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ORIGINAL PAPER

Regulation of Endocytic Sorting by ESCRT–DUB-Mediated Deubiquitination

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Abstract Endocytosis of cell surface receptors mediates cellular homeostasis by coordinating receptor distribution with downstream signal transduction and attenuation. Post-translational modification with ubiquitin of these receptors, as well as the proteins that comprise the endocytic machinery, modulates cargo progression along the endocytic pathway. The interplay between ubiquitination states of cargo and sorting proteins drives trafficking outcomes by directing endocytosed material toward either lysosomal degradation or recycling. Deubiquitination by specific proteinases creates a reversible system that promotes spatial and temporal organization of endosomal sorting complexes required for transport (ESCRTs) and supports regulated cargo trafficking. Two dubiquitinating enzymes—ubiquitin-specific protease 8 (USP8/Ubpy) and associated molecule with the SH3 domain of STAM (AMSH)—interact with ESCRT components to modulate the ubiquitination status of receptors and relevant sorting proteins. In doing so, these ESCRT–DUBs control receptor fate and sorting complex function through a variety of mechanisms described herein.

Keywords Endocytosis · Ubiquitin · Receptor · Protease · Deubiquitylase

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Introduction

Homeostatic regulation of receptor abundance at the plasma membrane is largely accomplished though endocytosis, and malfunctions along this pathway are associated with a plethora of pathologies, including cancer [1, 2]. Following their uptake into endocytic vesicles, cell surface receptors are sorted either to the lysosome for degradation or recycled back to the plasma membrane. Modification of receptor cargo by mono- and poly-ubiquitination constitutes a sorting signal for transport toward degradation [3–7]. These trafficking signals are interpreted and executed by the sorting machinery organized around the ESCRT complexes. Modification of specific ESCRT proteins by both phosphorylation and ubiquitination provides cellular control over functional complex assembly and disassembly [8, 9], thereby influencing cargo progression between endocytic sub-compartments.

Within the endocytic system, ubiquitin acts as a transferable interaction module that is recognized via low affinity interactions by various ubiquitin-binding domains (UBDs), including UIM, CUE, NZF as well as certain VHS and SH3 domains [10]. As discussed in greater detail elsewhere in this issue, ubiquitin is conjugated to a target protein via an isopeptide bond between the ε-amino group on an internal lysine (K) residue (or at the amino terminus) of the target protein and glycine (G)-76 of ubiquitin. The selective interaction of E3 ligases with their substrates allows for precise transfer of ubiquitin that confers specificity to the ubiquitination cascade [11–14].

Removal of ubiquitin moieties is accomplished by specific proteinases referred to as deubiquitylases (DUBs). As ubiquitination mediates a wide range of cellular processes, DUBs are implicated in diverse cellular pathways [15, 16]. This review focuses on the role of deubiquitination in

endocytic trafficking, with particular emphasis on the ESCRT-associated DUBs. The vast majority of DUBs conform to the paradigm of multifunctional modular proteins, encoding a catalytic domain as well as a variety of protein–protein interaction domains and motifs [17]. The non-catalytic modular protein interaction domains promote association of enzymes with substrate adaptors and scaffolds. Such interactions may drive specificity, allow regulation of catalytic activity and promote the assembly of larger multi-protein machines. The human genome encodes approximately 100 DUBs that comprise two classes and five families of proteolytic enzymes [18, 19]. A papain-like cysteine protease class includes four families: the ubiquitin C-terminal hydrolase (UCH), the ubiquitin-specific protease (USP), the ovarian tumor domain (OTU), and the Josephin domain (MJD) [18, 19]. JAB1/MPN/Mov34 metalloenzyme (JAMM) domain DUBs form the fifth family and represent the second class of DUBs belonging to the zinc-dependent metallopreoteases [15, 17, 20].

Protein ubiquitylation and subsequent recognition by UBDs act as a reversible means of assembling multi-protein complexes via ubiquitin-specific interactions [21, 22]. Inherent in this model is that these multi-protein complexes are controlled in part through the opposing signals supplied by the DUBs. This may be viewed as analogous to the recruitment of SH2 domain containing proteins to activated receptor tyrosine kinase complexes [23]. The recruitment and action of E3 ligases and DUBs thus controls both the spatial and temporal aspects of ubiquitin modification of target proteins, which in turn constitutes the basis for association/disassociation of UBD-dependent protein complexes. By controlling the reversible step in the reaction, DUBs determine the longevity of the modification and the disassembly of affected protein complexes and thus occupy an essential regulatory position.

