyproline-containing protein, a domain containing multiple SH2 binding sites, a Serine-rich domain and a conserved C-terminal domain containing a helix-loop-helix (HLH) motif [21]. HEF1 is processed in a complex manner since at least four protein species (p55<sub>HEF1</sub>, p65<sub>HEF1</sub>, p105<sub>HEF1</sub> and p115<sub>HEF1</sub>) can result from a single cDNA expressed <i>vivo</i> in a cell cycle-dependent manner. p105<sub>HEF1</sub> and p115<sub>HEF1</sub> represent different phosphorylation states of the full length HEF1 and are more predominantly cytoplasmic whereas p55<sub>HEF1</sub> arises through cleavage of the full length HEF1 during mitosis [17,22]. HEF1 has been implicated in many pathways including the signaling pathway of integrin, T-cell antigen receptor (TCR), B-cell receptor (BCR), the G protein coupled calcitonin receptor, cell adhesion as well as in the progression of the cell cycle through mitosis [22-26]. HEF1 has also recently been described as an apoptotic mediator at focal adhesion sites [21]. However the exact nature of the signaling events associated with HEF1 is still unknown. In TGF-β induced signaling events mediated by Smad3, it has been shown that Smad3 interacts with both the N-terminus and the C-terminus domains of HEF1 via its MH1 and MH2 domains respectively [17]. Such an interaction appears to trigger rapid proteasomal degradation of HEF1. A similar example is the ability of Smad3 to bind to the nuclear transcriptional co-repressor SnoN, which is also degraded by the proteasome pathway upon binding to Smad3 [18,19].

The 26S proteasome is a large proteolytic complex present both in the nucleus and the cytosol of eukaryotic cells. Most known proteasome substrate proteins require poly-ubiquitination to be targeted to the proteasome for degradation. Ubiquitination occurs through the sequential action of three enzymes [27]. First, the activating enzyme E1 activates ubiquitin. Ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme or Ubc. Finally, an ubiquitin protein ligase or E3 enzyme covalently attaches the ubiquitin to lysine residues present on the substrate protein. There are several classes of E3 ligase which appear to be responsible for substrate selectivity by binding directly or indirectly to the protein substrate: 1) N-end rule E3s; 2) the HECT (Homologous to E6-AP C-terminalis) domain family which includes the Smurf family and 3) the RING finger containing E3s. The latest are often part of a large protein complex such as the SCF (Skp1 cullin E-box protein) or the APC (Anaphase-promoting complex) which both belong to the cullin subfamily [28].

The mechanism of Smad3-binding-dependent proteasomal degradation of HEF1 is unknown. For SnoN, it has been shown to involve at least two different mechanisms. First, Smad3 has been shown to recruit the HECT family E3 ligase Smurf2 to regulate SnoN ubiquitination [18]. Smad3 has also been shown to recruit the APC complex to regulate SnoN degradation [18,19]. The APC or cyclosome is a cell cycle-regulated complex composed of at least eleven subunits in mammalian cells, APC11 being the RING finger containing subunit. It is required for the metaphase-anaphase transition and cyclin degradation. Two
Smad proteins (Smad1, Smad2, Smad3, Smad4) were transiently expressed in 293 cells together with T7-tagged APC10. The Smads were immunoprecipitated with anti-HA antibody and the co-precipitated T7-tagged APC10 was detected by Western blot using anti-T7 antibody. T7-APC10 was detected to co-precipitate with Smad2, Smad3, but not with Smad1 or Smad4 (Fig. 1A). Thus, the interaction between Smad3 and APC10 is specific and can occur in mammalian cells.

Next we tested the interaction between APC10 and the Smad proteins by GST pull-down assay using GST-APC10 expressed and purified from Escherichia coli BL21 and Flag-tagged Smads expressed in 293 cells. In this assay, APC10 again binds to the two R-Smads involved in the TGF-β signaling pathway, Smad2 and Smad3 (Fig. 1B, lane 2 & 3), but not with the Co-Smad, Smad4 (Fig. 1B, lane 4).

The interactions were further tested via in vitro binding assays. 35S-labeled in vitro translated Smad3 or APC10 proteins were incubated with GST-APC10 or GST-Smad3, respectively. GST alone was used as a negative control. 35S-labeled Smad3 was detected to bind GST-APC10 (Fig. 1C, lane 6); 35S-labeled APC10 was detected to bind GST-Smad3 (Fig. 1C, lane 5). Labeled HEF1 also binds to GST-Smad3 (Fig. 1C, lane 4). These data suggest that Smad3 can interact with both HEF1 and APC10 in vitro, most likely via direct interaction.

Smad3 interacts with APC10 via Smad3 MH2 domain and requires both N- and C-terminus of APC10

