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CHAPTER 2

Functional understanding of the versatile protein copper metabolism MURR1 domain (COMMD1) in copper homeostasis

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Functional understanding of the versatile protein copper metabolism MURR1 domain 1 (COMMD1) in copper homeostasis

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Copper is an important cofactor in numerous biological processes in all living organisms. However, excessive copper can be extremely toxic, so it is vital that the copper level within a cell is tightly regulated. The damaging effect of copper is seen in several hereditary forms of copper toxicity in humans and animals. At present, Wilson's disease is the best-described and best-studied copper-storage disorder in humans; it is caused by mutations in the *ATP7B* gene. In dogs, a mutation in the *COMMD1* gene has been found to be associated with copper toxicosis. Using a liver-specific *Commd1* knockout mouse, the biological role of Commd1 in copper homeostasis has been confirmed. Yet, the exact mechanism by which COMMD1 regulates copper homeostasis is still unknown. Here, we give an overview of the current knowledge and perspectives on the molecular function of COMMD1 in copper homeostasis.

Keywords: copper homeostasis; COMMD1; intracellular trafficking; vesicle transport

Hepatic copper toxicity has been described in several mammals, including humans, rats, mice, dogs, and sheep. Wilson's disease (OMIM 277900), the hereditary copper-storage disorder in humans, is caused by mutations in the *ATP7B* gene. *ATP7B* encodes the copper-transporting P-type ATPase protein, ATP7B. Spontaneous mutations in the *Atp7b* gene have also been identified in rats (LEC rat) and mice (toxic milk mouse).[1,2] However, at present, no mutations in the *ATP7B* gene have been described in dogs or sheep. In dogs, the best-described copper-storage disorder is copper toxicosis (CT) in Bedlington terriers.[3] CT in Bedlington terriers is an autosomal recessive disorder characterized by massive lysosomal copper accumulation in the liver of affected dogs. This is due to a defect in the excretion of copper into the bile.[4] A positional cloning approach identified a genomic deletion of 39.7 kb linked with CT, comprising exon 2 of the *MURR1* gene.[5–7] The name *MURR1* was changed into copper metabolism gene MURR1-containing domain 1 (COMMD1) after Burstein and colleagues identified the COMMD protein family,[8] with COMMD1 being the founder of this family. All 10 COMMD family members are widely expressed and characterized by a specific domain called the COMM domain, located in the carboxy terminus of these proteins.[8] The fact that COMMD1 protein was undetectable in liver homogenates of CT-affected Bedlington terriers suggests that the *COMMD1* exon 2 deletion results in a loss-of-function protein. In contrast to dogs, a *Commd1* loss-of-function mutation in mice results in embryonic lethality.[9] *Commd1* knockout mice die in utero between 9.5 and 10.5 days postcoitum (dpc). The development of *Commd1* knockout embryos is generally delayed, and the placental vascularization is abnormal. This latter observation has been suggested to be caused by aberrant activity of the transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 protein levels and its activity in *Commd1* knockout embryos were increased compared to wild-type embryos. The role of COMMD1 in HIF-1 signaling was further supported by various *in vitro* studies.[9–11] Although the reason for the phenotypic differences between dogs and mice is still unknown, it could be explained by the fact

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that COMMD1 in dog fetuses may be partially redundant and is compensated by the expression of other COMMD family members. In mice, the Commd proteins might have a distinct function or a different expression pattern compared to dogs, and therefore this compensation does not happen. We also speculated that the differences in the placental development between dogs and mice could be an additional explanation for the contradictory phenotypes of these two species.[9] To investigate the contribution of placental Commd1 to the defective embryonic development, we used *Commd1* floxed conditional knockout mice[12] and *Mox2-Cre* transgenic mice[13] to generate Commd1-deficient embryos with functionally normal placenta. We observed that restoration of *Commd1* expression in extraembryonic lineages was not sufficient to rescue the lethal phenotype of *Commd1* knockout embryos (unpublished data). Although this experiment did not prove that Commd1 expression is not important for proper placental development, it certainly indicated that Commd1 expression in the embryonic tissue is essential for normal murine embryogenesis. This is supported by reconstituting COMMD1 expression in Commd1-deficient mice by crossing *Commd1* knockout mice with mice expressing human COMMD1 protein. Expression of human COMMD1 in *Commd1* knockout mice rescues the lethal phenotype, and these mice are born healthy and do not show any overt phenotype.[14]