Versatility of cellular deubiquitination extends beyond limiting or reversing ubiquitin conjugation to include active contribution to ubiquitin chain assembly and dynamics [24]. By virtue of their ability to selectively deconjugate specific ubiquitin chain linkages within poly-ubiquitin chains, DUBs promote chain editing or even alter the type of ubiquitin chain assembly. The OTU family DUB A20, downregulates nuclear factor-κB (NF-κB) activation through selective deconjugation of K63-linked ubiquitin chains from RIP1, modified downstream of TNFR1 [25]. A20 also contains Zinc Finger domains that comprise an E3 ligase specific for polymerization of K48-linked chains known to target modified substrates for proteasomal degradation [26]. The opposition between the ligase and DUB functionalities thus allows A20 to edit ubiquitin chains from protective K63- to K48-linked chains, thereby directing RIP1 proteolysis. In the Toll- and TNF-receptor pathways, K63-linked ubiquitination nucleates assembly of signaling complexes ultimately leading to activation of NF-κB transcription factors, so that A20 effectively acts to dampen signaling [26]. Consequently, targeted gene disruption of A20 in the mouse results in sustained activation of NF-κB and immune signaling hyperactivity [27, 28]. As chronic activation of the immune system is known to drive cancer, it is not surprising that A20 has also been described as a tumor suppressor for human B-cell lymphoma [26–29]. The phenomenon of DUBs acting to edit ubiquitin chains and promote the assembly of alternative chains is not limited to the Traff/A20 system, and an increasing number of DUBs have been reported to control both the extent as well as linkage of ubiquitin modification [18].

Thus, DUBs act as much more than simple "off" switches in opposing E3 ligases. Rather, they are critical participants in the spatial and temporal control of protein ubiquitination, playing essential roles in the disassembly of UBD/ubiquitin-mediated complexes through removal of ubiquitin as well as chain editing and linkage switching. In endocytosis, DUBs AMSH and USP8 interact with and deubiquitinate various cargo and ESCRT machinery proteins. Herein, we discuss the role of these ESCRT–DUBs as critical modulators of cargo trafficking and sorting machinery function.

ESCRTs and the Associated DUBs

Attenuation of signaling originating at the cell surface requires that the activated receptors be removed from the plasma membrane and either trafficked for degradation or recycled. Sorting of proteins destined for the lysosome is coordinated by reversible ubiquitination of both cargo and ESCRT proteins [30–32]. Ubiquitination of cargo has been reported to occur at various points along the endocytic pathway and generally serves to target cargo to the multivesicular body (MVB) compartment (Fig. 1) [33–38]. MVBs constitute morphologically distinct late endosomal structures that receive cargo in transit to the lysosome. Once committed for degradation, cargo is deubiquitinated and deposited into intraluminal vesicles, which are then released to fuse with the proteolytic organelle [39] (Fig. 1). Deubiquitination of endocytosed receptors either prior to or following their delivery into the MVB may therefore profoundly alter receptor trafficking, ultimately affecting substrate turnover rate.

At key trafficking control sites, specialized ESCRTs 0, I, II, and III decode ubiquitination signals and mediate consequent sorting events, appropriately directing cargo along the endocytic pathway [40–43]. These waypoints present effective opportunities for recruitment of regulatory proteins responsible for modulating sorting outcomes. Two