To understand how APC10 interacts with Smad3, we carried out deletion analyses to determine the domains of interaction on both Smad3 and APC10. First, GST pull down assays were carried out to test the interaction between purified GST-APC10 and Flag-tagged Smad3 and Flag-tagged Smad3 deletion mutants expressed in 293 cells. The tested Smad3 deletion mutants include Smad3NL, Smad3LC, Smad3 MH1 (Smad3N) and Smad3 MH2 (Smad3C) (Fig. 2A, top panel). Only Smad3LC and Smad3C exhibited the ability to bind APC10 (Fig. 2A, bottom left panel, lanes 2 & 4). The interaction was also tested in a mammalian overexpression system 293 cells, which were co-transfected with T7-APC10 and Flag-Smad3 deletions. APC10 was immunoprecipitated from the cell lysates and the co-precipitated Smad3 and its deletion mutants were detected by Western blot with anti-Flag antibody. Again, only Smad3LC and Smad3C were detected to co-precipitate T7-APC10 (Fig. 2A, bottom right panel, lanes 7 & 9). Thus, the MH2 domain of Smad3 is necessary and sufficient to bind APC10, although a possible role of the linker region of Smad3 in assisting the interaction between Smad3 and APC10 is not ruled out.
Further experiments were carried out to determine Smad3 binding domain on APC10. A set of deletion constructs of APC10 (Fig. 2B, top panel) were made and tested for expression in 293 cells (Fig. 2B, bottom left panel). All of the three N-terminal deletion constructs (D2, D3, & D4) and one C-terminal deletion construct (D7) failed to express stable proteins (Fig. 2B, bottom left panel, lanes 2–5), while two C-terminal deletion constructs (D8 & D9) were expressed (Fig. 2B, bottom left panel, lanes 6 & 7). Thus, the N-terminal 41 amino acids and an internal domain (a.a.60–a.a 82) play roles in maintaining the stability of APC10. Both APC10 D2 and APC10 D9, when expressed in yeast as fusion proteins of B42, failed to bind the LexA fusion protein of Smad3 in the yeast-two hybrid system.

Figure 1
Smad3 interacts with APC10 specifically. (A) Co-immunoprecipitation of Smad3 and APC10 from mammalian overexpression system. 293 cells were transiently transfected with T7-APC10 and Smad proteins as indicated. The cell lysates were subjected to Western blot analyses directly or immunoprecipitation followed by Western blot as indicated. (B) Smad3 binds to APC10 in GST pull down assays. Flag-tagged Smad constructs were transfected into 293 cells and tested against GST-APC10 purified from E. Coli. Total input Smad protein levels (100 ug) were detected by immunoblotting of the cell lysates with anti-Flag antibody (top panel). Smad proteins bound to the GST-APC10 beads were eluted and analyzed by immunoblotting with anti-Flag antibody (middle panel). As a control, F-Smad proteins were incubated with the GST alone (bottom panel). (*) We noted that GST-APC10 was recognized by the anti-Flag antibody on Western blot. (C) Smad3 binds to HEF1 and APC10 in in vitro binding assay. In vitro translated 35S-labeled HEF1, 35S Smad3 and 35S APC10 were incubated with GST-Smad3, GST-APC10 or GST as indicated. The in vitro translated proteins before and after binding were detected by electrophoresis and autoradiography.
Domain mapping studies of the interaction between Smad3 and APC10 reveal distinct domains involved in binding. (A) The Smad3C (MH2 domain) is necessary and sufficient for binding to APC10. Top panel: a cartoon to illustrate the deletion constructs of Smad3. The APC10 binding activities of each truncated Smad3 as detected by assays in the bottom panels are summarized at the right side of the panel. Bottom left panel: MH2 domain is necessary and sufficient for Smad3 to bind APC10 in GST pull down assay. Flag-tagged full-length or truncated Smad3, as indicated, was transfected into 293 cells and tested against GST-APC10 and GST (negative control). Bottom right panel: MH2 domain is necessary and sufficient for Smad3 to bind APC10 in 293 cells. Different Smad3 truncations tagged with Flag were co-transfected with T7-APC10 into 293 cells. The expression of these proteins was detected by Western Blot using anti-Flag and the interaction between APC10 and Smad3 truncations was detected by immunoprecipitation of T7-APC10 followed by Western blot using anti-Flag. "L.C." represents the antibody light chain. (B) The C-terminal domain of APC10 is necessary for binding to Smad3. Top panel: a cartoon that illustrates the deletion mutants of APC10. The top three deletion constructs are amino-terminal deletion mutants. The number of amino acids deleted in each construct is indicated. For example, the D2 construct lacks the N-terminal 41 amino acids, thus it is also labeled as D41N. Bottom left panel: the six deletion constructs of APC10, each tagged with Flag, were transfected into 293 cells. The expression of these deletion mutants was detected by Western blot using anti-Flag. Stable protein expression was detected only in cells transfected with two C-terminal deletion constructs (D8 and D9). Bottom right panel: the C-terminal 66 amino acids of APC10 are required for Smad3 binding. T7-tagged APC10 and Flag-tagged APC10 D9 were transfected into 293 cells. Cell lysates were subjected to immunoblotting with anti-T7 (lane 1) and anti-Flag (lane 2) antibody. APC10 and APC10 D9 were tested against GST-Smad3 (lanes 3 & 4) and GST alone as a control (lanes 5 & 6).
system (data not shown). GST pull-down assay using GST-
Smad3 fusion protein against APC10 D9 confirmed the
defect of this deletion mutant of APC10 in binding to
Smad3 (Fig. 2B, bottom right panel, lane 4). These studies
revealed that the C-terminal 66 amino acids of APC10 is
required for Smad3 interaction while the N-terminal 82
amino acids of APC10 is required for the protein stability.

