Caspase-10, despite its FADD-dependent but caspase-8-independent death receptor signalling was controversial until it was shown that DED-containing caspase besides caspase-8, caspase-10. Its role in caspase cascade leading to apoptosis [3,4]. There is one other to and activation at the DISC is the decisive step for the initiation of the crucial for triggering apoptosis via death receptors since its recruitment (DISC) via a homotypic interaction with FADD. Indeed, caspase-8 is important for its recruitment to the death-inducing signalling complex.

1.1. Structure
Caspases belong to a family of highly conserved aspartate-specific cysteine proteases [1]. In humans, the caspase gene family consists of 11 members that are grouped into two major sub-families, namely the apoptotic and the inflammatory caspases [2]. The apoptotic caspases are further subdivided into two sub-groups, initiator and executioner caspases which together orchestrate the apoptotic cell death programme. Caspases are synthesised as zymogens which are inactive precursors consisting of an N-terminal pro-domain and a C-terminal protease domain. The C-terminal domain is subdivided into the large (20 kDa) α subunit and the small (10–12 kDa) β subunit. The pro-domain of caspase-8 consists of two death effector domains (DEDs) important for its recruitment to the death-inducing signalling complex (DISC) via a homotypic interaction with FADD. Indeed, caspase-8 is crucial for triggering apoptosis via death receptors since its recruitment to and activation at the DISC is the decisive step for the initiation of the caspase cascade leading to apoptosis [3,4]. There is one other DED-containing caspase besides caspase-8, caspase-10. Its role in death receptor signalling was controversial until it was shown that caspase-10, despite its FADD-dependent but caspase-8-independent activation at the TRAIL and CD95 DISCs, is neither required for apoptosis induction in the presence of caspase-8 nor capable of functionally substituting caspase-8 in its absence [5].

1.2. Recruitment to the DISC and the different models for caspase-8 activation
Two distinct signalling pathways trigger apoptosis: the extrinsic pathway – also known as the direct, or type I pathway – triggered by the binding of specific pro-apoptotic ligands belonging to the TNF superfamily (SF) to a subfamily of TNF receptor (TNFR) SF members also referred to as “death receptors”, and the intrinsic pathway activated by cellular damage in which the mitochondrion plays a central role.

The best-known mode of caspase-8 activation is by triggering of five of the six death domain (DD)-containing members of the TNFR SF, also referred to as death receptors. These are CD95 (APO-1, Fas) [6,7], TRAIL-R1 (DR4) [8,9], TRAIL-R2 (DR5) [10–13], TNFR1 (p55/p60 TNFR) [14,15] and TRAMP (DR3) [16,17]. These receptors are activated by respective ligands which belong to the TNF SF of cytokines, namely CD95L (APO-1L/FasL) [18], TRAIL (Apo2L) [19,20], TNF [21] and TL1A [22]. For DR6, the sixth DD-containing receptor, a specific amino-terminal cleavage fragment of the β-amyloid precursor protein (APP), N-APP, was recently described as a ligand [23]. No ligand for DR6 belonging to the TNF SF has been identified, at least so far, and caspase-8 does not seem to be involved in its signalling [23]. Therefore we will concentrate on the five caspase-8-activating death
receptors. These receptors come in two flavours as they are either direct recruiters of FADD, as is the case for CD95, TRAIL-R1 and [24] TRAIL-R2, or of TRADD, as in the case of TNFR1 and TRAMP. Whilst the primary output of the FADD recruiters is the induction of apoptosis, the TRADD recruiters primarily activate gene induction that is often referred to as being pro-inflammatory. However, the gene-inducing capacity of TRADD-recruiting receptors derived from the primary complex which forms around the cross-linked receptor (complex I) regulates the apoptosis-inducing output of a secondary complex (complex II) which forms subsequently to the receptor-associated signalling complex in the cytosol [25]. Consequently, under physiological conditions the gene- and cell death-inducing signals are in balance and it is only when this balance is perturbed that TNF and most likely also TL1A induce cell death. When signals from complex I of TRADD-recruiting complexes are blocked, then this complex II induces apoptosis in a manner quite similar to the events that occur when the death-inducing signalling complex (DISC) forms upon cross-linking of the FADD-recruiting receptors by CD95L or TRAIL. Following ligand binding to their cognate receptors the DD- and DED-containing adaptor protein FADD (MORT1) is recruited. This recruitment is due to a homotypic interaction of the FADD DD with the cytoplasmic DD of CD95, TRAIL-R1 or TRAIL-R2. Following its recruitment to the activated receptors, FADD, in turn recruits caspase-8, caspase-10, and cFLIP, a caspase-8- and -10-inhibiting protein, to the TRAIL and CD95 DISCs [5,26]. The recruitment of caspase-8, caspase-10 and cFLIP requires homotypic interactions between the DD of FADD and the N-terminal DD of the caspases or cFLIP [27]. Activation of caspase-8 and -10 occurs via DISC-recruitment-induced homodimerisation of the caspases which induces an activating conformational change. But even if recruitment of caspase-8 to the DISC initiates its activation, full caspase-8 activation requires complex molecular events which are so far not completely understood.