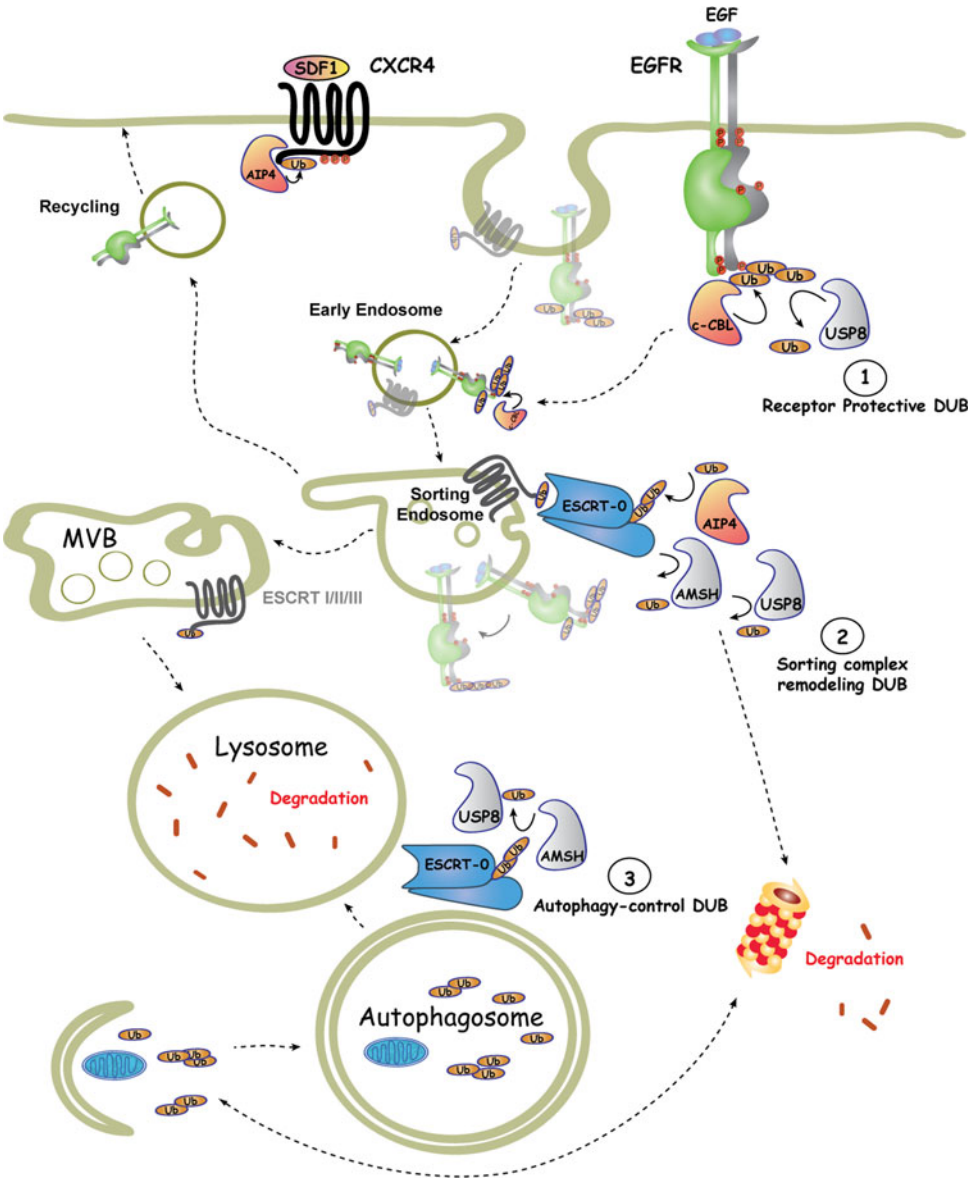

Fig. 1 Model of DUB orchestrated deubiquitination in endocytosis. The ESCRT–DUBs AMSH and USP8 physically interact with STAM proteins that together with Hrs form the ESCRT-0 complex. Both ESCRT–DUBs have been reported to interact with CHMP proteins that interact with ESCRT-III, and AMSH may directly interact with clathrin. These interactions allow the ESCRT–DUBs to regulate various steps in endocytic trafficking and progression. USP8 has been shown to directly deubiquitinate Receptor Tyrosine Kinase cargo acting to protect it from degradation. Both AMSH and USP8 appear to deubiquitinate ESCRT-0 components and in doing so act to regulate their assembly and function. This is manifest by changes in the sorting endosome. Finally, ESCRT–DUBs may play a role in autophagy, a parallel process that is also regulated by the ESCRT machinery