Using liver-specific *Commd1* knockout mice, we provided evidence for a biological role of COMMD1 in hepatic copper homeostasis.[12] Although affected dogs progressively accumulate copper in their livers, mice only show hepatic copper accumulation when being fed a copper-rich diet. However, the mice had no liver pathology nor increased mRNA levels of the metallothioneins *Mt-I* and *Mt-II*, both gene transcripts encoding a protein that chelates copper to prevent toxicity. Despite the progressive hepatic copper accumulation in this mouse model (up to a 20-fold increase compared to wild-type littermates), the copper values did not reach the toxic levels seen in CT-affected dogs.[12] Nevertheless, this animal model clearly supports the biological role of COMMD1 in copper homeostasis, although the exact molecular mechanism of how COMMD1 regulates biliary copper excretion still needs to be identified.

Molecular mechanism of COMMD1 in copper homeostasis

The identification of the interaction between COMMD1 and ATP7B[15,16] strongly suggests that COMMD1 positively regulates the copper-transporting activity of ATP7B and thereby the excretion of copper into the bile. Under basal conditions, ATP7B is located within the trans-Golgi network (TGN), where copper can be incorporated in cupro-enzymes. In the event of high copper levels, ATP7B is distributed to cytoplasmic vesicular compartments from which copper can be excreted out of the cell (Fig. 1).[17] When copper is

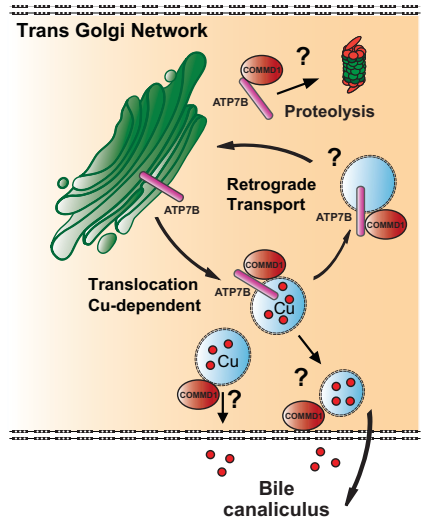

Figure 1. Current overview of the possible mechanisms by which COMMD1 maintains copper homeostasis. Under normal conditions, ATP7B localizes to the *trans*-Golgi network (TGN). Upon elevated copper levels, ATP7B relocalizes to vesicular compartments at the cell periphery from which copper can be excreted from the hepatocytes into the bile. Numerous studies proposed different mechanisms of COMMD1 in regulating copper homeostasis, indicated with question marks. Various studies suggested a role for COMMD1 in regulating the protein stability of ATP7B. Another study showed that COMMD1 deficiency attenuates the relocation of ATP7B back to the TGN when copper returns to normal physiological levels. Since the incorporation of copper in cuproenzymes is not affected in CT-affected dogs and liver-specific Commd1 knockout mice, it is very likely that COMMD1 acts downstream of ATP7B, and is required at this final step to excrete copper into the bile canaliculus.

Table 1. Overview of proteins and organelle markers that colocalize with COMMD1

Pathway studied	Colocalization	Marker	Cell type	Reference
Copper metabolism	Cd63	Lysosomes	HeLa	Ref. 6
	TFR	Early/recycling endosomes		
ATP7B/copper metabolism	ATP7B	*Trans*-Golgi	HEK293T	Ref. 18
ATP7B/copper metabolism	Rab7	Late endosomes	HeLa	Ref. 19
	Rab9	Late endosomes/TGN		
	Rab11[a]	Recycling endosomes		
Biochemical analysis of COMMD1 and PtdIns(4,5)P_2	EEA1	Early endosomes	Polarized HepG2	Ref. 20
	CHMP2B	Late endosomes/multi-vesicular bodies		
	Lamp1[b]	Lysosomes		
	Golgin-97[b]	Golgi		
Sodium transport	δENaC	Early/recycling endosomes	Cos7	Ref. 21
	TFR			
CFTR trafficking	Rab11	Recycling endosomes	HeLa	Ref. 22
	EHD1	Recycling endosomes		
	Tfr	Early/recycling endosomes		