**Different subdomains within the MH2 domain of Smad3 are involved in binding to APC10 and HEF1**

In order to map more precisely the domain on Smad3
necessary for APC10 binding, we tested several Smad3
deletion mutants within the MH2 domain against GST-
APC10. In addition, we also tested the binding property of
a Smad3/Smad1/Smad3 chimera, which contains N-ter-
minus a.a.1–236 and C-terminus a.a. 277–424 but with
part of the MH2 region of Smad3 (a.a. 237–276) replaced
with the co-responding region of Smad1 (a.a. 276–317),
as previously described [18]. Since Smad1 does not inter-
act with APC10 in this assay system, any binding to
APC10 is due to the Smad3C portion of the chimera.
These deletion constructs of Smad3 are illustrated in Fig-
ure 3A. These deletion constructs were transfected into the
293 cells and the expression of each protein was moni-
tored by Western blot analyses with anti-Flag monoclonal
antibody, since they are all tagged with the Flag epitope
(Fig. 3B, top panels). The cell lysates were incubated with
purified GST-APC10 proteins, or control GST proteins
both of which were absorbed onto Glutathione Sepharose
4 Fast Flow beads. The bead bound proteins were eluted
and analyzed by Western blot (Fig. 3B, middle and bot-
tom panels). Smad3 d2, which contains a deletion of the
C-terminal a.a.362–424, still binds strongly with APC10
(Fig. 3B, lane 1). Further deletion of a.a.330–362 (Smad3
d6) led to a significant reduction of interaction (Fig. 3B,
lane 3), and more deletion of a.a. 301–330 (Smad3 d4)
further weakened the interaction (Fig. 3B, lane 2). Densi-
tometry analyses of the protein signals were used to calcu-
late the relative percent of APC10-bound proteins in total
input proteins, by dividing the protein signals in GST-
APC10 panel by those in lysates panel. The derived rela-
tive percentages are indicated at the top right side of Fig.
3B. An abrupt drop of percentage occurs between Smad3
d2 (95%) and Smad3 d6 (34%), with an additional small
reduction between Smad3 d6 (34%) to Smad3 d4 (17%).
These data suggests that the amino acid residues 301 to
362, especially 301 to 330, are important for Smad3/
APC10 binding. The Smad3/Smad1/Smad3 chimera
(20%) exhibited much reduced binding ability compared with
Smad3 d2, suggesting that the region of Smad3
replaced by Smad1, a. a. 237 to 276, is also critical for
APC10 binding. Taken all these data together, we have
mapped residue 237 to 362 within Smad3MH2 to be
involved in APC10 binding.

Since Smad3 MH2 domain has also been shown to bind
HEF1 [17], we decided to compare Smad3 interaction
with both APC10 and HEF1, in order to better understand
the way APC10, Smad3 and HEF1 form a potential ternary
complex. Thus, the same set of cell lysates from 293 cells
transfected with Smad3 deletion constructs were tested
against bead-bound, purified GST-HEF1 and GST, the
later served as a negative control. The bead-bound pro-
teins were eluted and detected by Western blot (Fig. 3C,
middle and bottom panels). The percentage of bound-
protein signals over the lysate protein signals was also
calculated and presented at the top right side of Figure 3C.
The interaction between Smad3d2 with GST-HEF1 (21%)
is not as strong as the interaction between Smad3d2 and
GST-APC10 (95%). Also, there is almost no difference
between Smad3 d2 (21%) and Smad3 d6 (23%) in bind-
ing to GST-HEF1. Thus, the Smad3 MH2 region between a.
a. 330 to 362 is not critical for HEF1 interaction. How-
ever, there is a major difference between Smad3d6 (23%)
and Smad3 d4 (13%) in binding to HEF1, suggesting that
the MH2 region between a.a.301 and 330 is very impor-
tant for Smad3 interaction with HEF1. As for the Smad3/
Smad1/Smad3 chimera, its binding to HEF1 (24%) is as
strong as that for Smad3 d2 (21%), thus suggesting that
the region between a.a. 236 and 276 is not involved in the
interaction between HEF1 and Smad3. By comparing the
different interaction properties of these Smad3MH2
domain mutants exhibited during their binding to APC10
and HEF1, we concluded that different subdomains
within Smad3 MH2 domain are involved in Smad3 bind-
ing to APC10 and HEF1. For binding to APC10, the entire
region between residues 237 to 362 might be involved
(Fig. 3B), while a more localized region between residues
301 to 330 appears to bind HEF1 (Fig. 3C). Combined
with the information reported in our previous studies
[17], we propose a ternary complex formation between
HEF1, Smad3 and APC10, as illustrated in Fig. 3D.

**The CDH1 protein in APC complex binds to HEF1**

To be ubiquitinated and thus degraded by the proteasome
pathway, APC substrates need first to be recognized by
either CDC20 or CDH1 which targets the ligase to specific
substrates during metaphase-anaphase transition as well as
during late anaphase, respectively [29,35,36]. Recent
studies have demonstrated the direct binding between
APC substrates and CDC20 or CDH1 [37]. CDH1 has also
been shown to bind to the other Smad3 interactor SnoN
in Smad3-regulated SnoN ubiquitination [18,19]. We
thus also examined whether CDH1 interacts with HEF1,
by the direct binding test *in vitro*. Either GST-HEF1 or GST
alone as a control were incubated with *in vitro* translated
35S-labeled CDH1. As shown in Figure 4A, *in vitro* translated
CDH1 binds to GST-HEF1, suggesting a possible
role of CDH1 in targeting HEF1 for APC-mediated ubiqui-
utination of HEF1.
Smad3 MH2 domain contains overlapping but distinct binding sites for HEF1 and APC10. (A) A Cartoon to illustrate the deletion constructs of Smad3 (Smad3d2, d4 and d6) as well as a hybrid protein S3/S1/S3. (B) GST pull-down assay to map APC10 binding site on Smad3C. Flag-tagged Smad3 deletion mutants were transfected into 293 cells and the cell lysates were tested against GST-APC10. Cell lysates (top panel) and proteins bound to the beads (middle and bottom panels) were analyzed by Western blot with anti-Smad1/2/3 from Santa Cruz. The percentage listed at the right side of the listed constructs is derived from dividing the signals (measured by ImageQuant) of bound proteins in the middle panel by the signals of lysate proteins in the corresponding lane of the top panel and then times 100 percent. (C) GST pull-down assay to map HEF1 binding site on Smad3C. Same as in (B), with GST-APC10 replaced by GST-HEF1. (*) endogenous Smad1 and Smad3 recognized by anti-Smad1/2/3 antibody. (D) A cartoon to illustrate the complex of Smad3, HEF1 and APC10 and their potential interaction with the APC ligase core complex.
Figure 4
CDH1 interacts with HEF1 at the HEF1 C-terminal M2 domain. (A) In vitro binding test. In vitro translated $^{35}$S labeled CDH1 was incubated with GST-HEF1 or GST alone as a control in modified lysis buffer. $^{35}$S labeled CDH1 was separated by electrophoresis and detected by autoradiography. (B) A cartoon that illustrates putative D boxes on HEF1. (C) A cartoon that illustrates the deletion constructs of HEF1 used in (D). (D) Co-immunoprecipitation of HEF1/HEF1 deletions with CDH1 in 293 cells. Full-length HEF1 and T7-tagged HEF1 deletions were co-transfected with myc-tagged CDH1 into 293 cells. Myc-CDH1 was immunoprecipitated with anti-myc antibody and CDH1 bound HEF1 or HEF1 deletions were detected by immunoblot with either anti-p130Cas antibody (top panel, lanes 1 & 2) or anti-T7 antibody (top panel, lanes 3–5). The expression level of CDH1 was detected with anti-myc antibody (second panel). The amount of HEF1 (third panel) or T7-HEF1 deletions (bottom panel) expressed was detected by anti-p130Cas for HEF1 or anti-T7 antibody. (E) A cartoon that illustrate the putative complex of HEF1, Smad3, APC and CDH1.
CDH1 binds to HEF1 C-terminal M2 domain containing two D boxes