Currently, a number of models exist on how initiator caspases are activated and it is not yet clear which one, if any of them, will prevail. The so-called induced-proximity model was the first model to be proposed [28]. In this model the recruitment to the receptor complex by the adaptor protein FADD leads to clustering of initiator procaspases which in turn results in self-activation via a cross-proteolysis mechanism. Thereby, initiator caspases are auto-processed when brought into close proximity of each other. This model has led to the description of the so-called interdimer processing model of procaspase-8 activation by Chang et al. in 2003. In this study, the authors highlight the distinction between procaspase-8 and caspase-8 enzymatic activities [29]. Indeed, they showed that upon oligomerisation, individual procaspase-8 molecules associate with each other through their protease domain to form dimers. Those dimers are enzymatically active but can only recognise and cleave others dimers resulting in cross-cleavage. Procaspase-8 activation involves two sequential cleavage events, i.e., the separation of the large and the small subunit followed by the separation of the large subunit from the prodomain, which lead to the fully active caspase-8 which can cleave apoptotic substrates like caspase-3 and Bid as its best studied apoptosis substrates.

This first model was re-interpreted into the “proximity-induced dimerisation” model [30]. This model proposes that dimer formation drives activation of initiator caspases and the adaptor protein complexes serve to promote dimerisation by increasing the local concentrations of initiator caspases. Thus, in this model dimerisation, and not zymogen cleavage, is crucial for initiator caspase activation, even though caspase processing stabilises the active dimers. Finally the “induced conformation model” was proposed by Chao and collaborators [31] in which the conformation of the active site of the initiator caspase, attained through direct interaction with the adaptor protein complex, is crucial for activation. The latter model was based on studies with caspase-9 activation at the apoptosome, the other initiator-caspase-activating platform besides the DISC. The active site of caspase-9 is stabilised by the apoptosome which induces activation. However, caspase-9 can also be activated as a homodimer, as proposed by the proximity-induced dimerisation model and later its active site conformation is stabilised by dimer formation and dimer interactions with the apoptosome. The third possibility is that caspase-9 is activated as a higher order homo-oligomer and its active site conformation is stabilised both by the oligomer and the apoptosome [30,32]. Although these studies were performed with the initiator caspase-9 at the apoptosome, similar molecular concepts might be valid for activation of the DISC-associated initiator caspase-8 and -10.

Based on these existing models, a study published by MacFarlane and colleagues [33] introduced interesting new elements on caspase-8 activation at the DISC and on the downstream signalling outcomes. Using a reconstituted functional DISC with purified CD95, FADD, and procaspase-8, they propose a two-step activation mechanism involving both dimerisation and proteolytic cleavage of procaspase-8. Even more interestingly, they identify this as a key regulatory step whereby activated death receptor complexes decide between signal for death or survival. Indeed, they show that the initial dimerisation of procaspase-8 provides proteases only with a limited substrate repertoire limited to itself (probably caspase-10) and cFLIP, and that a secondary proteolytic cleavage is then required to fully activate caspase-8 so that it can cleave apoptotic substrates like caspase-3 and Bid.