[a]Used in study but shows no COMMD1 colocalization
[b]Minimal COMMD1 colocalization

normalized to physiological levels, ATP7B is recycled back to the TGN. We demonstrated that the subcellular localization of COMMD1 partially overlaps with ATP7B in HEK293T cells.[18] We showed that COMMD1 localizes to cytoplasmic vesicles with a perinuclear distribution, consistent with other reports.[6,19–22] Nevertheless, not all the studies were able to confirm the colocalization of COMMD1 with ATP7B, which may be explained by the different cell systems used, and by the fact that the interaction between ATP7B and COMMD1 might be transient. Although the identity of the cytoplasmic vesicles associated with COMMD1 is still not well defined, the above studies demonstrated that the cellular distribution of COMMD1 partially overlaps with markers of the lysosomal and endosomal pathways (early and recycling endosomes) and multivesicular bodies (Table 1). Despite the fact that these data imply an involvement of COMMD1 in the vesicular trafficking of ATP7B, none of the studies found evidence to show that reduced COMMD1 function affects copper-induced ATP7B trafficking.[18,19] Of note, changes in cellular copper levels do not lead to a different subcellular distribution of COMMD1, but can lead to reduced levels of COMMD1.[6,23] However, using a mouse hepatoma cell line, Miyayama and colleagues reported that the retrograde transport of ATP7B back to TGN is impaired in Commd1-insufficient cells.[24] This observation indicates that COMMD1 facilitates the relocation of ATP7B back to the TGN when the copper returns to normal physiological levels (Fig. 1). It is not known whether ATP7B also continuously cycles between the TGN and the cell periphery under basal conditions, similar to the highly homologous Menkes disease protein ATP7A,[25] but it would be interesting to investigate the contribution of COMMD1 to this recycling pathway. Nonetheless, if COMMD1 is required for shuttling ATP7B back to the TGN, we would expect the incorporation of copper into cuproenzymes to be affected as well. CT-affected Bedlington terriers and the liver-specific *Commd1* knockout mouse do not show reduced ceruloplasmin activity,[12,26] which implies that COMMD1 acts downstream of ATP7B and may be required at the final step of copper excretion into the bile (Fig. 1). This secretory pathway might be facilitated by the interaction of COMMD1 with the membrane phosphatidylinositol PtdIns(4,5)P_2, which has been shown to have a role in vesicular transport, acting as a membrane-anchoring molecule.[20]

Another hypothesis, proposed by de Bie and colleagues,[18] suggested that COMMD1 is involved in the quality control of newly synthesized ATP7B protein (Fig. 1). This concept was based on the observation that overexpression of COMMD1 enhanced the proteolysis of newly synthesized ATP7B, and that mutations in the N-terminal region of ATP7B increased its binding to COMMD1. Several of the described mutations were associated with mislocalization and decreased half-life of ATP7B.

Table 2. Overview of the literature on the effect of COMMD1 on ATP7A/B levels

Reference	COMMD1	ATP7A	ATP7B	Model	Copper
18	Overexpression	ND	Increased proteolysis	HEK293T	ND
24	Knockdown	ND	Decreased endogenous protein levels	Mouse hepatoma cell line	Increased
12	Knockout	ND	Decreased endogenous protein levels	Liver-specific knockout mice, 6 weeks old	Increased
	Knockout	ND	Not changed	Liver-specific knockout mice, 9–58 weeks old	Not changed, but increased upon a copper-enriched diet
31	Overexpression	Increased transiently overexpressed protein levels	ND	HEK293T	Improved copper-transporting activity
	Knockdown	Decreased endogenous and stably overexpressed protein levels	ND	HEK293T	Decreased copper-transporting
	Knockout	Decreased endogenous protein levels	ND	Mouse embryonic fibroblasts	activity
16	Overexpression	Decreased endogenous and stably overexpressed levels	Decreased endogenous protein levels	HEK293T	ND
		Increased transiently overexpressed protein levels		HEK293T	ND
	Knockdown	Increased endogenous protein levels	Increased endogenous protein levels	HEK293T	ND

ND = not determined.