Upon examining HEF1 amino acids sequence, we found five putative D box [RxxLxxxx(N)]. These putative D boxes are located within the SH3 domain (box 1), the SH2 binding sites domain (box 2), after the Ser rich domain (box 3) and within the M2 domain of HEF1 (box 4 & 5) (Fig. 4B). D box and KEN motif are the two motifs recognized by CDH1 on APC substrates [30].

To determine which D box interacts with CDH1, full-length HEF1 as well as T7-tagged HEF1 deletions, as illustrated in a cartoon in Figure 4C, were co-transfected with myc-tagged CDH1 into 293 cells. Cell lysates were subjected to immunoprecipitation using anti-myc antibody. Full-length HEF1, HEF1 d113 (114–834) and HEF1 M2 domain (654–834) were found to bind to CDH1 (Fig. 4D, top panel, lanes 2, 4, & 5). HEF1 1–154 did not show the ability to bind to CDH1 (Fig. 4D, top panel, lane 3). Therefore, we predict that CDH1 binds to a D box located within the M2 (C-terminal) domain that contains two putative D box, one located between residues 705 and 714 within the HLH motif, the other between amino acids 826 and 834. Since CDH1 is a substrate recognition component for APC ligase complex, the ability of CDH1 to bind to HEF1 suggests that HEF1 is a substrate for the APC ligase. The detected interactions between Smad3, HEF1, APC10 and CDH1 is illustrated in a cartoon in Figure 4E.

Smad3 interaction with APC10 is regulated by TGF-β1 receptor activation while CDH1 interaction with HEF1 is constitutive

To further test the effect of TGF-β receptor activation on the interaction between Smad3 and APC10 or between CDH1 and HEF1, these proteins were expressed in 293 cells in the presence or absence of a constitutively activated TGF-β1 type I receptor R4T204D (R4TD). As shown in Figure 5A, APC10 was detected only in the immunoprecipitates of Smad3 from cells with the coexpression of both Smad3 and R4TD (Fig. 5A, lane 6). We noted that anti-Smad3 precipitates two forms of Smad3 in lane 6 while only one form of Smad3 in lane 5. The higher molecular weight form is a phosphorylated version of Smad3, as confirmed by Western blot with anti-phospho-