Recently, Jin et al. proposed that caspase-8 polyubiquitination by the E3 ligase cullin-3 and its subsequent p62-dependent aggregation stabilises active caspase-8 and thereby positively regulates apoptosis [34]. They observed that stimulation of TRAIL-sensitive cells resulted in polyubiquitination of a small fraction of DISC-associated caspase-8. They identify cullin-3 as the E3 ubiquitin ligase responsible for this modification and as a new TRAIL DISC component. In their model, following its recruitment to the DISC, cullin-3 is able to ubiquitinate caspase-8 which in turn recruits the ubiquitin-binding protein p62. This newly formed caspase-8/cullin-3/p62 complex is then thought to translocate to the cytosol from where it induces apoptosis in a p62-dependent manner [34–36]. Yet, even though a fraction of caspase-8 indeed appears to be polyubiquitinated at the DISC (Jin et al. and our unpublished observation), the proposed molecular mechanisms for this modification and its functional consequences have to await confirmation before being fully integrated into our current model of the molecular events that occur at the DISC. It is noteworthy that in p62-deficient cancer cells caspase activity and apoptosis appear to be diminished, yet they are not abrogated. This suggests that p62 is not essential for extrinsic pathway activation but required for its full activity, an effect that can turn out to be highly relevant under physiological conditions since the signalling output of death receptors is tightly controlled.

2. Bid: the bridge between caspase-8 activated at the DISC and pro-apoptotic activation of mitochondria

2.1. The role of mitochondria in caspase-8-mediated apoptosis in type II cells

The mitochondria-dependent (or intrinsic) apoptosis pathway is activated by diverse stimuli including growth factor withdrawal, DNA damage, heat shock, UV- or γ-radiation, and chemotherapeutic drugs [37]. The signalling pathways activated by these stressors culminate in mitochondrial outer membrane permeabilisation (MOMP), enabling release of proteins from the mitochondrial intermembrane space [37]. As previously mentioned, in type II cells activation of the mitochondrial arm of the apoptosis pathway is required for the induction of apoptosis following a death receptor stimulus. The bridging element between the two arms of the apoptosis pathway is the caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member Bid [38] (Fig. 1).
2.2. Cleavage of Bid

Mitochondrial apoptosis is primarily regulated by molecular interactions between different members of the Bcl-2 family \[39,40\]. The common feature of members of this family is that they contain one or more Bcl-2 homology (BH) domains. Based on the number of regions of sequence homology with Bcl-2 (BH regions 1–4) they contain, as well as based on their function, members of the Bcl-2 family can be subdivided into pro- and anti-apoptotic members. The anti-apoptotic proteins of this family are Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1. All of them contain all BH domains, i.e., BH1 to BH4. The pro-apoptotic Bcl-2 family can be subdivided into two subgroups. The first subgroup consists of Bax, Bak and Bok, the three multi-domain pro-apoptotic proteins. The second subgroup, the so-called BH3-only proteins, so far has about a dozen or so members including proteins like Bim, Bmf, PUMA, NOXA, Bad and Bid \[41\]. The current models of BH3-only protein function at the mitochondria following their activation are eloquently described in a review by Andrews \[73\]. Therefore our main focus in the following paragraphs will be on the events that lead to the activation of Bid, the BH3-only protein that links death receptor cross-linking to pro-apoptotic events at mitochondria. This link is provided by active caspase-8. In its uncleaved form Bid is generally thought to be inactive as an apoptosis inducer. Following death receptor stimulation Bid is cleaved by caspase-8. This cleavage results in the generation of a 15 kDa protein termed truncated Bid (tBid). tBid is capable of inducing mitochondrial outer membrane permeabilisation (MOMP) in cells in which the ratio of pro- and anti-apoptotic Bcl-2 family members allows it to do so. Thereby, caspase-8-mediated cleavage of Bid into a pro-apoptotically active, truncated form provides the link between death receptor stimulation and mitochondrial apoptotic events \[38,42\].