Although this study[18] did not provide evidence that COMMD1 directly mediates the proteolysis of ATP7B, other studies support a role for COMMD1 in protein degradation.[9,10,16,27–29] COMMD1 acts as a hub to promote ubiquitination and proteosomal degradation of the NF-κB subunit p65[27] and the ubiquitination of the epithelial sodium channel ENaC,[28] enhances the proteolysis of HIF-1,[9,10] and is associated with several cullins.[29]

However, there are some discrepancies in the reported effect of COMMD1 on ATP7B protein levels (for overview, see Table 2). Miyayama *et al.*[24] reported that knockdown of Commd1 in a mouse hepatoma cell line reduced the protein levels and function of Atp7b, resulting in an increase in the intracellular copper concentration and the cytotoxicity to cisplatin. Cisplatin is also a substrate for ATP7B,[24] and a recent study supported the association between COMMD1 insufficiency and increased cisplatin sensitivity, but it is not known whether this can be explained by changes in ATP7B function or the interaction of COMMD1 with the BRCA1 C-terminal (BRCT) domain containing DNA damage–response proteins.[30] In line with the observation that COMMD1 is required for proper ATP7B levels, we recently reported that COMMD1 also enhances the protein levels and function of ATP7A.[31] Protein–protein interaction was demonstrated between COMMD1 and ATP7A.[16,31] This interaction improves the expression, cellular distribution, and copper-exporting activities of transiently expressed wild-type and mutant ATP7A in HEK293T cells.[31] The reduced levels and function of endogenous ATP7A in COMMD1-deficient HEK293T cells and mouse embryonic fibroblasts corroborated the observation seen in cells overexpressing ATP7A and COMMD1.[31] However, others could not completely confirm these findings. For example, Materia *et al.* reported opposite results, and they proposed that COMMD1 facilitates degradation of ATP7A and ATP7B.[16] This study could confirm the results described by Vonk *et al.,* but only when both proteins (i.e., COMMD1 and ATP7A) were transiently overexpressed in HEK293T cells. However, Materia *et al.*

did not investigate the effect of increased ATP7A/B on their copper-transporting activities, subcellular localization, or copper retention in COMMD1 knockdown cells. Since loss of COMMD1 is related to copper accumulation, the observation of elevated ATP7A/B levels in COMMD1-insufficient cells sounds counterintuitive, but was explained by an imbalance of copper efflux and copper sequestration, with copper sequestration being the primary function of ATP7B.[16,17] Despite these contradictory results demonstrated by different cellular models, deletion of *Commd1* in murine hepatocytes did not result in significant changes in Atp7b levels, except in the livers of the hepatic *Commd1* knockout mice at an age of 6 weeks.[12] Here, a significant reduction in Atp7b levels was observed, which correlated with an increase in the hepatic copper content of the liver-specific *Commd1* knockout mice. Thus, by using both cellular and mouse models, the role of COMMD1 in copper homeostasis has been confirmed, although the exact mechanism of its action needs further investigation.

COMMD1 function in other pathways

Since 2002, when we identified the *COMMD1* mutation in affected Bedlington terriers, the network of COMMD1-interacting proteins (for overview, see Table 3) has increased enormously, suggesting pleiotropy of COMMD1. Indeed, besides its role in copper metabolism, COMMD1 has been linked to the regulation of sodium transport via ENaC, enhancing the basolateral expression of the sodium–potassium–chloride cotransporter (NKCC1), regulating cystic fibrosis transmembrane conductance regulator (CFTR) trafficking, inhibiting Cu,Zn superoxide dismutase (SOD1) activity, and modulating HIF-1 and NF-κB signaling.[8,9,22,32–34] The role of COMMD1 in NF-κB signaling has been discussed in detail by Bartuzi *et al.*[14] A common theme in these pathways is COMMD1's role in the ubiquitination and proteolysis of its targets. It enhances the proteolysis of the NF-κB subunit p65 and HIF-1α and increases the ubiquitination of ENaC and NKCC1, but prevents the ubiquitination of CFTR.[10,21,22,27,34] These changes in ENaC, NKCC1, and CFTR ubiquitination are not correlated with proteasomal degradation, but with changes in the localization of ENaC, NKCC1, and CFTR at the cell membrane. In these studies, the authors suggested a role for COMMD1 in the trafficking of transmembrane proteins and targeting them to a specific cellular compartment. Altogether, these data advocate a function of COMMD1 in the vesicular transport and recycling of membrane proteins. A better knowledge of the function of COMMD1 in these pathways will be valuable in developing a fuller understanding of its molecular mechanism in copper homeostasis.