DUBs, AMSH and USP8, have been shown to associate directly with the early ESCRT-0 complex, consisting of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the adaptor protein signal transducing adaptor molecule (STAM) [34, 35, 38], as well as with chromatin-modifying protein (CHMP) components of the late ESCRT-III machinery [44–46]. Due to these associations, AMSH and USP8 will herein be collectively referred to as the ESCRT–DUBs. The JAMM metalloprotease AMSH preferentially cleaves K63-linked ubiquitin chains in vitro [47], suggesting a role for this DUB in non-proteasomal-targeting processes [48, 49]. Loss of AMSH activity is correlated with increased GPCR abundance under basal conditions with a concurrent loss of sensitivity to the coordinate ligand [50–52]. In agreement with this paradigm, we have recently shown that the GPCR CXCR4 is stabilized by loss of AMSH [38]. Similarly, AMSH appears to promote the movement of growth factor receptors through the sorting endosome and on to the MVB, as defects in AMSH result in EGFR accumulation [34]. Our studies on AMSH suggest that it constitutes a key positive regulator of cell surface receptor trafficking at the ESCRT-0 positive endosome through modulation of the Hrs/STAM ubiquitination status [38].

A cysteine protease DUB, USP8 shares a number of ESCRT-dependent interactions with AMSH, but exhibits distinct effects on receptor endocytosis. USP8 accumulates in response to growth stimulation and is downregulated under growth arrest conditions [53]. Targeted disruption of the USP8 gene in mouse embryonic fibroblasts causes progressive loss of growth factor receptors, including EGFR, c-Met, and ErbB3 [54]. Consequently, inducible

loss of USP8 results in gradual decrease in cellular proliferation, while its deficiency constitutes an embryonic lethal [54].

USP8 acts in endocytic trafficking by a number of different mechanisms that produce what at first glace appear to be contradictory phenotypes. USP8 deubiquitinates the δ-opioid receptor (DOR) and protease-activated receptor 2 (PAR_2) and is important for efficient turnover of these substrates [55, 56]. While CXCR4 does not appear to be a direct target for USP8, it is also stabilized in response to depletion or catalytic inactivation of the DUB [34]. Contrary to its roles in GPCR trafficking, deubiquitination of EGFR by USP8 protects the receptor from lysosomal proteolysis [35] and prolongs relevant signaling cascades [57]. Collectively, these results may be explained by the ability of USP8 to act on multiple substrates that impinge differently on the fate of various cargoes. In the case of EGFR, ubiquitination of the receptor at the plasma membrane and on early endosomes serves as a trafficking cue directing it to the MVB, which USP8 directly opposes. Conversely, PAR_2 requires deubiquitination by USP8 to proceed toward degradation, while DOR is subject to this DUB's activity following endocytic sorting. In all of these cases, the receptor constitutes the substrate, but de-ubiquitination elicits an outcome specific to that receptor's trafficking pathway. By contrast, CXCR4 is likely regulated indirectly through deubiquitination of the ESCRT-0 complex, without which trafficking stalls and receptor degradation is reduced.

The ESCRT–DUBs exhibit certain overlapping functions, yet as is evident from the contrasting effects of USP8 and AMSH manipulation on CXCR4 and EGFR stability, they clearly also fulfill distinct roles. In the CXCR4 system, AMSH depletion affords roughly the same degree of total receptor accumulation as loss of USP8 [34]. However, a key difference implicating the two DUBs in distinct aspects of CXCR4 trafficking and distribution is revealed through the comparison of surface receptor levels. By contrast to the effects observed with USP8 ablation, where the accumulated receptor resides largely on the cell surface [34], a substantial portion of CXCR4 stabilized due to AMSH depletion resides intracellularly [58]. Taken together with the opposing effects of USP8 and AMSH on EGFR trafficking and stability, these observations support non-redundant functions of the ESCRT–DUBs with respect to receptor sorting at the ESCRT-0-positive endosome.

ESCRT–DUB Interactions

A number of clues to the broad roles of the ESCRT–DUBs can be gleaned from their modular organization. These domains and motifs promote a variety of protein–protein interactions that advance the relevant cellular functions of the ESCRT–DUBs.