To confirm a functional role of CDH1 in proteasomal degradation of HEF1, we also tested the effect of CDH1 on HEF1 protein level, in the presence or absence of Smad3 and APC10, in 293 cells. Like APC10, overexpression of CDH1 alone reduced the protein levels of both forms of HEF1 (Fig. 6B, compare lane 5 with lane 11). The coexpression of the constitutively activated TGF-β type 1 receptor mutant R4T204D (R4TD) caused further reduction of the p115HEF level in the absence of overexpressed CDH1 or APC10 (Fig. 6B, compare lanes 11 & 12), but such a reduction was not very apparent when CDH1, Smad3, or APC10 was co-expressed either alone or in different combinations (Fig. 6B, compare lanes 1 & 2, 3 & 4, 9 & 10 in top panel). The coexpression of Smad3 with CDH1 and HEF1 caused significant reduction of HEF1 protein levels, as expected, but also did not alter the complex formation of CDH1 and HEF1 (Fig. 5A, lanes 11 & 12). When HEF1 was coexpressed with CDH1, APC10 and the MH2 domain of Smad3, which harbors the binding sites for both HEF1 and CDH1, we detected at four proteins in the immunoprecipitates of HEF1 (Supplementary Fig. 1). These data further confirms the complex formation of Smad3, HEF1, CDH1 and APC10 in mammalian cells and also suggest that TGF-β signaling may regulate the complex formation via regulating the interaction between Smad3 and APC10.
TGF-β type I receptor activation enhances Smad3 interaction with APC10 but does not alter CDH1 interaction with HEF1. (A) Smad3 interaction with APC10 is positively regulated by the activation of TGF-β type I receptor. Flag-Smad3 and T7-APC10 were co-expressed in the presence or absence of a constitutively active TGF-β type I receptor mutant R4T204D (R4TD). The interaction between Smad3 and APC10 was detected by immunoprecipitation of Smad3 with an anti-Smad3 polyclonal antibody followed by Western blot with anti-T7 (Top panel, lanes 4–6). The immunoprecipitated Flag-Smad3 was detected by anti-Flag antibody (middle panel). The expression of R4TD was detected by anti-TGF-βRI polyclonal antibody from Santa Cruz (Bottom panel). R4TD-p represents a potential cleavage product of R4. (B) HEF1 interaction with CDH1 is not regulated by TGF-β type I receptor activation. The 293 cells were transiently transfected with myc-CDH1 and HEF1 as indicated. The interaction between HEF1 and CDH1 was detected by immunoprecipitation with anti-p130Cas followed by Western blot with anti-myc (Top panel, lanes 7–13). The expression of myc-CDH1 and Flag-Smad3 was detected by Western blot with anti-myc and anti-Flag (Top panel, lanes 1–6). The expression levels of HEF1 were detected by anti-HEF1 (middle panel) and the levels of R4TD detected by Western blot with anti-TGF-βRI antibody (bottom panel).
Both APC10 and CDH1 can alter the steady state protein levels of HEF1 (A) Overexpressed APC10 caused the reduction of the steady-state protein levels of HEF1 and such an activity is compromised in a deletion mutant of APC10 (APC10 D8) that is defective in binding to Smad3. HEF1 was co-transfected with ubiquitin, Flag-Smad3, T7-APC10 or T7-APC10 deletion mutant APC10 D8. The expression level of HEF1 was detected by Western blot using anti-p130Cas antibody, as shown in the top panel; the expression of Smad3, APC10 and APC10D8 were detected by Western blot using anti-Flag and anti-T7, as shown in the bottom panel. (B) CDH1 enhances Smad3-regulated HEF1 degradation. HEF1 was co-expressed in different combinations with myc-CDH1, T7-APC10, Flag-Smad3 and R4T204D (R4TD), a constitutively active type I receptor in 293 cells. The steady state levels of HEF1 were detected by anti-HEF1 antibody (top panel) and the expression levels of Smad3 and APC10 were detected with anti-Flag and anti-T7 antibodies, respectively (second and third panels), while the expression levels of myc-CDH1 were detected by anti-Myc (fourth panel). To detect R4-TD, cell lysates were blotted with anti-TGF-β RI antibody (bottom panel).
When CDH1 was co-expressed with Smad3, or with Smad3 and APC10, the p105 HEF1 form appeared to be less reduced (Fig. 6B, compare lane 5 with lanes 7, 8 & 9). These data pointed out that Smad3, APC10 and CDH1 are all capable of altering the protein levels of HEF1 when they are coexpressed with HEF1.

Discussion

HEF1 has been implicated in many different signaling pathways such as those mediated by integrin, TCR and BCR where it plays the role of an adaptor protein. Recent studies have shown that HEF1 degradation is regulated by Smad3 via the proteasomal degradation pathway and is further enhanced by TGF-β stimulation [17]. These findings reveal a novel ability for Smad3, which has been primarily considered as a DNA-binding transcriptional factor, and also suggest a novel cross-talk mechanism between TGF-β/activin pathways and multiple HEF1-involved pathways. The molecular mechanisms underlying Smad3-regulated proteasomal degradation are not clear. Our current studies revealed the ability of Smad3 to bind to APC10, which is a regulatory component of the APC ligase core complex required for substrate interaction, as well as the ability of HEF1 to bind to CDH1, which is a co-activator of APC ligase for specific substrate recognition. The interaction between Smad3 and APC10 is subjected to the regulation by the TGF-β type I receptor, while the interaction between CDH1 and HEF1 is constitutive. Both APC10 and CDH1 exhibit the ability to regulate the steady state levels of HEF1 upon co-expression with HEF1. These data suggest a novel mechanism for Smad3 to regulate the proteasomal degradation of HEF1 via assisting the recognition of HEF1 by the APC E3 ligase.

The interaction between APC10 and Smad3 was first observed in the yeast two-hybrid system. This interaction was confirmed in mammalian over-expression system by co-immunoprecipitation, then by in vitro GST pull-down assay, and finally via in vitro binding assays to demonstrate a potential direct interaction between Smad3 and APC10. Domain mapping studies showed that Smad3 MH2 domain is necessary and sufficient to bind APC10, whose C-terminal domain is required for Smad3 binding, while the N-terminal domain for APC10 stability. These data strongly suggested a direct and domain-specific interaction between Smad3 and APC10. Previously Smad3 has been shown to recruit APC complex to ubiquitinate its nuclear interactor SnoN, but it was not clear how Smad3 recruits APC complex [18,19]. The ability of Smad3 to bind directly to APC10 suggests that this interaction could be the missing link for Smad3 to recruit APC complex in the ubiquitination of SnoN. Future studies will be carried out to directly test this possibility.

Our previous studies have shown that Smad3 interlocks with HEF1, with the MH1 domain binds to the N-terminal domain of HEF1, while the MH2 domain of Smad3 binds to the C-terminal domain of HEF1 [17]. Considering the involvement of the MH2 domain of Smad3 in binding to APC10, we compared the ability of various deletion constructs of Smad3 MH2 domain in their ability to bind to APC10 and HEF1. These studies showed that residues located within 237 to 362 on Smad3 are involved in Smad3 binding to APC10, while a more localized region between residues 301 to 330 is involved in binding to HEF1. These data suggests that Smad3, APC10 and HEF1 can potentially co-exit in one single complex.