Bid was first cloned in 1996 as a novel death agonist that heterodimerises with either agonists (BAX) or antagonists (BCL-2) \[43\]. Whilst most studies find that truncation of Bid is crucial for Bid-induced MOMP \[38,41,42,44,45\], there are also reports suggesting a pro-apoptotic role for full-length Bid \[43,46\]. Interestingly, in a model for anoikis – i.e., cell death by loss of attachment – endogenous Bid was shown to translocate to the mitochondria without cleavage \[47\]. Although the significance of these findings has yet to be assessed in terms of possible endogenous activation/cleavage events that could

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**Fig. 1.** Schematic representation of the role of mitochondria in caspase-8 signalling. Binding of CD95 or TRAIL to their respective receptors leads to receptor trimerisation and formation of the death-inducing signalling complex (DISC). The adaptor protein FADD is recruited to the DISC where the death domains (DD) of both proteins interact. Subsequently, procaspases-8 and -10 are recruited to the protein complex where they interact with FADD via the death effector domains (DEDs). cFLIP can compete with caspase-8 for the binding to FADD. Therefore, high levels of cFLIP can abrogate caspase-8 activation at the DISC. DISC-activated caspase-8 and -10 trigger a caspase cascade by cleavage of caspase-3. In type I cells, activation of the extrinsic pathway is sufficient to induce TRAIL- and CD95-induced apoptosis whereas in type II cells, Bid cleavage is required for apoptosis induction by TRAIL and CD95L. Caspase-8 cleaves Bid into tBid which initiates the mitochondrial apoptosis pathway leading to release of cytochrome c (CytC) and Smac/DIABLO from the mitochondria. MTC8/MMP facilitates the translocation of tBid to the outer mitochondrial membrane (OMM). Recently it was proposed that caspase-8 integration into Cardiolipin (CL)-rich domains of the OMM results in full activation of this caspase which can then directly access and cleave its substrate Bid. After release from mitochondria, CytC, together with Apaf-1 forms the apoptosome, an activation platform for caspase-9. Smac/DIABLO counteracts the inhibitory function of XIAP thereby allowing for full activation of caspase-3 and -9, ultimately leading to cell death.
follow translocation of full-length Bid to mitochondria, these studies suggest that cleavage of Bid may not be an absolute requirement for Bid's pro-apoptotic function. However, in most cases activation by truncation seems to be required. During apoptosis Bid can be cleaved by caspase-8 [38], yet several other proteases including granzyme B [38,48], cathepsins [49,50], and calpains [51], but also caspase-10 [52,53] and caspase-3 [54], have been shown to be capable of cleaving, and thereby activating, Bid.

The fact that Bid is a caspase-8 substrate and that its cleavage can be crucial for CD95-induced apoptosis was first reported in 1998 [38,42]. Caspase-8 cleaves Bid at aspartic acid residue 60 (Asp60) [38], leading to the release of a truncated form containing the carboxy (C)-terminal part of the protein. The resulting 15 kDa product (p15) is tBid which has the capacity to rapidly accumulate at mitochondria and to initiate MOMP. Cleavage of Bid by caspase-8 removes the N-terminal portion of the protein which, when still linked to the C-terminal part inhibits the pro-apoptotic function of the latter. Accordingly, when Bid is not cleaved it normally does not accumulate at the outer mitochondrial membrane (OMM). The inhibitory role of the N-terminal fragment was elegantly demonstrated by Tan et al. [55]. They showed that the N- and C-terminal fragments of Bid are able to bind to each other via interaction of the BH3-B domain of the N-terminal and the BH3 domain of the C-terminal portion of the protein. Accordingly, mutations in the BH3-B region of Bid that prevent interaction with the BH3 domain in its C-terminal portion show apoptosis activity similar to that of tBid when expressed alone. The authors could thereby conclude that the amino-terminal portion of Bid contains a region which negatively regulates the exposure of the BH3-domain and consequently its binding to the OMM and/or factors included in such membranes. Mc Donnell et al. reported [56] that this cleavage changes the conformation of the protein, leading to an exposition of hydrophobic residues, thereby allowing the insertion of tBid into the membrane and binding of its BH3 domain to other Bcl-2 family members. In view of these findings it is tempting to speculate that in cases when full-length Bid is thought to act pro-apoptotically, the interaction between the BH3-B and BH3 domains of Bid may be prevented in a manner different from proteolysis, e.g., by post-translational modifications of Bid or by the interaction of the BH3-B domain with another factor.

2.3. Translocation of Bid to the mitochondria and initiation of MOMP

In 1999 Gross et al. reported that cytosolic inactive p22 BID is cleaved by caspases at internal aspartic acid residues to yield a major p15 fragment and two minor fragments, p13 and p11. The Bid p15 fragment then translocates to mitochondria and inserts into the OMM [44]. Following binding of Bid to mitochondria Bak and/or Bax oligomerise in the OMM, and enable cytochrome c release [57,58]. Bid or tBid alone is unable to permeabilise the OMM without Bak and Bax [41,59] but as recently reviewed [41], Bid seems to be more closely related to multi-domain Bcl-2 family members like Bak than to other BH3-only proteins and therefore its insertion into the OMM is likely to be more “Bax-like” as well.