AMSH contains a N-terminal microtubule interacting (MIT) domain, a nuclear localization sequence, a clathrin binding domain (CBD), a single consensus P-X-V/I-D/N-R-X-X-K-P (RXXK) peptide motif and a catalytic JAMM domain. The AMSH CBD, located between amino acid residues 139 and 171 binds the terminal domain of the clathrin heavy chain [59]. This interaction promotes AMSH recruitment to early endosomes [44, 60], although AMSH may also be recruited there indirectly through an association with Hrs [61]. The endosomal clathrin coat may thereby act as a scaffold increasing the efficiency of AMSH-mediated deubiquitination of cargo sorted by the Hrs/STAM complex [59].

The high affinity RXXK motif of AMSH [62] specifies an association with the SH3 domains of adaptor proteins STAM1/2 [63, 64]. By virtue of this interaction, AMSH is recruited directly to the ESCRT-0 sorting complex on endosomes, which is essential for the deubiquitination of STAM1 and Hrs as well as potentially other substrates found on ESCRT-0 positive endosomes [38]. AMSH is also able to bind the SH3 domains of the Grb2 family of adaptors in vitro, albeit possible cellular implications of such complexes remain unknown [38]. Interestingly, changes in the ubiquitination status of Hrs and STAM as a function of AMSH activity do not alter their stability. As Hrs and STAM function is dependent on UIM-mediated interactions [54, 60, 65], these observations may indicate an effect of AMSH on ESCRT complex remodeling.

In addition to its relationship with the ESCRT-0 proteins, AMSH also interacts with several CHMP components of the ESCRT-III, including CHMP1A, CHMP1B, CHMP2A, and CHMP3, via its MIT domain [66]. The MIT domain in AMSH is similar to that of the Vps4 AAA-ATP, which recruits Vps4 to the MVB for the disassembly of the ESCRT-III machinery [67].

USP8 shares several of the endosomal recruitment determinants with AMSH. At the N-terminus USP8 contains a domain of unknown function 1873 (DUF-1873), followed by a Rhodanese-like domain known to interact with the ubiquitin ligase Nrdp1, which mediates ErbB3 ubiquitination. DUF-1873 has been reported as a possible MIT domain capable of binding to CHMP1A/B, CHMP4C, and CHMP7 components of the ESCRT-III complex [45, 68]. Since AMSH also binds to some of these proteins, this may represent a competitive binding interaction between the two DUBs. The precise role of the USP8 DUF-1873/MIT domain nevertheless remains unclear, as deletion of this region does not alter the extent of ligand-mediated EGFR deubiquitination by USP8 [35].

Perhaps, the most appreciated commonality between the ESCRT–DUBs lies in their ability to bind STAM adaptors.

The middle portion of USP8 encodes an extended region that contains three RXXK motifs, which constitute low-affinity binding partners for the atypical SH3 domains [35] and may function in a cooperative manner to promote USP8/ESCRT-0 complex formation in vivo (P.D. Nash unpublished data). The decision to recycle cargo back to the plasma membrane or to direct it toward the lysosome is largely a function of ESCRT-0 positive endosomes. Indeed, the interaction between USP8 and STAM is essential to mediate EGFR deubiquitination and drive activated receptors into the recycling pathway [35].

Besides targeting specific receptor cargo, USP8 also protects the Hrs and STAM proteins from proteasomal degradation [54], which is required to maintain the integrity and bulk flow of cargo through the ESCRT-0 sorting endosome [34]. Moreover, USP8 may serve an additional function with respect to ESCRT-0 ubiquitination analogous to that afforded by AMSH, as it exhibits specificity toward both K48 and K63 ubiquitin linkages in vitro [69].

Their association with the early, middle as well as late endosomal sub-compartments through different interaction elements allows these enzymes to not only impinge on diverse substrates, but also control spatiotemporally distinct checkpoints in endocytosis. The same interaction domains place the ESCRT–DUBs at the intersection between ESCRT-mediated control of endocytic trafficking and the broader processes of cell growth and division.