Several mechanisms have been described as regulating the function of COMMD1 (reviewed in Ref. 14), including cellular copper levels.[23] One of the regulators of COMMD1 that is also linked to copper homeostasis is the X-linked inhibitor of apoptosis (XIAP). Burstein *et al.* have demonstrated that fibroblasts derived from Xiap-deficient mice have reduced copper and increased Commd1 levels.[35] Later, it was shown that when copper levels are elevated, Xiap levels are markedly decreased both in inherited and acquired CT.[36] It has been suggested that XIAP might be involved in regulating the expression of COMMD1 in a copper–dependent manner.[36,37]

Other members of the COMMD family also have the ability to interact with copper-transporting ATPases. COMMD2, COMMD8, and COMMD10 can interact with ATP7A and ATP7B, but COMMD3, COMMD4, and COMMD5 interact only with ATP7A (de Bie, Wijmenga, and Klomp, personal communication). However, it is still unclear if other COMMDs are also involved in regulating copper homeostasis, and whether they act in concert with COMMD1, since COMMD proteins can interact with themselves or with each other.[8,38]

Concluding remarks

Since *COMMD1* exon 2 deletion is linked to the hereditary CT in Bedlington terriers, compelling evidence has been provided for a biological role of COMMD1 in copper homeostasis by a mouse model and numerous *in vitro* studies. One of the most noteworthy findings is the interaction between COMMD1 and the Wilson disease protein ATP7B. This protein–protein interaction was reported by various research groups and points to ATP7B requiring COMMD1 to excrete copper efficiently into the bile. Various studies suggest that COMMD1 insufficiency affects the protein levels of ATP7B, although this is not fully supported by the observation that neither the Atp7b levels, nor the copper transport into the TGN, are affected in the livers of adult hepatic Commd1-deficient mice. This suggests that

Table 3. COMMD1 interactome

Pathway/group of proteins	Interacting partner	Expression of COMMD1	Expression of interacting partner	Method	Reference
Copper transport	ATP7A	o/e	o/e	PD	31
		endog.	endog.	IP	16
	ATP7B	o/e	endog.	IP, PD	15
		o/e	endog.	*in vitro* interaction	
		o/e	o/e	IP, PD	18
		endog.	endog.	IF	
		endog.	endog.	IP	16
Free radical scavenging	SOD1	endog.	endog.	IP	33
		o/e	o/e	PD	
	CCS	endog.	endog.	IP	
		o/e	o/e	PD	
COMMD family	COMMD1-10	endog.	o/e	PD	8
	COMMD6	o/e	o/e	Y2H, BFC	38
		endog.	endog.	IP	
		endog.	endog.	IP	39
Nuclear factor-κB (NF-κB) signaling	RELA (p65)	endog.	endog.	IP	40
		endog.	endog.	IP	8
		o/e	o/e	IP	41
		o/e	o/e	PD	42
	RELB	o/e	endog.	PD	8
	c-REL	o/e	endog.	PD	
	NF-κB2/p100	o/e	endog.	PD	
	NF-κB2/p100	o/e	endog.	PD	
	IκBα	o/e	endog.	IP	40
		endog.	endog.		
	GCN5	o/e	o/e	IP	43
	CCDC22	endog.	endog.	IP	39
		o/e	endog.	PD	
Ubiquitin ligase complex	CUL1	endog.	endog.	IP	40
		data not shown	data not shown	?	27
		endog.	endog.	IP	29
		o/e	o/e	PD	
	CUL2	endog.	endog.	IP	27
		o/e	o/e	PD	
		o/e	endog.	PD	39
		endog.	endog.	IP	29
		o/e	o/e	PD	
	CUL3	endog.	endog.	IP	
		o/e	o/e	PD	
	CUL4A, 4B, 7	o/e	o/e	PD	
	CUL5	o/e	o/e	PD	
		data not shown	data not shown	?	27
	ELONGIN C	o/e	o/e	PD	
	SOCS1	o/e	o/e	PD	
		endog.	endog.	IP	
Inhibitor of apoptosis family of proteins (IAP)	XIAP	endog.	endog.	IP	35
		o/e	o/e	IP, PD	44
	c-IAP1	o/e	o/e	IP, PD	
	c-IAP2	o/e	o/e	IP, PD	
		o/e	o/e	IP, PD	35
	NAIP	o/e	o/e	PD	