However, even though certain aspects of the molecular mechanism leading to MOMP following tBid translocation are already quite well established, others are not. As an example, in a recent study the Gross laboratory identified MTH2/MIMP as a major player in the events resulting in MOMP. In this study Zaltsman et al. showed that MTH2/MIMP facilitates recruitment of active tBid to mitochondria [45]. They used different experimental systems including MTH2/MIMP conditional knockout ES cells and MEFs to demonstrate the importance of MTH2/MIMP for both, recruitment of tBid to mitochondria and its pro-apoptotic role. Finally they establish that this interaction is also relevant in vivo as they found that MTH2/MIMP absence in hepatocytes decreased the sensitivity of MTH2/MIMP mice to anti-CD95-induced hepatocellular damage.

Hence, this study expands our knowledge about Bid-induced pro-apoptotic signalling. Yet, at the same time it exemplifies that even though we now have a fairly good understanding of this process, we are still far from grasping all the intricate and complex molecular alterations and interactions that lead to activation of Bid, MOMP and apoptosis via the mitochondrial pathway following death receptor stimulation.

2.4. The Cardiolipin enriched “mitosome”: a new mitochondrial activation platform for caspase-8?

The current view on why mitochondria are important for death-receptor-mediated apoptosis in type II cells is because their pro-apoptotic programme is triggered by tBid. The role of caspase-8 activity in the current model is confined to the TRAIL or CD95 DISC and it is thought to provide the Bid activatory signal from this platform only. However, in a recent study Gonzalez et al. challenge this notion by showing that caspase-8 integration into Cardiolipin (CL)-rich domains of the OMM results in full activation of this caspase which can then directly access and cleave its substrate Bid [60]. Several molecules have been suggested in the past to be involved in caspase-8 translocation to or sequestration at the mitochondria. In 2002, Stegh et al. showed that in MCF7 cells the bifunctional apoptosis regulator (BAR) binds to, and thereby sequesters and neutralises, active caspase-8 in a Bcl-2-regulated manner [61]. Others suggested that, following anti-CD95-induced translocation of caspase-8 to mitochondria, the caspase-8-binding protein FLICE-associated huge protein (FLASH) would form a molecular complex with caspase-8, thereby presumably activating the mitochondrial apoptosis pathway by regulating caspase-8 activity [62]. Another protein suggested to play a role in caspase-8 translocation to mitochondria is the mitochondrial membrane protein Cardif [63,64]. Gonzalez et al. are, however, the first to show that the anionic mitochondria-specific phospho-lipid CL acts as a mitochondria-associated platform that is actually required for caspase-8 translocation, oligomerisation and activation after CD95 stimulation, and hence CD95-induced death in type II cells. The authors first show a correlation between synthesis of mature CL and sensitivity to CD95-induced apoptosis since cells obtained Barth syndrome patients and tafazzin-deficient HeLa cells are resistant to CD95-induced apoptosis. Barth syndrome is a genetic disorder involving loss of tafazzin expression, a transacylase which is required for maturation of CL [65]. In CL-deficient cells, resistance is mediated by a defect in tBid production with the consequence that cytochrome c and SMAC/DIABLO release from mitochondria and caspase-3 activation are inhibited. Importantly, the defect was not due to tBid being incapable of inducing MOMP in these cells as tafazzin-knockdown cells transiently transfected with a tBid expression vector were as sensitive to MOMP induction as control cells. As the next logical step the authors then tested whether caspase-8 was properly activated in CL-deficient cells and found that this was indeed not the case; in CL-deficient cells, both caspase-8 translocation to the mitochondria and its subsequent activation were abrogated. In summary, Gonzalez et al. show that Cardiolipin is crucial for caspase-8 activation and that it allows the insertion of procaspase-8 into the OMM, where it then homodimerises leading to its autoactivation. Thus, in type II cells this mechanism is crucial for cell death induction. Finally the authors suggest the existence of a “mitosome” in CL-enriched regions of the OMM, bringing together caspase-8 and its substrate Bid. It is tempting to speculate that MTH2/MIMP and its role in Bid recruitment may synergise with CL-induced mitosome formation to facilitate MOMP.