Other ESCRT–DUB Functions

ESCRT machinery has been implicated in multiple cellular processes beyond endocytic trafficking, which include cytokinesis and autophagy. During cytokinesis, some ESCRT proteins are found at the mid-body, and recent evidence indicates their requirement for abscission in late mitosis [70, 71]. Hrs (ESCRT-0), TSG101, and VPS37 (both ESCRT-I) interact with microtubule bundling proteins, such as CEP55, that are recruited to the mid-body during cell division [71]. Mutation or depletion of Vsp4 or other ESCRT-III proteins results in multipolar spindles, defects in chromosome segregation and incomplete abscission [71], suggesting that ESCRT-III proteins may fulfill multiple roles during mitosis and cytokinesis. Both of the ESCRT–DUBs are also found at the mid-body during cytokinesis, with AMSH present in the early phases of cytokinesis and USP8 colocalizing with ubiquitated proteins at the mid-body later in the process [72]. AMSH contains a nuclear localization signal [73], but does not directly co-localize with ubiquitinated species, mapping instead immediately adjacent to the ubiquitin-positive patches on the mid-body [72]. During mitosis, AMSH deubiquitinates v-SNARE and VAMP8 [72], further implicating it as a factor regulating cellular proliferation and survival [70, 71].

USP8 also contains sequence determinants consistent with a potential role in mitosis. One such site found in the region of amino acid sequence $R_{677}SYSSP_{682}$ matches the binding motif of 14-3-3. Physiologically, phosphorylation of USP8 at serine-680 during M-phase induces 14-3-3ε binding that in turn affects stability and activity of the DUB [74]. USP8 can also be phosphorylated on threonine-907 in an Akt-dependent manner, contributing to the stabilization of the DUB, and suggests that Akt-mediated growth and survival signals may utilize USP8 as a downstream effector. USP8 is also tyrosine phosphorylated by Src downstream of EGFR-ErbB2 activation and this stimulates USP8-mediated deubiquitination of the receptor [75]. Collectively, the unique spatial and temporal localization of each of the ESCRT–DUBs at the mid-body during cytokinesis suggests that each performs a distinct regulatory function in cell division [72].

Autophagy uses much of the same endosomal trafficking machinery to carry damaged organelles, protein inclusions and other cell content for lysosomal destruction. The autophagosome is a close relative of the MVB and can fuse with existing MVBs [42, 76]. Autophagasomes form around cellular aggregates and the phagaphore is extended by two ubiquitin-like conjugating systems—the Apg12/LC3 and the Apg8 systems, each of which employs a distinct cascade of ubiquitin conjugating enzymes [77]. Once the autophagasome has united with the endosomal pathway it is regulated by the ESCRT complexes and their mediator, ubiquitin. Depletion or inhibition of ESCRT components does not generally prevent the formation of autophagasomes but rather appears to halt autophagic flux toward the autolysosome [42, 76, 78, 79]. These observations indicate that ESCRT components may function to promote progression of autophagy in much the same way as endosomal maturation. It remains to be resolved whether the ESCRT–DUBs constitute key modulators of autophagy, and if so, whether the conserved means of recruitment employed by AMSH and USP8 in endocytosis are also involved.

Concluding Remarks

While the ESCRT–DUBs have been described largely for their role in modulating trafficking and stability of a handful of cell surface receptors, it is increasingly clear that they, and the ESCRT machinery which they control, play broad cellular roles connecting endocytosis with ubiquitin homeostasis, cellular stress, autophagy, and cytokinesis. Much of our understanding of the ESCRT–DUBs comes from studying their interaction domains and

motifs. Interactions with the RXXK motifs of ESCRT–DUBs promote the dynamic assembly of functional complexes between ESCRT-0 and the ESCRT–DUBs on sorting endosomes. These complexes act to regulate trafficking at the early sorting subcompartment. During later sorting events, ESCRT–DUBs may interact with ESCRT-III components to promote deubiquitination of cargo which for certain cargo may be required for MVB internalization or delivery into the lysosome. Yet, the role of ESCRT proteins extends beyond endocytosis to control a range of cellular processes including cytokinesis and autophagy, and preliminary evidence suggests that the ESCRT–DUBs likewise impinge on these processes. As proteolytic enzymes, the ESCRT–DUBs are potential therapeutic targets of small molecule inhibition and may represent attractive drug design avenues. USP8 inhibition in particular may provide a novel means of depriving cancer cells of essential growth factor signals. Indeed, examination of USP8 and/or AMSH inhibitors in combination with existing therapeutic modalities—particularly kinase inhibitors and drugs such as Herceptin—may provide substantial synergistic benefits and reduced rates of failure due to resistance.

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