Continued

Table 3. ***Continued***

Pathway/group of proteins	Interacting partner	Expression of COMMD1	Expression of interacting partner	Method	Reference
Hypoxia adaptation	HIF-1α	o/e	o/e	IP, PD	9
		endog.	endog.	IP	
		endog.	endog.	IP	11
		o/e	o/e	PD	
	HIF-1β	o/e	o/e	PD	
	HIF-2α	o/e	o/e	PD	
Protein folding	HSP70	o/e	o/e	IP	10
Nuclear export	CRM1	o/e	o/e	IP	45
Ion (co) transporters	βENaC	o/e	o/e	PD	32
		o/e	o/e	IP	28
	δENaC	o/e	o/e	IP, Y2H	32
		o/e	o/e	IP, PD	21
	γENaC	o/e	o/e	PD	32
	NKCC1	o/e	o/e	Y2H, *in vitro* interaction	34
		endog.	endog.	IP	
AGC kinase family	SGK1	o/e	endog.	IP	28
	Akt1/PKBα	o/e	o/e	IP	
Cystic fibrosis	CFTR	o/e	o/e	Y2H	22
		endog.	endog.	IP	
COMMD1 ubiquitination	ARF	o/e	o/e	IP, Y2H, IF	46
		endog.	endog.	IF	
	HSCARG	o/e	o/e	IP, Y2H	47
		endog.	endog.	IP, IF	
Secretory clusterin/ apoptosis	sCLU	o/e	o/e	Y2H	48
		endog.	endog.	IP, IF	
		endog.	o/e		
		endog.	endog.	IP	16
DNA damage response	CHK2	o/e	o/e	Y2H	30
	LIG4	o/e	o/e	Y2H	
	BRCA1	o/e	o/e	Y2H, PD	
	BARD1	o/e	o/e	Y2H, PD	

endog. = endogenous; o/e = overexpression; BFC = bimolecular fluorescence complementation; IF = immunofluorescence; IP = immunoprecipitation; PD = pull-down; Y2H = yeast two-hybrid.

COMMD1 acts downstream of ATP7B. There is still controversy as to whether ATP7B directly pumps copper into the bile canaliculus or whether ATP7B-containing vesicles are only to be found at the periphery of the hepatocytes, and in close proximity to the biliary canaliculus, when hepatic copper levels are high. Since there is only a partial overlap between COMMD1 and ATP7B localization, and it may be transient, it is tempting to speculate that COMMD1 acts as a hub in this final step to facilitate the fusion of the copper-containing exocytic vesicles with the bile canalicular membrane to release copper into the bile. However, since COMMD1 colocalizes with early and recycling endosomal markers, it cannot be ruled out that it is involved in directing the proteins to the correct vesicular compartment within a cell. Mislocalization of proteins can result in enhanced proteolysis, either proteasomal- or lysosomal-dependent, and this may explain the reduced ATP7B levels, as shown by various studies. The current data on COMMD1 function have been obtained mainly from different kinds of tumor cells, which may not be the appropriate cellular models for studying the hepatic function of COMMD1. With the generation of the conditional *Commd1* knockout mouse, an excellent and novel tool has become available to further delineate COMMD1's exact molecular mechanism

in copper homeostasis in hepatocytes and other cell types. In the near future, this mouse model will also allow us to investigate the role of Commd1 in various other biological processes, such as ATP7A-dependent copper transport; sodium, potassium, and chloride transport; HIF-1 signaling; and NF-κB mediated inflammation.

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Conflicts of interest

The authors declare no conflicts of interest.

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