3. Discrimination between type I and type II cells

Dependency of cells on the mitochondrial pathway to undergo apoptosis following a death receptor stimulus, defines two cell types,
namely type I and type II cells. In type II cells, Bid cleavage is required for apoptosis induction by TRAIL and CD95L whereas type I cells do not require Bid cleavage and activation of the extrinsic pathway is sufficient to induce TRAIL- and CD95-induced apoptosis. The differentiation into type I and type II cells was identified by, and first thought to be solely due to, differences in formation of the death-inducing signalling complex (DISC). Indeed it was shown that in type I cells caspase-8 activation at the DISC was very efficient, resulting in direct activation of caspase-3 and the caspase cascade. By contrast, DISC formation in type II cells was found to be weaker and it was shown that in such cells with weak capability of DISC formation the mitochondrial arm of the apoptosis pathway was required for CD95 to kill cells [66,67]. Elegant in vivo proof for the correctness of the biochemically defined distinction into type I and type II cells was provided by the finding that Bid-deficient mice survived a single bolus injection of anti-CD95 antibodies that killed wild-type mice [68].

Although this study provided genetic proof for the correctness of the type I–type II distinction and the biochemical differences at the DISC led the way to its discovery, the fact that the molecular differences in DISC formation were responsible, or even required for this distinction, were subsequently challenged. An important role in this challenge was played by the subsequent discovery of the X-linked inhibitor of apoptosis protein (XIAP) and its role as an inhibitor of caspase-3, -7 and -9 [24], in combination with the finding that the second mitochondrial activator of caspases (SMAC), also known as direct inhibitor of apoptosis-binding protein with low PI (DIABLO), when released from mitochondria following MOMP can neutralise the caspase-inhibitory role of XIAP [69]. In a number of biochemical studies it was shown that the distinction between type I and type II cells could also be made on the basis of the level of expression of XIAP. It was found that when XIAP expression was low or absent, cells were usually of type I and in its presence usually of type II, i.e., in the latter case Smac/DIABLO release from mitochondria was required to remove XIAP from effector caspases, thereby enabling their autocatalytic activation [70].

In 2009, Wilson et al. showed that silencing of cFLIP which resulted in increased caspase-8 activation was not necessarily sufficient to convert a type II cell into a type I cell but that combined inhibition of cFLIP and XIAP enabled Bax-independent apoptosis in formerly mitochondria-dependent type II colorectal cancer cells [71]. Finally, the role of XIAP in discriminating between type I and type II was recently also proven genetically in an elegant study by Jost et al. [72].

Hence, triggering of the DISC leads to caspase-8 activation. Active caspase-8 cleaves caspase-3 which, in type I cells, leads to cell death induction. However in type II cells this is blocked by XIAP. However, active caspase-8 also cleaves Bid and this can then induce MOMP. MOMP in turn results in the release of pro-apoptotic factors from the mitochondrial intermembrane space, including cytochrome c and SMAC/DIABLO. Cytochrome c release triggers apoptosome formation and activation of caspase-9, whereas release of SMAC/DIABLO results in neutralisation of XIAP, thereby enabling full maturation and activation of the effector caspase-3, -7 and -9 and, consequently, induction of apoptosis. Therefore, cells which express high levels of XIAP cannot directly activate caspase-3 following DISC-induced caspase-8 activation which is why in these cells Bid cleavage and subsequent MOMP are required for apoptosis to occur. Thus, XIAP expression or lack thereof is an important factor in the classification of cells as type I versus type II with respect to death receptor-mediated apoptosis.

This being said, given that the differences in DISC formation between type I and type II cells exist and that these differences were in fact the defining element in this distinction, one should not easily rule out that the DISC-forming capacity of a particular cell type contributes to its type I versus type II affiliation. It is quite possible that there are still unidentified differences at the DISC between type I and type II cells that may co-segregate with presence and/or absence of XIAP expression. It may therefore be rewarding to investigate the relationship between these two different modes of pathway control in more detail and whether crosstalk between them may even exist.

References