Since APC10 is known to play a role in regulating substrate recognition and ubiquitination by mammalian APC [Hartmut C. et al., Current Biology 13:1459, 2003] the interaction between Smad3, APC10 and HEF1 suggests that HEF1 and Smad3 are potential ubiquitination substrates for APC ligase. Our finding of the direct interaction between HEF1 and the WD40 repeat protein CDH1 further qualifies HEF1 as a substrate for APC, since interaction with CDH1 or CDC20 has been shown to be a prerequisite for APC substrates [37]. The role of APC10 and CDH1 in regulating HEF1 degradation was confirmed by the ability of overexpressed APC10 and CDH1 in enhancing Smad3-regulated HEF1 degradation in 293 cells (Figure 6). The physical interaction between HEF1 and CDH1, a regulator at late anaphase of cell cycle, could be functionally linked to a previous observation of the ability of a processed form of HEF1, p55HEF1, in interaction with mitotic spindles [22]. The exact role of HEF1 in cell cycle regulation and how Smad3, via interacting with the co-activators of APC complex to regulate such a role is also an important subject for future studies.

While Smad3 can regulate the proteasomal degradation of both HEF1 and SnoN, phosphorylation of Smad3 by the activated type I receptor is required only for SnoN degradation but not for HEF1 degradation [17-19]. The mechanism for such a difference warrants future investigation. One apparent difference between HEF1 and SnoN is their intracellular localization. While HEF1 is predominantly cytoplasmic, SnoN is primarily a nuclear protein. It has been shown that the inactive Smad3 is primarily cytoplasmic, and its nuclear translocation is triggered upon its phosphorylation by the type I receptor of TGF-β at the C-terminal SSVS motif. Thus, the dependence of SnoN degradation on Smad3 phosphorylation at the SSVS motif could be at least partially due to the dependence of Smad3 phosphorylation for Smad3 accumulation into the nucleus. However, a recent study (Feng L. et al., manuscript submitted) indicated that the in vitro translated Smad3 can induce HEF1 degradation in an in vitro degradation assay, which does not involve the issue of nuclear...
localization, thus suggesting that additional component(s) involving phosphorylation-dependent structural changes of Smad3 may be involved in regulating SnoN ubiquitination and degradation. One such structural change has been recently revealed. Previously it was shown that inactive unphosphorylated Smad3 is bound to proteins such as SARA (Smad anchor for receptor activation) in the cytoplasm and that phosphorylation of Smad3 by the type I receptor decreases its affinity for SARA thus allowing Smad3 to interact with the Co-Smad Smad4 before entering the nucleus [38]. In a recent study, molecular details were revealed regarding the interaction between inactive Smad3, SARA and the TGF-β type I receptor. The inactive Smad3 exists in a monomer form in a complex with SARA and the TGF-β type I receptor, at the cell membrane or at early endosome [39,40]. The phosphorylation of Smad3 releases it from SARA and allows Smad3 to adopt a different conformation that favors trimer formation. Interestingly, the later conformation is preferred by the nuclear oncoprotein Ski [41]. Thus, SARA serves as a molecular guardian of Smad3 to prevent it from forming aberrant trimmers for constitutive activation. Therefore, one possible explanation for the dependence of Smad3 phosphorylation for SnoN degradation, is that SnoN, like Ski, only interacts with Smad3 in oligomers, which could be either Smad3 homo- trimmers or Smad3 (2)/Smad4 (1) hetero-trimmers, and that the ubiquitination of SnoN occurs in such a complex. For HEF1, since neither the interaction nor the degradation is dependent upon Smad3 phosphorylation, we therefore consider it likely that Smad3 binds to HEF1 in monomeric form before its phosphorylation and that such a complex is sufficient for assisting HEF1 ubiquitination and degradation. It also remains a possibility that HEF1 could function like SARA to keep Smad3 in an inactive conformation, thereby assisting Smad3 recognition by the type I receptor kinase and directly regulating Smad3 trimer formation. Our preliminary data have suggested such an inhibitory role of HEF1 [17]. Future studies of the crystal structures of the complex of Smad3 and HEF1 in the presence or absence of TGF-β type I receptor cytoplasmic domain, as well as the structural studies of the complex of Smad3, HEF1 APC10 and CDH1, as demonstrated here, will significantly advance our current understandings of the mechanism and regulation of HEF1 degradation in TGF-β pathway.

The physiological regulation of the complex formation between Smad3, APC10, HEF1 and CDH1 is likely very dynamic and complex. Since all these observations are made via in vitro systems, we do not know the detailed regulation of these interactions in specific cell types under different conditions. The physiological complex formation between Smad3, HEF1 and APC10 is likely subjected to constant changes, depending upon the expression levels of these proteins. It is also possible that the formation of the complex involves sequential steps. For example, Smad3 could first bind to the APC10 before binding to HEF1 or vise versa and such binding could stabilizes Smad3 in a favorable conformation for subsequent interaction with other proteins, such as CDH1. The regulation of the formation of this complex could also via phosphorylation of HEF1 and Smad3 and via competition involving other interacting proteins of Smad3 and HEF1. Here we gain a glimpse of the complexity of the regulation of HEF1 ubiquitination and degradation in vivo. In 293 cells, we observed an enhancement effect of TGF-β receptor activation on Smad3 interaction with APC10, but not on HEF1 interaction with CDH1 (Fig. 5).

In Figure 7, a cartoon is presented to summarize our data and present a model for the role of Smad3 interaction with APC10 in recruiting APC complex for ubiquitination and degradation of HEF1, downstream of TGF-β type I receptor activation. In this model, we propose three possible ways for HEF1 to be ubiquitinated by APC ligase via the formation of the multimeric complex of Smad3, HEF1, APC10, CDH1, the latter two of which bring in the entire APC ligase. The first scenario is that the phosphorylated Smad3, released from SARA, forms a complex with APC10, while CDH1 interacts with HEF1 constitutively. The two complexes come together via Smad3 interaction with HEF1. APC10 assists CDH1 to bring Smad3-bound HEF1 to APC ligase for the subsequent ubiquitination. The second scenario is that HEF1 tethers Smad3 in a cytoplasmic complex and the type I receptor activation leads to phosphorylation of Smad3 and the subsequent conformational changes that can enhance its interaction with APC10, which recruits the APC ligase and assists CDH1 to bind the substrate HEF1. The third scenario is that monomeric unphosphorylated Smad3 and HEF1 forms a constitutive complex to further recruit APC10 and CDH1. Such a complex may mediate some basal level of constitutive ubiquitination of HEF1. We recognize that the current observations need to be followed up in more physiological relevant conditions, such that the physiological functions of the observed interactions between these proteins can be validated.

Conclusions

The studies presented here revealed a physical link between a key signal transducer of TGF-β (Smad3) and a component (APC10) of the cell cycle regulatory E3 ligase APC, and further suggest a molecular mechanism via which such a physical interaction contributes to the regulation of the protein stability of a multi-domain cytoplasmic docking protein HEF1, which is involved in a large network of signaling events. Since Smad3 interacts with many other nuclear and cytoplasmic proteins, this observation may have broad implications for the regulation of
Figure 7
A cartoon for possible signaling pathways that involve Smad3 interaction with APC10. A cartoon that illustrates possible pathways for Smad3-induced HEF1 degradation involving APC10 and CDH1. Please see text for details.
these other Smad3 interactors in a similar fashion. While previous studies of the APC complex have been primarily limited to cell cycle control, the observations reported recently extended the role of APC into non-cell cycle events associated with SnoN [18,19]. Our studies here provide the detailed link between Smad3 and APC and now add a cytoplasmic signaling adapter onto the list of APC substrates. The data imply that the cellular functions previously viewed as separate are intimately interwoven in a complex fashion: a cell cycle regulatory E3 ligase is physically and functionally connected to the vast signaling networks involving Smad3 and HEF1. Furthermore the biological consequences of the TGF-β-induced HEF1 degradation in lymphocytes where HEF1 is predominantly expressed have yet to be studied and their understanding could lead to new insights in the molecular events involved in the onset of complex diseases such as cancers.

Methods

Mammalian cell line

293 cells (human kidney cells transformed with adenovirus 5 DNA) were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco/BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS), 25,000 units of penicillin, 25 mg of streptomycin and 5 mL of 200 mM L-Glutamine at 37°C in the presence of 5% CO2.

Antibodies and reagents

Anti-myc (9E10), anti-Smad1/2/3 (H-2), anti-HEF1 (N-17) and anti-TGF-β RI (V-22) were purchased from Santa Cruz biotechnology. Anti-p130Cas monoclonal antibody was purchased from Transduction Laboratories. Anti-Flag was obtained from Sigma and anti-HA was purchased from Novagen. Anti-Flag was purchased from Transduction Laboratories. Anti-T7 (69522-4) was purchased from Novagen. Anti-Flag was purchased from Transduction Laboratories. Anti-p130Cas (1:1000), α-cdc27 (1:500), α-cdc16 (1:500), α-Flag (1:4000), α-T7 (1:1000), α-myc (1:1000), α-HA (1:1000) and α-TGF-β RI (1:4000).

GST pull-down assay

GST-APC10, GST-Smad3 and GST-HEF1 were expressed and purified from Escherichia coli strain BL-21. Briefly, the culture was induced at O.D. ~0.6 with 0.4 mM IPTG for 2 to 3 hours. Cells were collected by spinning at 5,000 rpm at 4°C for ~15 min. The pellet was then resuspended in Prep buffer (100 mM NaCl, 100 mM Tris-HCl pH 8.0, 50 mM EDTA, 2% TritonX-100) supplemented with 2 mM DTT and 1 mM PMSF. Lysis occurred using 10 µg/mL lysis buffer and debris was pelleted by spinning 30 min on ice and debris was pelleted by spinning 30 min at 14,000 g at 4°C. Cell lysates were then incubated with a 50% slurry of Glutathione Sepharose 4 Fast Flow (Amersham). Beads were then washed once using lysis buffer and 3 times with modified lysis buffer (lysis buffer containing 0.1% of TritonX-100). The precipitated proteins were eluted in 2× SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) + 10% β-mercaptopoethanol, loaded on SDS-PAGE and transferred onto PVDF membrane (Millipore). Membranes were analyzed by Western blotting [42]. Antibodies were diluted as followed: α-HEF1 (1:500), α-p130Cas (1:1000), α-cdc27 (1:500), α-cdc16 (1:500), α-Flag (1:4000), α-T7 (1:1000), α-myc (1:1000), α-HA (1:1000) and α-TGF-β RI (1:4000).

Immunoprecipitation and Western blotting

Cells were incubated 30 min on ice in HBS-Lysis buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% TritonX-100 supplemented with protease and phosphatase inhibitors just prior to use). Cell debris was pelleted by spinning in a microcentrifuge at 14,000 g at 4°C for 10 min and supernatant was saved for immunoprecipitation and western blot analysis. For immunoprecipitation, cell lysates were incubated with 2 µg of primary antibody for 2 h at 4°C followed by an additional 2 h-incubation with 40 µL of 50% slurry of protein G-sepharose 4 Fast Flow (Amersham). Beads were then washed once using lysis buffer and 3 times with modified lysis buffer (lysis buffer containing 0.1% of TritonX-100). The precipitated proteins were eluted in 2× SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) + 10% β-mercaptoethanol, loaded on SDS-PAGE and transferred onto PVDF membrane (Millipore). Membranes were analyzed by Western blotting [42]. Antibodies were diluted as followed: α-HEF1 (1:500), α-p130Cas (1:1000), α-cdc27 (1:500), α-cdc16 (1:500), α-Flag (1:4000), α-T7 (1:1000), α-myc (1:1000), α-HA (1:1000) and α-TGF-β RI (1:4000).

Abbreviations

- TGF-β: Transforming Growth Factor-β; TCR: T-cell receptor

Transfection

293 cells were transfected using the standard CaPO4 procedure [42] and cells were harvested 24 h after transfection into ice-cold phosphate-buffered saline (PBS).
- BCR: B-cell receptor
- HEF1: Human Enhancer of Filamentation I
- APC: Anaphase-Promoting Complex
- CDC20: Cell division cycle 20
- CDH1: Cdc20 homolog 1
- MG132: Nε-Benzylxocarbonyl-L-leucyl-L-leucyl-L-leucinal [Z-Leu-Leu-Leu-H]
- SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
- Mv1Lu cell: Mink Lung cell
- MH1, MH2 domain: Mad homology-1, -2 domain

Authors’ contributions

CN responsible for carrying out all key experiments, data collection and organizing the data into a thesis format. LM was in charge of the in vivo interaction tests of the interaction between Smad3 and APC10. XL carried out the initial yeast two-hybrid test to isolate APC10 as a Smad3 interactor. Mona Peng contributed to Figure 4D and Figure 5A, after CN left our lab. TW was responsible for providing advice for all the experiments, coordination between members who worked on this project and for the editing and submission of the text and figures.

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References

1. Roberts AB, Sporn MB: Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors 1993, 8:1-9.
2. Roberts AB, Sporn MB: Differential expression of the TGF-b isoforms in embryogenesis suggests specific roles in developing and adult tissues. Mol Reprod Dev 1992, 32:91-98.
3. Hogan BL: Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 1996, 10:1580-1594.
4. Whitman M: Smads and early developmental signaling by the TGFbeta superfamily. Genes Dev 1998, 12:2445-2462.
5. Massague J: TGF-beta signal transduction. Annu Rev Biochem 1998, 67:753-791.
6. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J: TGF beta signals through a heteromeric protein kinase receptor complex. Cell 1992, 71:1003-1014.
7. Massague J, Andres J, Attisano L, Chelletz S, Lopez-Casillas F, Ohtsuki M, Wrana JL: TGF-beta receptors. Mol Reprod Dev 1992, 32:59-104.
8. Attisano L, Wrana JL, Montalvo E, Massague J: Activation of signaling by the activin receptor complex. Mol Cell Biol 1996, 16:1066-1073.
9. Wieser R, Wrana JL, Massague J: GS domain mutations that constitutively activate T beta-R1, the downstream signaling component in the TGF-beta receptor complex. EMBO J 1995, 14:2199-2208.
10. Wang T, Li BY, Danielson PO, Shah PC, Rockwell S, Lechleider RJ, Martin J, Mangano T, Donahoe PK: The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell 1996, 86:435-444.
11. Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massague J: The TGF beta receptor activation process: an inhibitor- substrate-binding switch. Mol Cell 2001, 8:671-682.
12. Huse M, Chen YG, Massague J, Kuriyan J: Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell 1996, 96:425-436.
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7. Massague J, Andres J, Attisano L, Chelletz S, Lopez-Casillas F, Ohtsuki M, Wrana JL: TGF-beta receptors. Mol Reprod Dev 1992, 32:59-104.
29. Fang G, Yu H, Kirschner MW: Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell 1998, 2:163-171.

30. Pfleger CM, Kirschner MW: The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev 2000, 14:655-665.

31. Grossberger R, Gieffers C, Zachariae W, Podtelejnikov AV, Schleiffer A, Nasmyth K, Mann M, Peters JM: Characterization of the DOC1/APC10 subunit of the yeast and the human anaphase-promoting complex. J Biol Chem 1999, 274:14500-14507.

32. Wendt KS, Vodermaier HC, Jacob U, Gieffers C, Gmachl M, Peters JM, Huber R, Sondermann P: Crystal structure of the APC10/DOC1 subunit of the human anaphase-promoting complex. Nat Struct Biol 2001, 8:784-788.

33. Pravtcheva DD, Wise TL: Disruption of Apc10/Doc1 in three alleles of oligosyndactylism. Genomics 2001, 72:78-87.

34. Au SW, Leng X, Harper JW, Barford D: Implications for the ubiquitination reaction of the anaphase-promoting complex from the crystal structure of the Doc1/Apc10 subunit. J Mol Biol 2002, 316:955-968.

35. Schwab M, Lutum AS, Seufert W: Yeast Hct1 is a regulator of Cdc2 cyclin proteolysis. Cell 1997, 90:683-693.

36. Visintin R, Prına S, Amon A: CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. Science 1997, 278:460-463.

37. Pfleger CM, Lee E, Kirschner MW: Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex. Genes Dev 2001, 15:2396-2407.

38. Tsukazaki T, Chiangi TA, Davison AF, Attisano L, Wrana JL: SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell 1998, 95:779-791.

39. Qin BY, Lam SS, Correia JJ, Lin K: Smad3 allostery links TGF-beta receptor kinase activation to transcriptional control. Genes Dev 2002, 16:1950-1963.

40. Moustakas A, Heldin CH: From mono- to oligo-Smads: the heart of the matter in TGF-beta signal transduction. Genes Dev 2002, 16:1867-1871.

41. Liu X, Sun Y, Weinberg RA, Lodish HF: Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev 2001, 12:1-8.

42. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: Current Protocols in Molecular Biology Edited by: John Wiley & Sons. New York, NY; 1994.